CUTTING WEDGE: BACTERIAL COMMUNITY DIVERSITY AND STRUCTURE ASSOCIATED WITH THE CHEESE RIND AND CURD OF SEVEN NATURAL RIND CHEESES

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ABSTRACT

The microorganisms that inhabit cheese contribute greatly to the flavor and development of the final product. While the rind and curd microbiota have been characterized separately, there is limited information on how the structure and function of microbial communities in rinds and curds vary within and amongst cheeses. To better understand the differences in community structure and function between communities of cheese rinds and curds, we combined culture-based methods with culture-independent community profiling of curds and rinds. Rinds contained greater taxonomic diversity than curds. *Lactobacillales* dominated curd communities while members from the order *Actinomycetales* were found in high abundance in rind communities. Communities varied more between rinds and curds than among cheeses produced from different milk types. To better understand microbial community functions, we cultured and assayed isolates for antibiotic susceptibility and carbon source utilization. Among European and U.S. cheeses, 70% of all susceptible isolates were cultured from U.S. cheeses. Overall, our study explored the differences within and between rind and curd microbial communities of natural rind cheeses, provided insights into the environmental factors that shape microbial communities, and demonstrated that at the community and isolate level the cheese microbiome was diverse and metabolically complex.

INTRODUCTION

Cheese production exemplifies a reproducible succession of microbial communities (10, 48, 49). Microbes execute the biochemical transformation of milk from a liquid suspension of lactose, casein, whey, and fat into cheese which is a solid aggregate of amino acids, lactate and volatile flavor compounds and pigments (6). Over centuries, humans optimized the cheese-making process to select for distinct and reproducible microbial communities that give cheeses their individual tastes, consistencies, colors and other desirable properties (6, 14, 43, 49). In natural-rind cheeses, endogenous microorganisms or the addition of bacterial starter culture to milk are needed to acidify and coagulate the milk into

curds and enable future colonization by fungi and bacteria on the cheese surface (26, 49).

The early process of curd formation is well characterized (11, 16) . Ripening begins in milk, where lactic acid bacteria (LAB) such as *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus casei* ferment lactose into lactate, acidify the medium and digest proteins and milk (11, 25, 35, 36). LAB can be introduced by a starter culture, or the milk can be permitted to acidify naturally from the microbial community in it (6). Regardless of the addition of starter culture, most curds become inhabited by a simple community, dominated by lactose fermenters that may include organisms belonging to genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Staphylococcus*, *Enterobacter*, and *Streptococcus* (1, 6, 16).

After the initial curd formation, the rind begins to form. Throughout the ripening, excess lactate accumulates and dissolves into the medium, eventually migrating upward to the curd surface (22). Once lactate becomes accessible at the outer surface of the cheese, aerobic fungal taxa including *Candida*, *Penicillium*, and *Scopulariopsis* colonize the surface and metabolize lactate, leading to an increase in pH at the surface environment (22, 49). De-acidification facilitates the colonization and succession of microbes that prefer more alkaline and salty conditions including coryneforms such as *Corynebacterium*, *Brevibacterium* and *Brachybacterium* (27, 37, 41).

The composition of the external rind communities is governed by factors beyond pH, including microbe-microbe interactions. Fungal species can contribute to cheeses' unique characteristics, such as the blue-vein appearance of Roquefort by the fungus *Penicillium roqueforti*, and can be crucial to the survival of rind bacteria like *Corynebacterium*, *Halomonas*, *Pseudomonas*, *Pseudoalteromonas*, and *Vibrio* spp. (4, 29, 49). Fungi such as those belonging to the spore-forming *Penicillium* species can also produce antibacterial compounds that can lead to selection for more antibiotic-resistant bacterial strains (28). Likewise, bacteria on the rind such as those belonging to the antibiotic-producing *Actinomycetes* group can have a similar effect (32). The possibility of cheese bacteria developing resistance mechanisms has warranted the characterization of antibiotic-susceptibility of bacteria on various types of cheeses (4, 29).

While many studies have characterized the succession of early cheese community development from curd to rind in individual natural rind cheeses (1, 16, 20, 21), less is known about the comparison of microbial composition of a mature rind to a mature curd within and across natural rind cheese varieties. Here we compare microbial community structure between the rinds and curds of seven natural-rind cheeses that differ by pH, moisture content, and milk source. To obtain a detailed understanding of the rind and curd microbial communities, we used culture-independent, high-throughput Illumina sequencing of 16S rRNA genes. We also sought to characterize specific bacteria cultured from the cheeses. These isolates were identified using Sanger sequencing of the 16S rRNA gene (40) and were assayed for antibiotic susceptibility and carbon utilization profiles using BIOLOG's Ecoplates. Through these analyses, we aimed to evaluate the following hypotheses: 1) cheese rind communities would exhibit higher taxonomical diversity than curd communities; 2) antibiotic susceptibility of cheese isolates would differ between cheeses and between the two regions where these cheeses originated, Europe and the U.S.; and 3) cheeses with higher taxonomical diversity would be more metabolically active and can utilize more carbon sources. This study contributes to the characterization of the curd- and rind-associated communities of natural rind cheeses and reveals patterns of microbial diversity according to cheese type as well as the overall metabolic and antibiotic resistance profile of isolates from the different cheeses.

METHODS

SAMPLE COLLECTION AND ENVIRONMENTAL PARAMETERS

Seven natural rind cheeses—Vermont Shepherd, Stichelton, Sonnet, Missouri Truckle, Maggie's Round, Comte, and Alpage Gruyere—were obtained from Wasik's Cheese Shop in Wellesley, Massachusetts. A 100 +/– 10 mg sample from each of the rinds and curds of the examined cheeses was collected aseptically and processed immediately. Rind samples were scraped using a sterile razor blade and curd samples were taken from the cheese center after scraping off the exposed curd layer. A solid-state pH meter (S175CD/BNC; Sensorex, Garden Grove, CA) was used to determine the pH of each cheese rind and curd. Samples were dried for 7 days and moisture was determined by subtracting the dry weight of the sample from the original wet weight of a 1 g sample.

BACTERIAL ISOLATION AND CHARACTERIZATION

The 100 +/– 10 mg of fresh rind and curd samples were crushed using a pellet pestle and diluted in sterile water to 10^{-3}, 10^{-4}, and 10^{-5} of their original concentrations. All diluted samples were grown aerobically at room temperature on three different media: Plate Count Agar (PCA) with Milk (PCAM: 5g Peptone, 2.5 g yeast extract, 1 g dextrose, 1 g of whole milk powder, 15 g agar), Nutrient Agar (NA: 0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl) and PCA with fermentation indicators for lactose (5 g Peptone, 2.5 g yeast extract, 1 g dextrose, 10 g lactose, 0.03 g neutral red, 15 g agar). Isolates were re-streaked from single colonies three times in order to obtain pure cultures.

DNA ANALYSIS OF ISOLATES

The genomic DNA of selected bacterial isolates were extracted by suspending a colony in polymerase chain reaction (PCR)–grade water and freezing for 20 min at –80°C and then thawing. A 1465 base pair (bp) sequence of the 16S rRNA gene was PCR-amplified from genomic DNA (10–30 ng). Mastermix reagents consisted of 1 µg/µL bovine serum albumin, 200 µM dNTP mix, 1x buffer w/ $MgCl_2$, 300 pM 27F primer (AGA GTT TGA TCC TGG CTC AG), 300 pM 1492R primer (ACG GCT ACC TTG TTA CGA CTT) (IDT Integrated DNA Technologies, Inc., Coralville, IA) (30), and 0.2 U Takara Ex. *Taq* polymerase (Takara Clontech, Mountain View, CA). DNA extracts from *Escherichia coli* and sterile PCR-grade water were used as positive and negative controls, respectively. The thermal cycler program ran for 34 cycles in the following order: 1 cycle of initial denaturation (3 min at 95°C); 32 cycles of denaturation (30 sec at 95°C), annealing (25–30 sec at 50°C), and extension (1.5 min at 72°C); 1 cycle of final extension (10 min at 72°C); and hold (4°C). Amplicons were detected from banding patterns on 1.5% agarose gel electrophoresis and quantified on the NanoDrop 2000 UV-Vis spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Inc., Wilmington, DE). Excess *TAQ* polymerase, primer and nucleotides were precipitated from the amplicon solution by adding 1µL USB ExoSap-iT reagent (Affymetrix, Inc., Santa Clara, CA) to 9µL of amplicon. Purified products were identified via Sanger sequencing of the 16S rRNA gene using 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3') (GeneWiz, Madison, WI, USA). From each sequence we extracted >750 consecutive nucleotides with quality score Q>20 and each chromatogram was visually inspected to insure there were no base caller errors (Supplementary Table 1).

