Microbial and biochemical characterization of three artisan British cheeses throughout the maturation process

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Microbial and Biochemical Characterization of Three Artisan British Cheeses throughout the Maturation Process

Sabrina Longley,* Glenn Gibson, and Anisha Wijeyesekera

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ABSTRACT: Cheese is a very popular fermented dairy product that has potential gut health benefits. To gain insight into possible mechanisms behind such effects, this study aimed to characterize three distinct types of British artisan cheeses and to assess microbial and biochemical changes throughout maturation up to the point of consumption. The three types of cheese used in this study were a soft bloomy-rind cheese, a semisoft washed-rind cheese, and a semihard cheese aged in hay. Cheese samples were collected at different stages of maturation and characterized microbially and biochemically using 16S amplicon sequencing and ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy, respectively. Profound differences were found between the different types of cheese and between the same types of cheese at different stages of maturation in a way that shows physical progression as measured by their appearance and sensory characteristics. With these results, we can hypothesize the effects that their consumption might have.

KEYWORDS: *fermented dairy, ^{1}H NMR spectroscopy, cheese-omics, amplicon sequencing, cheese composition*

1. INTRODUCTION

Cheese is a widely consumed and very popular food product that has been historically used as a method of preserving nutrients present in milk. It is now produced all over the world in many varieties, with differences in appearance, texture, taste, and aroma. Cheese is a fermented product that relies on lactic acid bacteria (LAB) to convert the lactose present in milk into lactic acid and is essential for the cheese-making process as well as other derivatives, which have roles in forming the taste, texture, and aroma characteristics of different cheeses. LAB is found in unpasteurized milk, but for cheesemaking, it is added in sufficient quantities to ferment the milk. In addition, other ripening bacteria and fungi may also be included, for example, *Brevibacterium linens*, which forms the orange aromatic rind associated with washed-rind cheeses, and *Penicillium candidum*, which is an important ripening mold for white-rind cheeses. Many of the bacterial cultures used in cheesemaking have probiotic potential, such as *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*.[1−3] It is believed that the cheese food matrix comprising fat and protein can have a protective effect on the cultures within,[4] making it more likely that they may survive through the digestive tract in order to reach the large intestine and thereby making cheese a potential candidate for probiotic delivery to the lower gut.

Several in vivo studies have investigated the potential of cheese to influence the gut microbiota. Cheese-derived cultures have been detected in the feces of humans consuming daily doses of Camembert or Parmesan, both during the consumption period and persisting beyond.[5,6] In mice, consumption of an Emmental-style cheese during DSS-induced colitis resulted in a change in microbial structure compared to control, with a concurrent increased immune response and reduced overall tissue damage.[7] Similarly, mice with induced dermatitis fed cream cheese enriched with *Lactococcus chungangensis* CAU 28 had altered gut microbial populations and a modulated immune response resulting in reduced dermatitis scores.[8] Sequencing of cultures present in cheese could shed light on the presence of potential probiotics in mature cheeses that are ready for consumption and could have a positive impact on health.

Cheese is sometimes not perceived as a "healthy" food product due to its high fat and salt content; however, it has previously been shown to confer health benefits such as reducing the risk of cardiovascular disease, improving bone mineral density, and reducing the rate of all-cause mortality.[9−11] Mechanisms proposed for the purported benefits to cardiovascular health include the production of biologically active peptides by LAB used in cheesemaking, which prevents the production of angiotensin 2, a vasoconstrictor, thus reducing blood pressure.[12] The presence of calcium in cheese also plays a role in improving cardiovascular health by binding with bile acids, which necessitates bile acid regeneration from cholesterol in the liver, leading to reduced cholesterol in the bloodstream.[13,14] Furthermore, calcium reduces fat absorption in the small intestine by the formation of soaps, thus increasing fecal fat excretion; this is thought to be the mechanism by which researchers have seen a lower BMI and reduced weight gain across 5 years in people who regularly consume

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cheese.[13,15,16] By establishing the metabolic profiles of certain cheeses and their relationship to microbial communities, we may establish mechanisms for such purported health benefits.