Supplementary Table 1

ISOLATE NAME	SEQUENCE IDENTIFICATION	MILK TYPE	CHEESE NAME	COUNTRY	GENUS
LWHW2-27F	Uncultured *Brevibacterium* sp. Clone PtS-1-cl11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW3-27F	Uncultured *Brevibacterium* sp. Clone PtS-1-cl11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW4-27F	*Brevibacterium aurantiacum*, isolate 0911TES25Y3	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW6-27F	*Brevibacterium* sp. EP11 Strain EP11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW7-27F	*Corynebacterium glycinophilum*	cow	Alpage Gruyere	Switzerland	*Corynebacterium*
ZMBB1-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
ZMBB2-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB3-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB4-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB5-27F	*Lactobacillus plantarum or casei*	cow	Stichelton	England	*Lactobacillus*
ZMBB6-27F	*Brevibacterium linens or epidermidis*	cow	Stichelton	England	*Brevibacterium*
ZMBB7-27F	*Staphylococcus simulans*	cow	Stichelton	England	*Staphylococcus*
ZMBB8-27F	*Staphylococcus pasteuri*	cow	Stichelton	England	*Staphylococcus*
ZMBB9-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
ZMBB10-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
AX1-27F	*Staphylococcus equorum*	cow	Missouri Truckle	USA	*Staphylococcus*
AX2-27F	*Brachybacterium aimentarium*	cow	Missouri Truckle	USA	*Brachybacterium*
AX5-27F	*Brachybacterium alimentarium*	cow	Missouri Truckle	USA	*Brachybacterium*
AX7-27F	*Staphylococcus warneri*	cow	Missouri Truckle	USA	*Staphylococcus*
RRSH1-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH3-27F	*Brachybacterium sp.*	cow	Maggies Round	Massachusetts	*Brachybacterium*
RRSH4-27F	*Bacillus sp.*	cow	Maggies Round	Massachusetts	*Bacillus*
RRSH6-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH7-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH8-27F	*Bacillus mojavensis*	cow	Maggies Round	Massachusetts	*Bacillus*
CH1-27F	*Staphylococcus* sp. U1371-101227-XH136	sheep	Vt. Shepherd	USA	*Staphylococcus*
CH3-27F	*Brevibacterium* sp. EP11	sheep	Vt. Shepherd	USA	*Brevibacterium*
CH7-27F	*Staphylococcus equorum*	sheep	Vt. Shepherd	USA	*Staphylococcus*

DIRECT DNA ANALYSIS OF CHEESE MICROBIAL COMMUNITIES

Total DNA from the cheese rind and curd samples was extracted using the MO BIO Power® Soil DNA Isolation Kit (MoBio, Carlsbad, CA). The 16S rRNA gene from community genomic DNA was amplified, detected, quantified, and purified as described for the isolates. Samples were sequenced on a MiSeq instrument at the Forsyth Institute, Cambridge, MA. The V3/V4 region of the 16S rRNA gene was amplified from each sample using the forward primer 341F 5'-*AATGATACGGCGACCACCGAGATCTACAC* **TATGGTAATT *GT*** CCTACGGGAGGCAGCAG-3'; where italicized text indicates Illumina adaptor, bold text indicates primer pad, italicized bold indicates primer linker and an underlined text a conserved bacterial primer 314F. The reverse primer was 806R 5'- *CAAGCAGAAGACGGCATACGAGAT* **XXXXXXXXXXXX AGTCAGTCAG *CC*** GGACTACHVGGGTWTCTAAT-3'; where italicized text indicates reverse complement of the Illumina adaptor, 12 X-letters in bold are the Golay barcode primer followed by barcode primer and primer linker (italicized bold letters). The conserved bacterial primer 806R is indicated by underlined letters. PCR products from respective samples were each tagged by a sample-specific 12-base barcode (9). All samples were amplified in triplicates with 5 Prime Hot Master PCR Mix (Five Prime) on an Eppendorf Master Cycler Pro PCR Thermocycler using 0.2 µM of each primer and 10 ng template. Reaction conditions were: 94°C for 3 min, followed by 35 cycles at 94°C for 45 secs, 50°C for 1 min and 72°C for 1.5 min. Following the 35th cycle, samples were incubated at 72°C for 10 min. Amplicons were purified using Ampure magnetic beads according to the manufacturer's instructions (Agencourt from Beckman Coulter, Danvers, MA), quantified by Nanodrop, and further purified using the Qiagen MiniElute Gel Extraction Kit (Qiagen, Valencia, CA). Libraries were quantified on a Bioanalyzer instrument according to the Bioanalyzer manual using a DNA High Sensitivity chip, pooled, and sequenced on a MiSeq Illumina sequencer (Illumina, San Diego, CA). Forward and reverse reads were joined using Flash software (31). Libraries were demultiplexed and filtered using a q-score cutoff of 20 using split_libraries_fasq.py in Quantitative Insights into Microbial Ecology (QIIME) v1.8.0 (9). Any reads that did not assemble or meet the q-score threshold were removed and were not used in subsequent analyses. Sequences were clustered into operational taxonomic units (OTUs) using the UCLUST algorithm (15) at 97% sequence identity level with the generation of new clusters with sequences that match the reference, and classified using the Greengenes 97% reference dataset released on May 2013 (12, 33). Raw sequence data were submitted to Sequence Read Archive in Genbank under accession number PRJNA354727.

ANTIBIOTIC DISC SUSCEPTIBILITY ASSAY

We used a modified version of antibiotic disc diffusion susceptibility test to compare fungal and bacterial-derived antibiotic susceptibility of cheese bacteria isolates, focusing on antibiotic susceptibility trends among isolates cultured from cheeses of different regions (5, 7). Ten morphologically diverse isolates were cultured in nutrient broth (NB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 40 – 48 hours. The liquid culture of bacteria was diluted using NB to match the turbidity of a 0.5 McFarland standard to ensure roughly equivalent densities of each inoculum. Bacterial culture was evenly streaked onto the dried surface of a nutrient agar plate using a sterile swab and allowed to be absorbed into the agar for at least 3 – 5 minutes. Six antimicrobial-

Table 1. Characteristics of the seven investigated natural rind cheeses. ND = Not Determined.

Cheese	*Origin*	*Country*	*Milk*	*Curd pH*	*Rind pH*	*Curd % Moisture*	*Rind % Moisture*
Alpage Gruyere	Gruyere	Switzerland	Cow	7.45	6.42	9.71	28.43
Comte	French Alps	France	Cow	7.14	6.5	21	26
Maggie's Round	MA	USA	Cow	5.34	5.04	4	26
Missouri Truckle	MO	USA	Cow	6.01	5.73	27	24
Vermont Shepherd	VT	USA	Sheep	ND	ND	ND	ND
Sonnet	VT	USA	Goat	6.44	5.55	ND	ND
Stichelton	Nottingham	England	Cow	8.17	Blue – 8.24 White – 6.96	ND	ND

impregnated discs (AM10: Ampicillin 10 μg; P10: Penicillin 10 IU/IE/UI; E15: Erythromycin 15 μg; RA5: Rifampin 5 μg; N30: Neomycin 30 μg; NB30: Novobiocin 30 μg) were evenly pressed onto the bacterial-containing agar surface using a disc dispenser. Plates were incubated for one week at room temperature prior to examination for antibiotic susceptibility. Diameter measurements in millimeters of the zone of clearance around the individual antibiotic discs for cheese isolates were used to categorize each cheese isolate into one of the three susceptibility levels based on the following zone clearance interpretation: Resistant (13 mm or less); Intermediate Susceptible (14 – 16 mm); and Susceptible (17 mm or more) (45).

CARBON SOURCE UTILIZATION PROFILING

Community-level physiological profiling (CLPP), a metabolic profile, of both the cheese rind and selected curd communities and individual bacterial isolates were analyzed using BIOLOG EcoPlate™ assay (Biolog, Hayward, CA). The capacity of either a bacterial community or a single bacterial isolate to utilize 31 distinct carbon sources over a 7-day period was examined and compared between the community sample and isolates of the same cheese and among different cheese types. The 100 +/– 10 mg of fresh rind and curd samples were crushed with a pestle and diluted in sterile water to 10^{-3} in 10 mM phosphate buffer. Subsequently, 100 μL of the solution was inoculated into separate BIOLOG Ecoplate™ wells. Individual isolates were incubated in NB overnight at room temperature and diluted to 10^{4} cells/ml and 100 μl was inoculated into each well of the new BIOLOG EcoPlate™. Growth was measured using the Molecular Devices SpectraMax 190 and the SOFTmaxPRO6.3™ program at A_{590} absorbance, for six consecutive days where day 1 was the day of inoculation. Metabolic diversity (CMD) was defined as the number of carbon sources utilized by the sample. Top carbon sources were defined as carbon sources that exhibited the maximum absorbance value for from fluorescence of tetrazolium salt reduction in the BIOLOG ECOPLATE™ assay.

RESULTS

ENVIRONMENTAL PARAMETERS OF NATURAL RIND CHEESES

To explore the relationship between the rind and curd within and between natural rind cheeses, and to determine how the physical factors of cheese environments (pH and moisture) correlate with microbial community diversity, we analyzed the microbial communities from seven different cheeses. The seven natural rind cheeses varied in appearance, place of origin, and type of milk used in the cheese-making process. Four cheeses came from the United States and three came from Switzerland, France, and England. All of the European cheeses and two of the U.S. cheeses were made from cow milk. The remaining two U.S. cheeses were made from sheep (Vermont Shepherd) and goat (Sonnet) milk. In each cheese the curd was slightly more acidic and contained more moisture than the corresponding rind (Table 1). Moisture in the curd varied slightly between cheeses, from 24% to 28%, while moisture in the rind had a larger range between cheeses, from 4% to 27% (Table 1).

To determine the types of bacteria that were present in the cheese communities, we analyzed the 16S rRNA gene using Illumina sequencing of the total extracted DNA from cheese curd and rind, and Sanger sequencing of cultured cheese isolates. There were more organisms identified to the genus level in the culture-independent approach than culture-dependent approach (see "other" category, Figure 1).

Unculturable organisms that were common to the cheese rinds included *Streptococcus* spp. (comprising 24% of total bacterial cells in the Comte rind and 55% of the Maggie's Round rind), *Lactococcus* spp. (39% of all cells in Missouri Truckle rind), *Yaniella* spp. (20% of Stichelton rind), *Prauseria* spp. (18% of Vermont Shepherd rind), *Halomonas* spp. (17% of Stichelton rind and 7% of Alpage Gruyere rind) and *Lactobacillus* spp. (16% of Missouri Truckle rind and 13% of Comte rind). In the curds, prevalent uncultured taxa included *Lactococcus* spp. (91% of Missouri Truckle rind and 89% of Stichelton rind), *Streptococcus* spp. (78% of Maggie's Round rind, 73% of Comte rind, and 26% of Alpage Gruyere rind), and *Lactobacillus* spp. (71% of Alpage Gruyere rind, 35% of Comte rind). In the curd, 43% (Figure 1B), and in the rind, 65% (Figure 1C) of bacteria were not culturable on PCAM agar. Thus, a significant fraction of bacterial species was not recovered on PCAM plates.

THE RIND HARBORS A MORE COMPLEX BACTERIAL COMMUNITY THAN THE CURD

In order to determine the bacterial diversity of the rind and curd, we carried out Illumina sequencing of the 16S rRNA gene. Rarefaction curve analysis, which assesses species richness from samples, showed all samples approached the asymptote and revealed that the overall bacterial diversity was well represented (Figure 2). The curds of Alpage Gruyere and Comte had the fewest unique OTUs (Figure 2A) while the largest number of unique OTUs was found in the rind of Alpage Gruyere cheese, followed by the rinds of Stichelton and Comte cheeses (Figure 2B). The Shannon diversity index, a measurement of overall diversity, of pooled data from the rinds and curds of the seven cheeses indicated more species richness and evenness in the rind communities than the curd (Figure 2C). Together, OTU distribution and richness data demonstrate higher alpha diversity in rind communities.

Through the identification of the organisms present in the cheese samples, we found that, with the exception of the Sonnet cheese, the only phylum represented in the curd communities was *Firmicutes* (Figure 3). In contrast, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were found in the rind communities. In a similar trend, no more than ten taxonomic units were found in each of the curd communities, while no less than ten genera were identified in each of the rind communities (Figure 3). In general, dominant genera in the rinds and curds were widespread among sampled cheeses. Cheese curds were dominated by lactic acid fermenters including *Lactobacillus*, *Streptococcus*, and *Lactococcus* while the rind communities showed a greater relative abundance of *Brevibacterium* and *Actinomycetaceae* (Figure 3; rind). However, variation was present within both the rind and curd communities. For example, while the Missouri Truckle, Sonnet, and Stichelton curds were almost completely dominated by *Lactococcus*, this organism made up less than 2% of the Maggie's Round, Comte, and Alpage Gruyere cheeses (Figure 3; curd, purple).

We next sought to investigate whether the differences in community composition could be associated with other abiotic factors such as moisture content, pH, or milk type. Principal coordinates of analysis (PCoA) demonstrated that communities were found to cluster by rind or curd (Figure 4), but not by milk type or moisture content (Supplementary Figure 1). A trend towards clustering was seen with pH (Supplementary Figure 1).

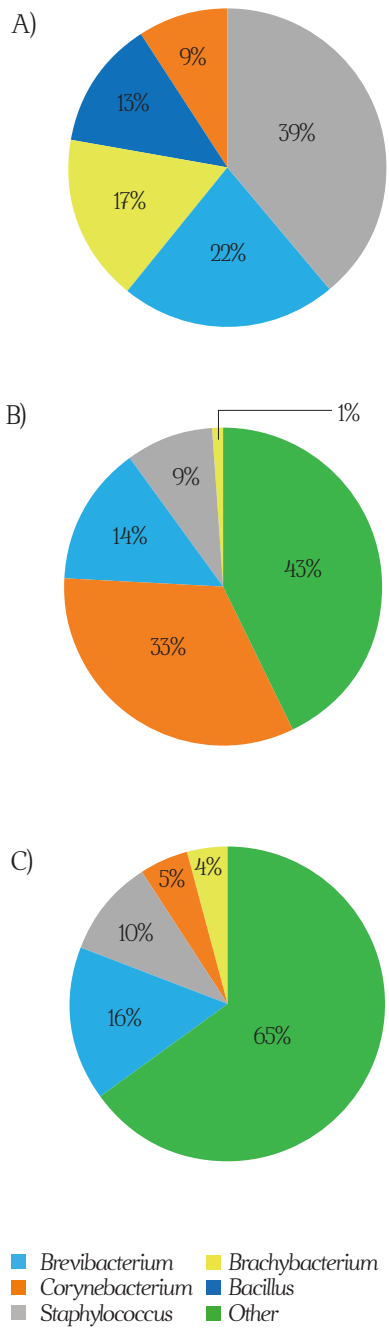


Figure 1. Prevalence (in % operational taxonomic units, OTUs) of cheese microbes successfully identified to the genus level through A) culture-dependent Sanger sequencing of the 16S rRNA gene of organisms isolated from both the rind and the curd, as compared to the prevalence of these organisms found through high-throughput community sequencing in either B) the curd or C) the rind. The green "other" category in Figure 1 represents bacteria identified by Illumina sequencing but not detected by culturing.