Different aspects of cheese ripening can be studied with multiple methods of omics, from characterizing the microbiota using metagenomic approaches to transcriptomic analysis, proteomics, and even recent developments in lipidomics.[17] 16S rRNA amplicon sequencing has been used for the microbial profiling of 62 Irish artisanal cheeses, which established distinct differences between different types of cheeses (soft vs hard) and unique colonies present on the rind, some of which had not been previously identified in cheese.[18] Metabolomic methods involving gas chromatography and mass spectrometry have been applied for biochemical characterization of cheese, with the presence of certain metabolites linked to different flavor profiles and quality.[19] A technique has been developed using secondary electrospray ionization mass spectrometry (SESI-MS) which can predict the quality of different batches of Cheddar, a tool that could be utilized to help artisan cheese producers maximize the value and quality of each batch.[20] High-resolution magic angle spinning nuclear magnetic resonance spectroscopy (HR-MAS NMR) has been used to characterize metabolic changes associated with ripening for Parmigiano Reggiano cheese, where ripening time is a crucial factor for the organoleptic quality of the cheese and is positively correlated with price.[21] Other protocols, such as the use of infrared spectroscopy, have been developed as an easy screening process for the authenticity of traditional cheeses;[22] this is especially important for cheeses certified with a Protected Designation of Origin (PDO) status. It is also possible to combine multiple different methods using multivariate statistical analyses to visualize how different aspects of cheesemaking can impact the microbial and chemical profile; particularly, cheeses made by artisan producers can be distinguished from those made by large industrial producers.[23] Characterizing cheeses in this way can help to identify bioactive compounds produced by the microorganisms present in order to predict benefits to the consumer.[24]

In what has been termed "cheese-omics", a combination of characterization methods has enabled correlations between different sets of data. For instance, a large study conducted by Wolfe et al. (2014) involved high-throughput sequencing of 137 different cheeses from 10 different countries, finding 24 genera to be dominant community members among these cheeses, with distinct differences in communities divided by rind type. This study also conducted shotgun metagenomics on all samples to identify key metabolic pathways taking place in the different varieties of cheese, which could be correlated with the microbial communities identified.[25] However, many of the starter cultures used in the production of many cheeses already have probiotic potential, and by investigating the microbial and biochemical characteristics of these cheeses and how they mature, we can hypothesize how they may function in the human gut and thereby speculate about mechanisms for the health benefits that have been associated with their consumption.

Here, we assessed three varieties of cheese produced by the artisan cheese producer Nettlebed Creamery, Nettlebed, Henley, UK. The cheeses studied were Bix, a soft, bloomy-rind cheese, Highmoor, a semisoft, washed-rind cheese, and Witheridge, a semihard cheese aged in hay. These represent three distinct types of cheese with different maturation procedures, enabling a comprehensive analysis of how different styles of cheese can develop throughout aging.

Samples of these cheeses at different stages of maturation were analyzed by 16S rRNA amplicon sequencing for bacterial content and by ^{1}H NMR spectroscopy for metabolic profiling. This study represents an initial investigation into the composition and associated potential health benefits of these three cheeses.

2. MATERIALS AND METHODS

2.1. Cheese Samples

Cheese samples were collected during production at Nettlebed Creamery (Henley, UK) and stored at −20 °C until use, conditions that have previously been verified for chemical and microbial analysis.[26] Samples were taken from all three types of cheeses at different stages of maturation. In addition, a sample of Witheridge from a batch made with unpasteurized (raw) milk was also collected to assess differences between the same type of cheese made with pasteurized milk and raw milk. Raw materials and manufacturing for each cheese are summarized in Tables 1 and 2.

Table 1. Production Dates and Ages of the Cheese Samples, Which Were Collected on 13/12/22

cheese name	young (age)	midmaturation (age)	mature (age)
Bix	12/12/22 (1 day)	N/A[a]	05/12/22 (8 days)
Highmoor	12/12/22 (0 days)	30/11/22 (13 days)	24/11/22 (19 days)
Witheridge	08/12/22 (5 days)	28/07/22[b] (4.5 months)	12/05/22[b] (7 months)
Raw Witheridge	18/10/22 (8 weeks)		

[a]A "mid-maturation" sample for Bix was not collected as the maturation period for this cheese is only 8 days. [b]The midmaturation and mature samples of Witheridge had been treated with hay, but the young sample and the raw sample were not.

Nutritional analyses of composite samples pertaining to three batches of each type of cheese at the point of sale were conducted by Premier Analytical Services (High Wycombe, UK). Samples were sent fresh, packaged with ice to keep the temperature below 8 °C, and analyzed using UCAS-accredited in-house procedures. Saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids were calculated using a 0.956 conversion factor for nonfatty acid material in the fat. Carbohydrate content was calculated by the difference of sugars and higher-molecular-weight sugars. The total sugar value shown is the sum of the glucose, sucrose, fructose, lactose, and maltose levels measured.

2.2. 16S Amplicon Sequencing

Samples of cheese were sent to Novogene (Cambridge, UK) on dry ice for DNA extraction and 16S RNA sequencing. DNA was extracted using a Magnetic Universal Genomic DNA kit (DP341-T4A) (Tiangene). Extracted DNA was amplified using specific primers for the V3 and V4 regions of the 16S rRNA gene; primers used were 341F (5′-CCTAYGGGRBGCASCAG) and 806R (5′-GGACTACNNGGGTATCTAAT). PCR was carried out using 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 2 μM each of forward and reverse primers, and 10 ng of template DNA. The following cycling conditions were used: 98 °C for 1 min; 30 cycles of 98 °C for 1 min, 50 °C for 30s, and 72 °C for 30s, finishing with 72 °C for 5 min.