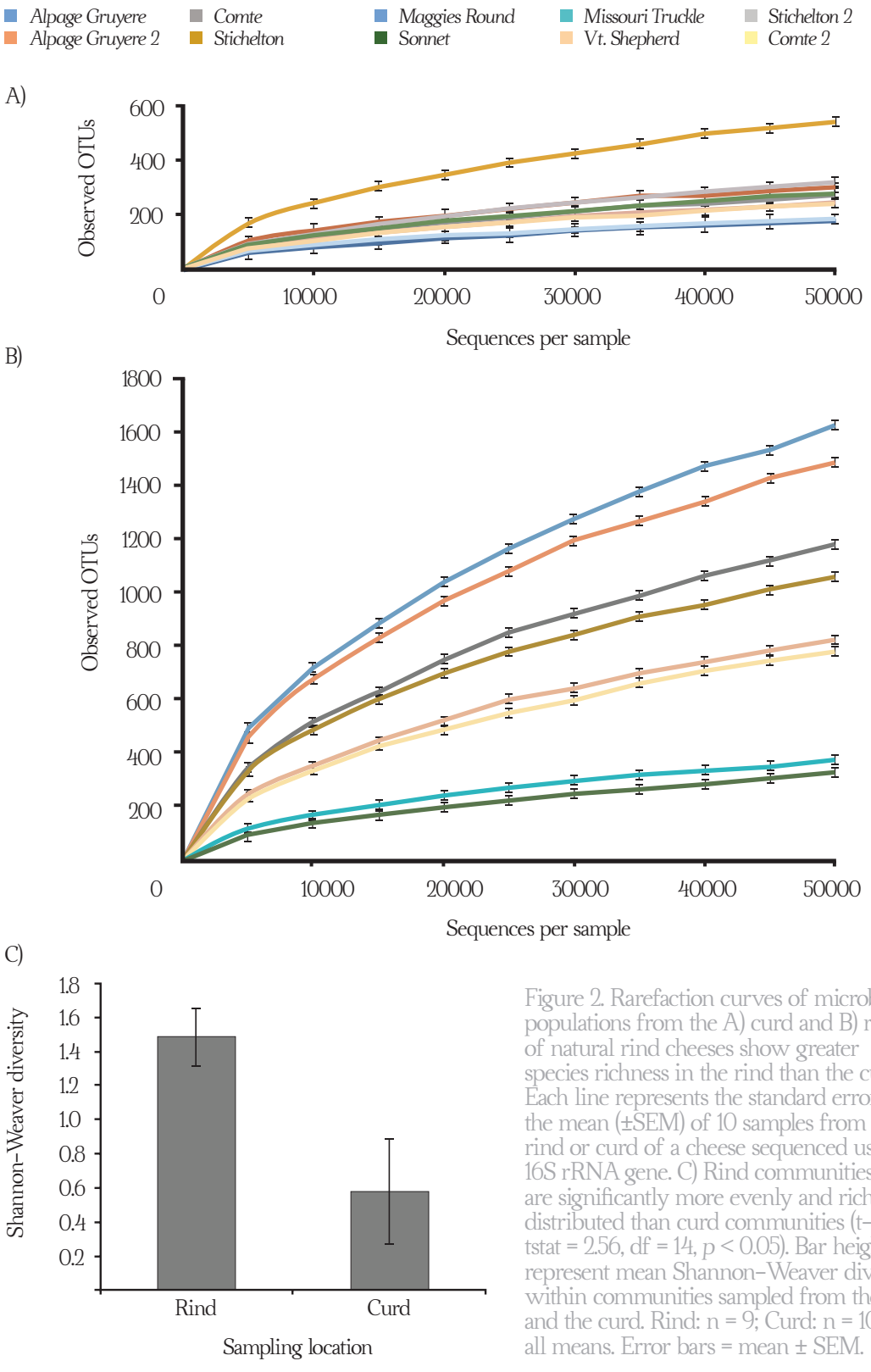


Figure 2. Rarefaction curves of microbial populations from the A) curd and B) rind of natural rind cheeses show greater species richness in the rind than the curd. Each line represents the standard error of the mean (±SEM) of 10 samples from the rind or curd of a cheese sequenced using 16S rRNA gene. C) Rind communities are significantly more evenly and richly distributed than curd communities (t-test, tstat = 2.56, df = 14, $p < 0.05$). Bar heights represent mean Shannon–Weaver diversity within communities sampled from the rind and the curd. Rind: n = 9; Curd: n = 10 for all means. Error bars = mean ± SEM.

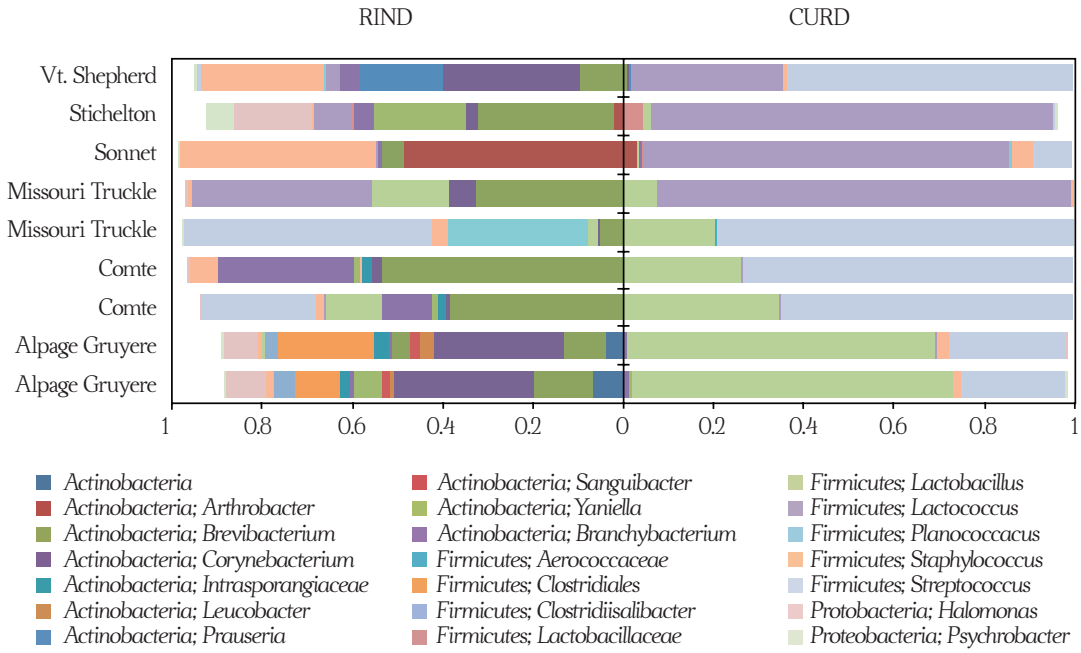


Figure 3. Relative abundance of microbial phyla and genera from the rind and curd of six natural rind cheeses. Horizontal bars represent microbiome samples from six cheeses in the rind and the curd and are colored according to the microbial phyla and genus found in these environments through Illumina sequencing of the 16S rRNA gene. Darker shades represent *Actinobacteria* while lighter shades represent either *Firmicutes* or *Proteobacteria*. Represented organisms were found at ≥ 2% relative abundance.

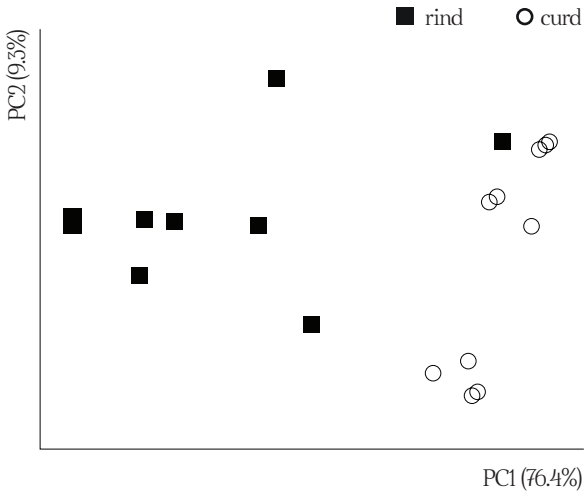
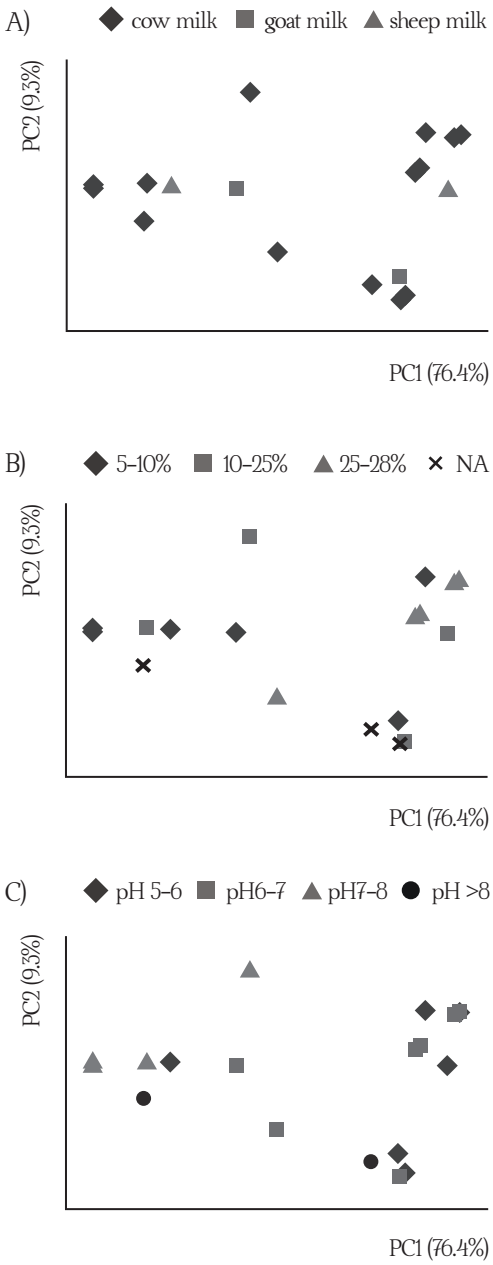


Figure 4. Principal coordinate analysis of rind and curd microbial communities. Clustering is seen with rind samples and curd samples, regardless of the cheese type. A 16S rRNA gene dataset was analyzed with QIIME and R was used to generate the principal coordinate analysis. Each white or black mark represents averaged community composition data of the rind or curd for the cheese sampled.



Supplementary Figure 1. Principal coordinate analysis of weighted Unifrac showing clustering based on other abiotic factors without consideration for rind or curd sampling location. A) Milk type B) Moisture and C) pH. Values range from 5.04 to 8.24.

ISOLATES FROM U.S. CHEESES DISPLAY HIGHER OVERALL SUSCEPTIBILITY TO ANTIBIOTICS THAN EUROPEAN CHEESES

To explore the outcomes of potential interactions between bacteria and fungi in the cheese communities from regionally diverse cheeses, inhibition of bacterial growth by fungal and bacterially derived antimicrobials was examined for cheese isolates. Isolate susceptibility levels were compared between cheeses and their two respective geographical regions of origin: the U.S. and Europe (Figure 5). Thirty-five bacterial isolates sampled from six cheeses were tested for their susceptibility or resistance to six antibiotics on nutrient agar plates: ampicillin (AM10), penicillin (P10), erythromycin (E15), rifampin (RA5), neomycin (N30), and novobiocin (NB30). Among the six cheeses examined, French Comte has the highest percentage of resistant isolates while U.S. Maggie's Round and Sonnet cheeses have the lowest percentage of resistant isolates (Supplementary Figure 2). Between the two geographical regions, fifty-five percent of the total resistant isolates belong to the European cheeses while bacterial isolates from the U.S. cheeses constitute seventy percent of the total susceptible isolates (Figure 5A and 5C). Compared to European cheese isolates, American cheese isolates showed larger percentages of intermediate susceptibility when exposed to ampicillin (AM10), penicillin (P10), and rifampin (RA5) (Figure 5B). Isolates from the three European cheeses exhibited higher percentages of resistant isolates, especially those isolated from French Comte (Supplementary Figure 2A). In general, American cheese isolates revealed higher percentages of susceptible isolates, especially Maggie's Round and Sonnet (Supplementary Figure 2). However, the small and uneven numbers of cultured isolates and cheese types for each of the regions limited the ability to further analyze the correlation between the cheese origin and susceptibility to antibiotics.

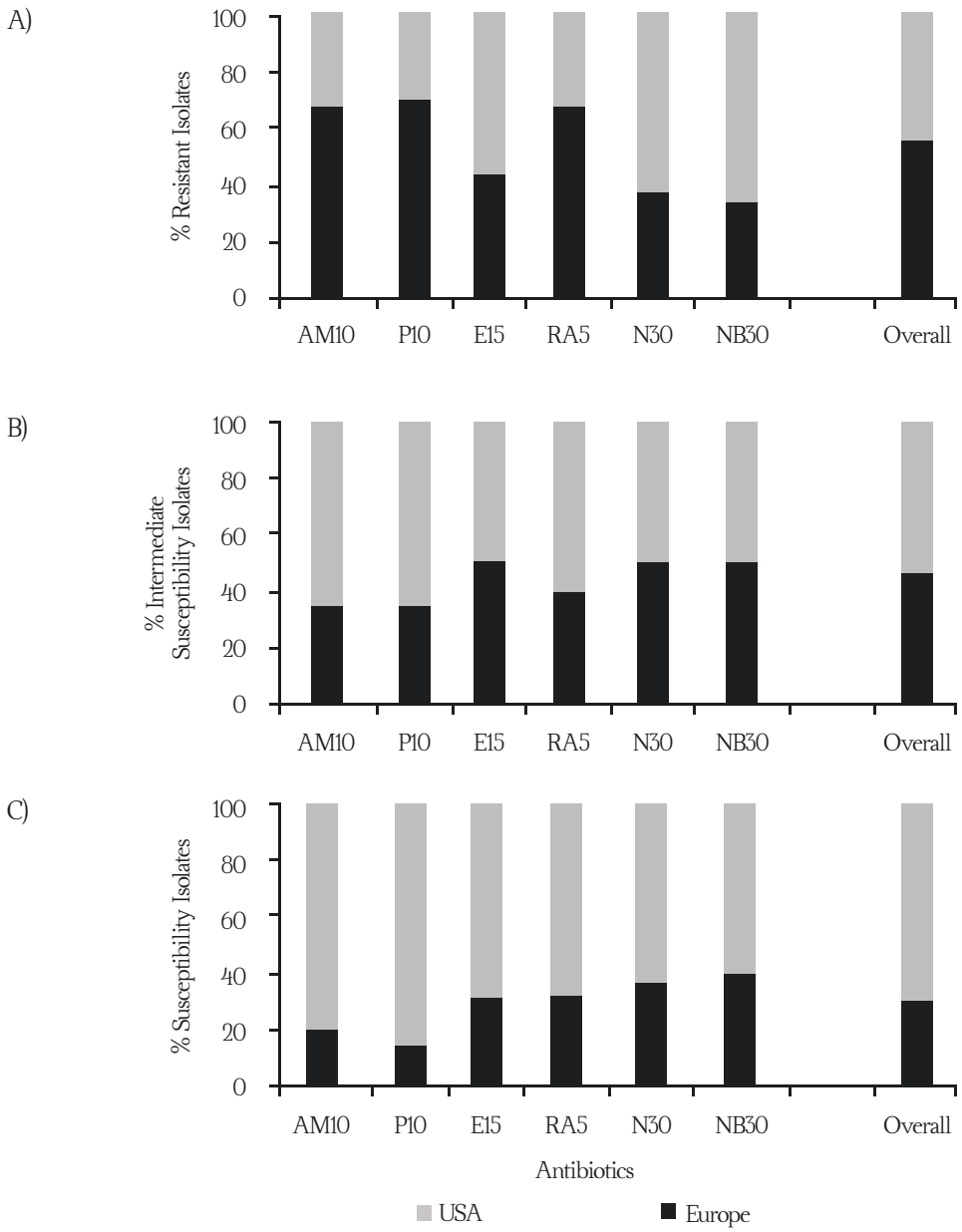
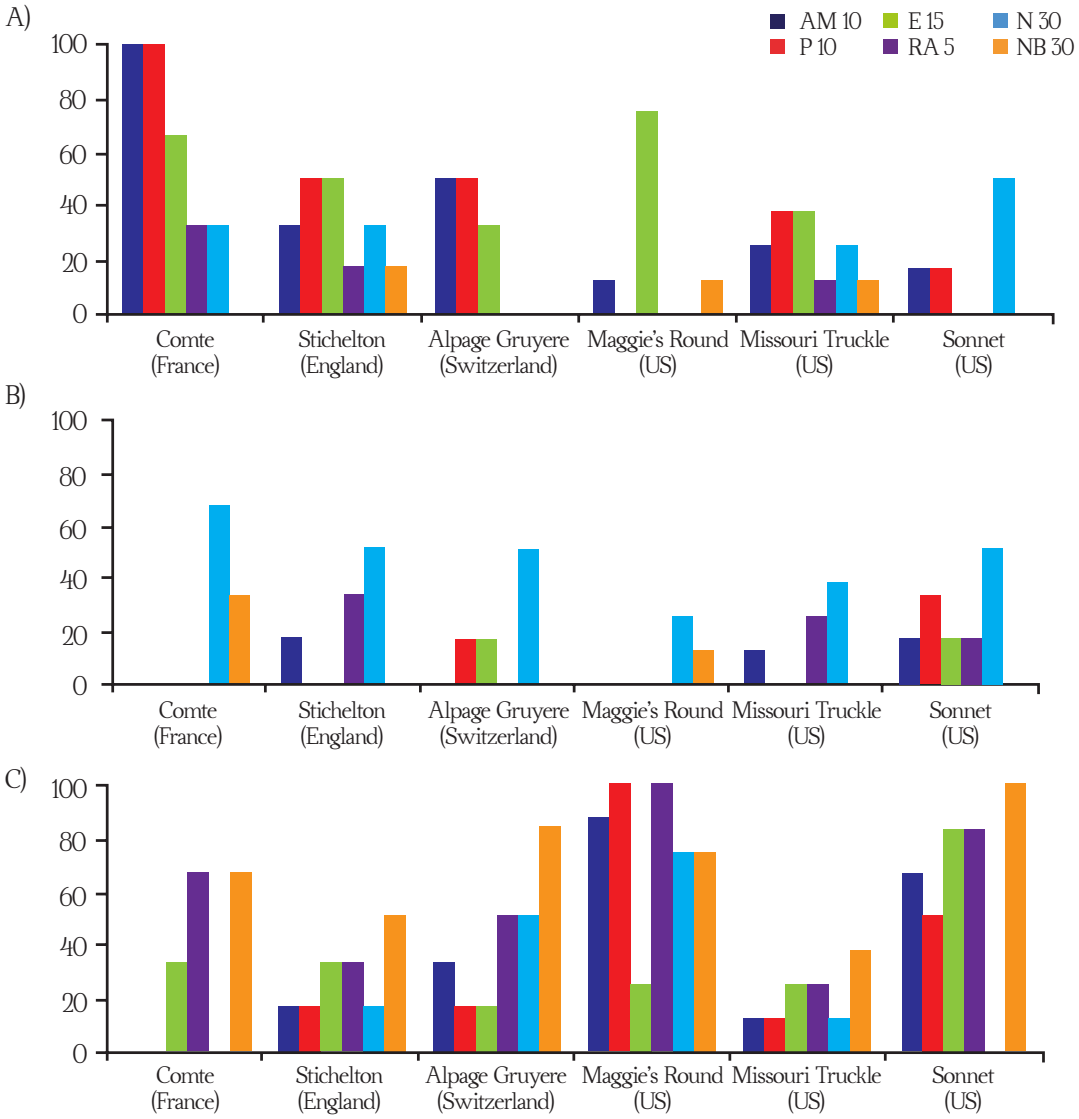