For DNA detection, PCR products were mixed with the same volume of 1× loading buffer with SYB green, and electrophoresis was run on a 2% (w/v) agarose gel. DNA was purified using a Qiagen Gel Extraction Kit (Qiagen, Germany).

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Table 2. Raw Materials and Production Procedures for Three Cheese Types

	raw materials	starter cultures	cheesemaking steps and conditions	ripening conditions
Bix	milk (pasteurized)	*Lactococcus lactis* subsp. *cremoris*	starters added 35 °C	daily turning for 3 days and a final turn at 7 days post production
	double cream (10%)	*Lactococcus lactis* subsp. *lactis*	rennet added 34 °C	storage at 18−20 °c for 4 days
	rennet (3.2 mL/10L milk)	*Lactococcus lactis* subsp. *lactis biovar. diacetylactis*	set 5× flocculation time	storage at 11 °C for 4 days
	salt (dry) (3.5 g/cheese)	*Leuconostoc pseudomesenteroides*	cut size 2.5 cm^2	pack at 8−9 days postproduction
		Dabromyces hansenii	45 min stir	
		Geotrichum candidum	hand ladle	
		Penicillium candidum	overnight drain	
			unmolding and salting by hand	
Highmoor	milk (pasteurized)	*Lactococcus lactis* subsp. *cremoris*	starters added 35 °c	warm aging (18 °c) 4 days
	rennet (3 mL/10L milk)	*Lactococcus lactis* subsp. *lactis*	rennet added 36 °c	cold aging (11 °c) 17 days
	salt (80% brine) (45 min)	*Lactococcus lactis subsp. lactis biovar. diacetylactis*	Set 5× flocculation time	5× brine wash with 3% brine
		Leuconostocpseudomesenteroides	cut size 0.5 cm^2 sq	pack at 18−21 days post production
		Streptococcus thermophilus	1 h stir	
		Lactobacillus delbrueckii subsp. *bulgaricus*	ladle temperature 36.5 °c	
		Brevibacterium spp.	ladled by gravity into a distributer	
		Brachybacterium spp.	2 h drain	
		Corynebacterium spp.	unmolding and brining	
		Hafnia alvei		
		Microbacterium spp.		
		Staphylococcus		
		Propionibacterium freudenreichii		
Witheridge	milk (pasteurized[a])	*Lactococcus lactis* subsp. *cremoris*	starters added 35 °c	cold aging (10−12 °c)
	rennet (2.8 mL/10L milk)	*Lactococcus lactis* subsp. *lactis*	rennet added 36 °c	vac-pack in hay at 7 days
	salt (80% brine) (18 h)	*Lactococcus lactis* subsp. *lactis biovar. diacetylactis*	set 2.5× flocculation time	weekly turning
	hay[a] (7.5 g/cheese)	*Leuconostoc pseudomesenteroides*	cut size 1 cm^2 sq	unwrap at 8 months
		Streptococcus thermophilus	1−1.5 h stir	addition of further dry hay
		Lactobacillus delbrueckii subsp. *bulgaricus*	ladle temperature 45 °c	pack 4 weeks later
			ladled by gravity into a distributer	
			2 h drain	
			unmolding and brining	

[a]The Raw Witheridge sample used in this study underwent the same production procedures; however, it used unpasteurized milk, and no hay was used.

DNA was prepared for sequencing with a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA). The prepared sequencing libraries were verified with Qubit and real-time PCR for quantification and a bioanalyzer for size distribution detection. Sequencing was then performed on an Illumina sequencing platform.

2.3. Sequencing Data Analysis

Paired-end reads were truncated by cutting off the primer sequence and then merged using FLASH (V1.2.11)[27] to create raw tags. Quality filtering on the raw tags was performed with fastp (Version 0.23.1) to create clean tags.[28] These were compared with the Silva database (16S) using the UCHIME algorithm for chimera sequence detection. Chimera sequences were removed,[29] and effective tags were obtained. Uparse (V7.0 1001) was used for sequence analysis.[30] Sequences with ≥97% similarity were assigned the same number of OTUs.

Species annotation of the OTUs was performed using the Silva database with the Mothur algorithm.[31] MUSCLE software (V 3.8.31) was used for multiple sequence alignment to determine phylogenetic relationships between OTUs and species dominance.[32]

Alpha diversity, including observed species, Chao1, Shannon, Simpson, ACE, and good-coverage, and beta diversity were calculated with QIIME (V 1.9.1). Data visualization was conducted using R software (Version 4.0.3).