Figure 5. Antibiotics assay reveals U.S. cheese bacterial isolates constitute a larger percentage of the total susceptible cheese isolates than European cheese bacterial isolates. Growth inhibition by six distinct antibiotics was tested among 35 bacterial isolates sampled from 6 cheeses, which represent two geographical regions, Europe (blue) and USA (red). A) Resistant isolates, B) Intermediate susceptible isolates and C) Susceptible isolates. Diameter measurements of the zone of clearance (in mm) were grouped into the following susceptibility categories: a) Resistant (13 mm or less); b) Intermediate Susceptible (14 – 16 mm); and c) Susceptible (17 mm or more). Cheeses: [France] Comte, [England] Stichelton, [Switzerland] Alpage Gruyere, and [USA] Sonnet, Missouri Truckle, and Maggie's Round. AM10 = Ampicillin 10 ug; P10 = Penicillin 10 IU/IE/UI; E15 = Erythromycin 15 ug; RA5 = Rifampin 5 ug; N30 = Neomycin 30 ug; NB30 = Novobiocin 30 ug.



Supplementary Figure 2. Antibiotic assay shows a higher percentage of European cheese bacterial isolates that are resistant to the antibiotics tested than U.S. cheese bacterial isolates. Susceptibility level to six distinct antibiotics was tested among 35 bacterial isolates sampled from 6 cheeses, which represent two geographical regions, Europe: France (Comte n=3 isolates), England (Stichelton n=6 isolates), Switzerland (Alpage Gruyere n=6 isolates), and USA: (Sonnet n=6 isolates, Missouri Truckle n=6 isolates, and Maggie's Round n=8 isolates). Diameter measurements of the zone of clearance (in mm) were grouped into the following susceptibility categories: A) Resistant (13 mm or less); B) Intermediate Susceptible (14 – 16 mm); and C) Susceptible (17 mm or more). Percentages of isolates belonging to one of the three susceptibility levels against each of the six antibiotics examined were plotted for all six cheese types. AM10 (blue) = Ampicillin 10 ug; P10 (red) = Penicillin 10 IU/IE/UI; E15 (green) = Erythromycin 15 ug; RA5 (purple) = Rifampin 5 ug; N30 (cyan) = Neomycin 30 ug; NB30 (orange) = Novobiocin 30 ug.

Community level physiological profiling (CLPP) on both cheese community samples and cultured isolates was performed to examine their metabolic potential and diversity through their utilization of 31 distinct carbon sources (46) (Supplementary Table 2). We expected that 1) cheeses with greater taxonomical diversity would also have community samples that are more metabolically active, with higher numbers of utilized carbon sources, than their counterparts; and 2) cheese community samples would be able to utilize higher numbers of carbon sources than their respective individual isolates within the same cheese. Metabolic diversity (CMD), defined as the number of carbon sources utilized by the sample, increased for all isolates and for whole cheese communities over time (Figure 6; Supplementary Figure 3). Alpage Gruyere community sample was found to be the most metabolically active, with the highest number of utilized carbon sources (23 carbon sources) (Supplementary Table 2). Consistent with our first hypothesis, Alpage Gruyere rind was also the most taxonomically diverse out of all the examined rind communities, containing the most observed OTUs (Figure 2B). However, the second and third most metabolically active cheese community samples, Missouri Truckle (21 utilized carbon sources) and Maggie's Round (19 utilized carbon sources), respectively, contained the least numbers of OTUs in their curd samples (Supplementary Table 2 and Figure 2A). This inconsistency may be attributed to the overall lower taxonomical diversity in the curd communities and that the community samples collected for metabolic analysis were mainly derived from cheese rinds, contributing higher diversity. In addition, with the exception of the Stichelton cheese, cheese microbial community samples utilized more carbon sources than their respective individual isolates at the end of the 7-day sampling period, partially confirming our expectation that, in general, community samples are more metabolically active compared to their respective isolates (Supplementary Figure 3). A closer examination of the top carbon sources utilized by the cheese community samples revealed that there was large diversity of top carbon sources utilized. Cyclodextrin, tween-40 and Alpha-D-Lactose were the top carbon sources most frequently found, though they were the top carbon sources in only two cheeses each. None of the cheeses metabolized the phosphate-activated substrates (glucose-1-phosphate and alpha glycerol phosphate).

DISCUSSION

Cheese is an excellent model system for studying the mechanisms and patterns of microbial diversity because the microbial communities form under controlled and easily manipulated conditions. While the succession of the microbial community during the early curd and rind formation has been well characterized, the differences between the microbial composition of the mature rind and curd are not well understood, especially when compared between different cheeses (1, 10, 16, 20, 21). We characterized the microbial communities present within the mature rinds and curds of seven different natural rind cheeses using culture-independent, high-throughput Illumina sequencing and culture-dependent Sanger sequencing of the 16S rRNA gene. These seven cheeses vary in their geographic region of production and milk type used in the cheese-making process. Additionally, we examined the antibiotic susceptibility and carbon source utilization of the microbial communities in each of the cheeses.

We found that sampling site (curd or rind) was a strong predictor of community structure but milk type (cow, goat, or sheep), geographic origin, moisture content, and pH had little influence on the microbial community structure, in contrast to Wolfe and colleagues, 2014. It was found that rinds of all seven of the cheeses had greater microbial richness, measured as the number of unique OTUs, compared to the respective curds of the seven characterized cheeses (Figure 4B). The only phylum represented within the curds of all seven cheeses, except for that of Sonnet, was *Firmicutes*. *Streptococcus*, *Lactobacillus* and *Lactococcus*, which are genera commonly used as starting cultures during cheese production, were the most abundant genera in the curd and have been previously shown to dominate the curd even in cheese made without starter cultures (1, 6, 16, 17). In contrast, the rinds of these cheeses hosted community members from the *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phyla and, compared to the curd, had a greater relative abundance of the known rind colonizers *Brevibacterium* and Actinomyceteacae (49).

The differences in community composition and complexity between the rind and the curd is likely due to differential exposure to environmental conditions during ripening. While the curd is an anaerobic environment that is largely protected from environmental exposures, the rind is exposed to ambient air and is in direct contact with the surface on which the cheese is aged, providing opportunities for colonization and succession of the rind microbial community by secondary microorganisms from the environment. The colonizing microbes from the environment can influence characteristics of the rind such as pH that can influence further colonization and succession. For example, de-acidification of the rind by certain species of yeast facilitates the colonization and succession of microbes that prefer more alkaline environments such as *Corynebacterium*, *Brevibacterium* and *Brachybacterium*, in keeping with our observation of an increase in relative abundance of *Brevibacterium* in rind samples (27, 37).

ANTIBIOTIC ASSAYS

Another contributing factor to cheese community structure is the interaction between bacteria and fungi. In many cases, interactions among bacteria and yeast may prevent pathogens (27, 29), and opportunistic pathogens like *Staphylococcus aureus* from dominating the rind community and spoiling food (3). A major means of interaction involves the release of antimicrobial chemical compounds from one microbe to the other (23). From the antibiotic susceptibility assays, we observed that isolates taken from microbial communities in the rind and curd of six of the seven cheeses were resistant to a variety of antibiotics. In order to survive on the rind, bacteria most likely develop resistance mechanisms to these antibiotics and mycotoxins produced by species known to inhabit cheese, such as that of *Penicillium nalgiovense* (2, 8, 13, 18, 19, 24, 38).

Our limited analysis suggested that American cheeses overall have a higher susceptibility to antibiotics than do European cheeses. We speculate that the difference we observed could be ascribed to the different mechanisms by which European and American cheeses are aged. European cheeses are more commonly aged on older surfaces than American cheeses, such as wooden shelves or caves constructed centuries ago. Thus, the microorganisms on such surfaces could have had an opportunity to develop resistance over a longer period of time. The documentation of antibiotic resistance within cheese microbiota is a public health concern due to the possibility of a transfer of resistance to pathogenic bacteria in the human colon upon consumption (42). Consequently, an understanding of the stability, diversity, metabolism, and antimicrobial resistance of rind and curd microbiota would advance cheese production and safety.

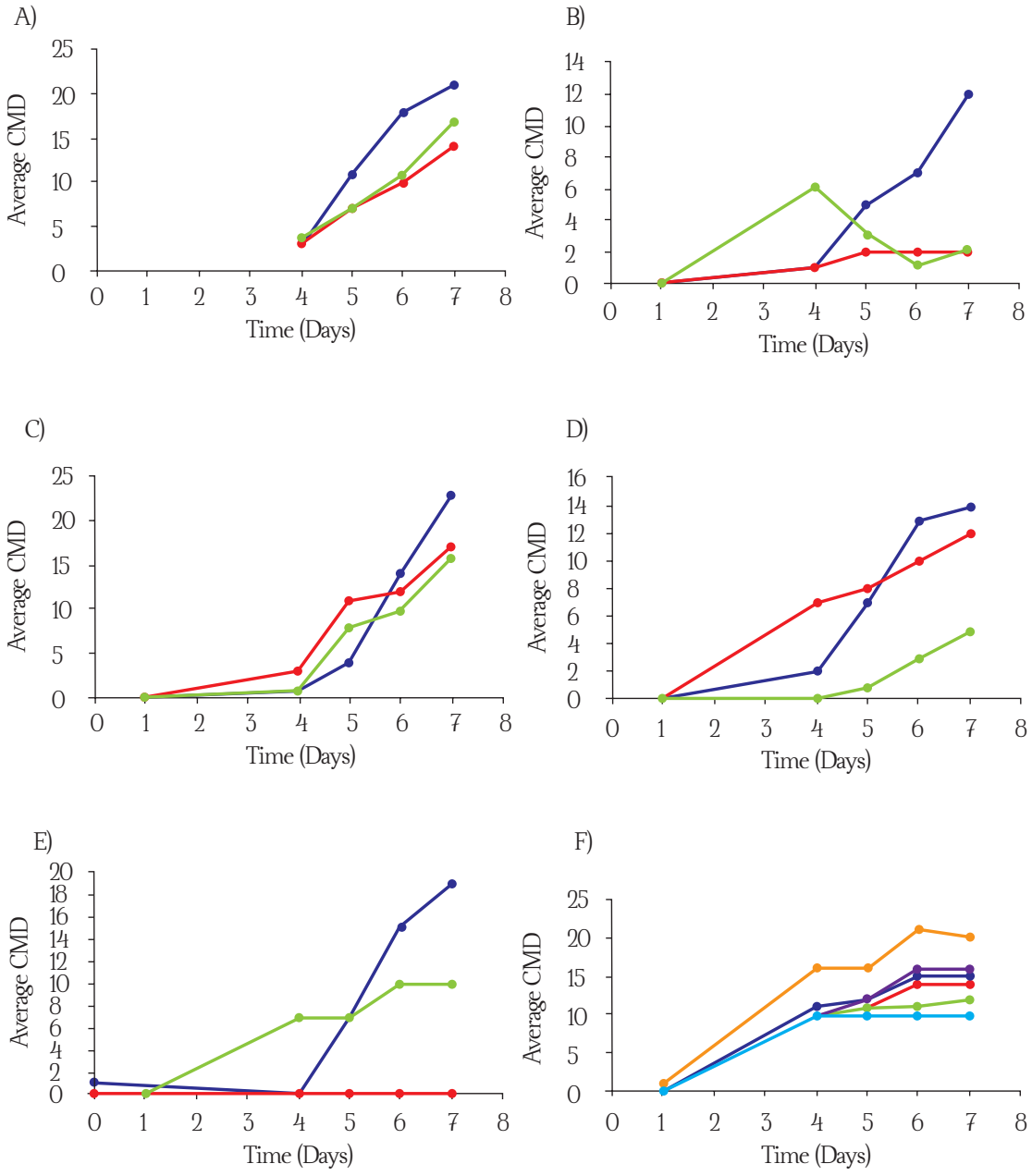
Supplementary Table 2

Cheese Community Sample
Isolate Sample
Curd Community Sample
Utilized Carbon Source

	MISSOURI TRUCKLE	*BRACHYBACTERIUM AIMENTARIUM*	*STAPHYLOCOCCUS WARNERI*	*ALPAGE GRUYERE*	*CORYNEBACTERIUM CASEI*	*BREVIBACTERIUM AURANTIACUM*	*COMTE*	*UNCLEAR*	*UNCLEAR*	*MAGGIE'S ROUNDS*	*BACILLUS MOJAVENSIS*	*UNCLEAR*	*STICHELTON*	*GREEN CURD*	*WHITE CURD*	*UNCLEAR*	*UNCLEAR*	*UNCLEAR*
Pyruvic acid methyl ester	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0
Tween-40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Tween-80	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1‡	1‡	0
Cyclodextrin	1	1	1	1	1	1	1	1	1	1	0	1	1*	1	0	0	0	0
Glycogen	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0
D-Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
alpha-D-Lactose	1*	0	0	1*	0	0	1	1	0	1*	0	0	0	1	0	1	0	0
Beta -Methyl-D-Dlucoside	1	1	1	0	1	1	0	1‡	0	0	1	0	0	0	0	0	0	0
D-Xylose	1	1	1	1	1	1	1*	0	0	1	1	1	0	0	0	0	0	0
I-Erythroitol	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1
D Mannitol	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1
N-Acetyl-D-Glucosamine	1	1	1	1	1	1	1*	0	0	1	1	1	0	0	0	0	0	0
D-Glucosaminic Acid	1*	0	0	0	0	0	0	1‡	0	0	0	1‡	1	1	1	1	1	1
Glucose-1-Phosphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-alpha-Glycerol Phosphate	0	0	0	0	0	0	0	1‡	0	0	0	0	0	0	0	0	0	0
D-Galactonic acid, gamma-Lactone	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
D-Galacturonic Acid	1	1	1	1	1	1	0	0	0	1*	0	0	1	1	1	1	1	1
2-Hydroxy Benzoic Acid	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-Hydroxy Benzoic Acid	0	0	0	1*	0	0	0	0	0	1*	0	0	1	1	1	1	1	1
Hydroxybutyric Acid	0	0	1‡	1*	0	0	0	1‡	0	0	0	0	0	0	0	0	0	0
Itaconic Acid	0	0	0	1*	0	0	0	0	0	1*	0	0	1	1	1	1	1	1
Alpha-Ketobutyric acid	0	0	0	1*	0	0	0	0	0	1*	0	0	0	0	0	1‡	0	0
D-Malic acid	0	0	0	1	0	1	1*	0	0	1*	0	0	1	1	1	1	1	1
L-arginine	1*	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
L-asparagine	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	1	1
L-Phenylalanine	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Serine	1	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1‡	1‡	0
L-Threonine	1	0	1	1	1	1	0	0	0	0	1‡	0	0	0	0	1‡	1‡	0
Glycyl-L-Glutamic Acid	1*	0	0	1	1	1	1*	0	0	1	0	1	1	0	0	1‡	1‡	0
Phenylethyl-amine	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1‡	0	0
Putrescine	0	1	1‡	1	1	1	1*	0	0	1*	0	0	0	0	0	0	0	0
TOTAL	*21*	*14*	*17*	*23*	*17*	*16*	*14*	*12*	*5*	*19*	*13*	*10*	*15*	*14*	*12*	*20*	*16*	*10*

** Carbon source utlized only in cheese community and/or curd community sample(s)*

‡ Carbon source utilized only in isolate sample(s)



Supplementary Figure 3. Carbon source utilization profiling shows that, in general, cheese community utilizes higher number of carbon sources at the end of the seven day sample period than the individual isolates, with the exception of two isolated from Stichelton. Blue = cheese community sample; Red = first isolate; Green = second isolate; A) Missouri Trucke; B) Vermont Shepherd; C) Alpage Gruyere; D) Comte; E) Maggie's ROund; F) Stichelton: Green Curd (Red), White Curd (Green), Yellow Isolate (Purple), White Isolate (cyan), Orange Isolate (Orange).

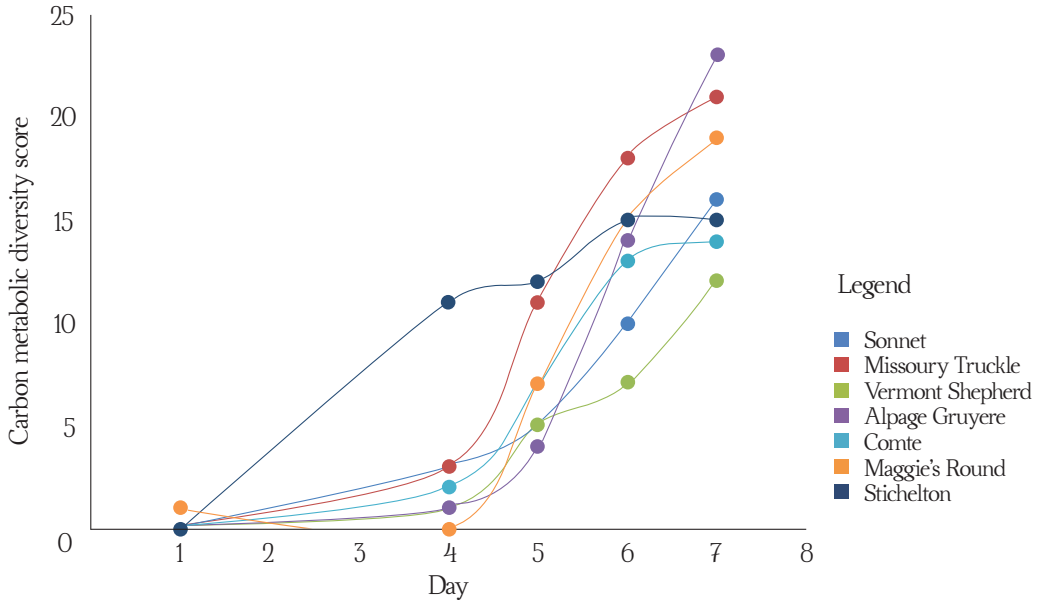


Figure 6. Diversity of carbon metabolism in cheeses over a seven-day period. Utilization of a carbon source was determined by measuring the reduction of tetrazolium salts WTS-1 and WTS-2 to fluorescent purple formazans with the BIOLOG Community-level physiological profiling (CLPP) kit and protocol. For a list of carbon sources see Supplementary Information.

CARBON SOURCE UTILIZATION

An alternative to measuring taxonomic diversity in a microbial community is measuring functional diversity, in this case the composite signature of various microbial metabolic pathways (34, 39). Community-level physiological profiling (CLPP) was conducted on microbiota and isolates from the rind and curd over a seven-day period using the BIOLOG EcoPlate™ assay (46). Sources included detergents, amino acids, and simple and complex sugars, among other compounds. The number of unique carbon sources utilized, here described as metabolic diversity (CMD), increased for all isolates and communities over time, albeit at different rates (Figure 6; Supplementary Figure 3).

It appeared that digestion by some enzymes occurred more quickly than others. Stichelton had the most unique metabolic signature, and was not able to metabolize any of the commonly used polysaccharides (cyclodextrin, xylose and N-acetyl-d-glucosamine). Perhaps this metabolic signature is related to the fact it is a blue cheese and there is contact with the mold inside the cheese. Further study is needed to elucidate a relationship between community composition and metabolic functions including mineralization. Various rind communities have shown to be largely culturable and reproducible (49) suggesting that such study is possible.

Table 2. Top carbon sources utilized by the cheese community samples and their respective isolates for five cheeses: Missouri Truckle, Alpage Gruyère, Comte, Maggie's Round, and Stichelton. Total CMD and Top Carbon Sources refer to that of the whole cheese microbial community samples; CMD in Isolates and Top Carbon Sources per Isolate refer to the individual isolate samples.

Cheese Type	*Isolate*	*Total CMD*	*CMD in isolate*	*Top Carbon Sources*	*Top Carbon Sources per Isolate**
Missouri Truckle	*Brachybacterium*	21	14	Glycogen Alpha-D-Lactose	D-Mannitol D-cellobiose Glycogen
	Staphylococcus	21	17	Glycyl-L-Glutamic	D-cellobiose D-mannitol Glycogen
Alpage Gruyère	*Corynebacterium*	23	17	Alpha-D-lactose Tween-80	D-cellobiose D-Mannitol N-acetyl glucosamine
	Brevibacterium	23	16		D-cellobiose Cyclodextrin Tween-80
Comté	(unclear) #3	14	12	D-cellobiose Cyclodextrin Tween-40	D, L-alpha-glycerol, Phosphate, Beta-methyl-D-glucoside, Cyclodextrin
	(unclear) #9	14	5		Tween-40, Cyclodextrin D-cellobiose
Maggie's Round	Bacillus	19	13	D-mannitol Cyclodextrin	Tween-80, Beta-mthyl-D-glucoside, D-mannitol
	unclear- #10	19	10	Tween-40	Cyclodextrin, Tween-40, N-acetyl-glucosamine
Stichelton	Green curd	15	14	D-Malic acid L-asparagine D-glucosaminic acid	D-malic acid, L-asparagine, D-glucosaminic acid
	Orange isolate Unkown		20		D-glucosaminic acid, D-malic acid, L-asparagine
	White curd		12		D-glucosaminic acid, D-malic acid, L-asparagine
	White isolate Unknown		10		L-asparagine, D-glucosaminic acid, D-malic acid
	Yellow isolate Unknown		16		D-glucosaminic acid, D-galactuonic acid, L-asparagine

Cheese communities maintained higher CMD than isolates (Supplementary Figure 3). It is likely that the purified isolates did not represent the majority of the community. Secondly, many isolates purified from cheese rinds were exposed to substrates typical of milk curds in the EcoPlate™ assay (Table 2). Rind microbes are not selected for in an environment with a predominance of substrates found in raw milk. Strikingly, in the Missouri Truckle cheese, isolates were able to digest two carbon sources that were inaccessible by the community (Supplementary Table 2). This suggests that those microbes that are best suited to aerobic growth on agar plates may not accurately represent the taxonomic or functional makeup of the community. Given that most CLPP of cheese microbes have used exclusively culture-dependent methods (44, 47) our findings suggest that future analyses of cheese community metabolism using CLPP should incorporate culture-independent methods in addition to culture-dependent methods.

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