2.4. ^{1}H NMR Preparation

Cheese samples were freeze-dried in a ScanVac Cool Safe (Labogene) for 5 days at −110 °C and at an average pressure of 1.7 mbar and subsequently stored at −20 °C until further use, an adaptation of a method previously used for the analysis of cheese samples.[33] A 20 mg amount of sample was agitated with 1 mL of D_2O and allowed to dissolve overnight at 4 °C. Amicon Ultra Centrifugal Filters (50 kDa) were used to separate larger molecular weight components from the samples and were first primed for use by washing through with 1 mL of D_2O (centrifuged at 12,175*g* for 5 min and then repeated, followed by another 1 mL of D_2O for 5 min and finally 10 min). Cheese samples were then added to centrifugal filters and centrifuged at 12,175*g* for 30 min at 4 °C. A 200 μL portion of filtrate was mixed with 400 μL of sodium phosphate buffer (comprising 0.2 M Na_2HPO_4, 0.04 M NaH_2PO_4, 1 mM sodium 3-(trimethylsilyl)-

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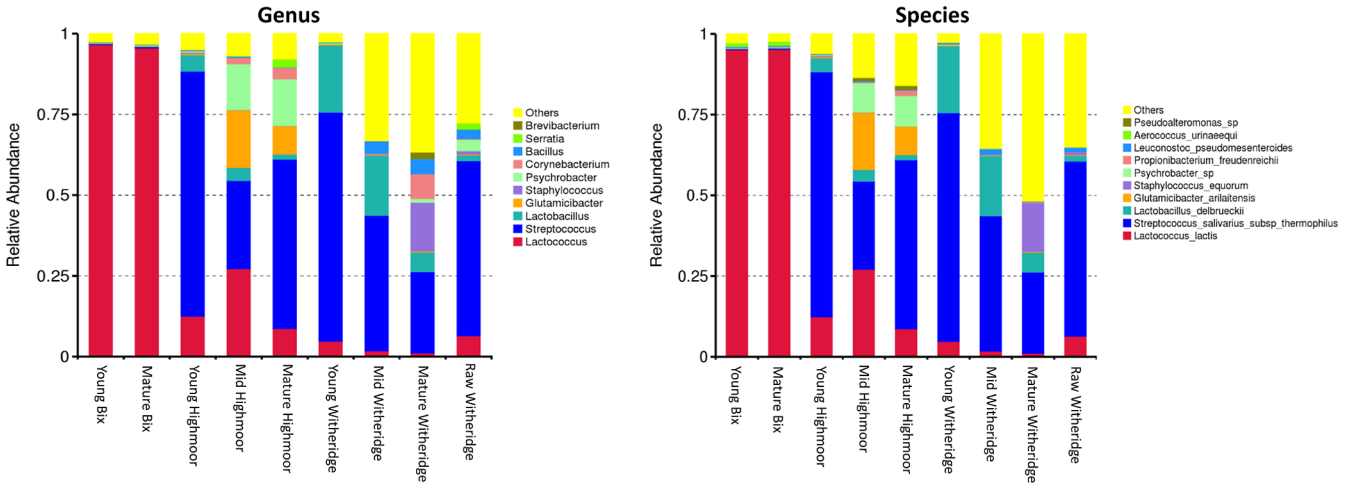

Figure 1. Relative abundance bar charts for genus and species of bacteria present in samples of the three cheeses: Bix (soft, mold-ripened cheese), Highmoor (semisoft, washed-rind cheese), and Witheridge (semihard cheese aged in hay). Data for each cheese at different stages of aging is shown to illustrate the changes in microbial profile as their rind and flavor develop. A sample of Witheridge cheese made with unpasteurized milk is shown and labeled as "Raw Witheridge". A "Mid"-maturation sample of Bix is not included as this cheese only matures for 8 days.

Table 3. Nutritional Analysis of Three Varieties of Cheese at Point of Sale

		unit	Bix	Highmoor	Witheridge
energy		kJ/100g	1612	1407	1645
energy		kcal/100g	391	339	397
fat		g/100g	38.2	29.4	33.6
of which	saturates	g/100g	25.2	32.7	21.7
	monounsaturates	g/100g	9.8	5.3	8.9
	polyunsaturates	g/100g	1.2	0.7	1.1
carbohydrate	g/100g	<0.1	<0.1	<0.1	
of which	total sugars	g/100g	<0.1	<0.1	<0.1
	starch	g/100g	<0.1	<0.1	<0.1
dietary fiber		g/100g	1.4	<0.5	0.5
protein		g/100g	13.2	19.2	23.5
omega-6		g/100g	0.9	0.6	0.7
omega-3		g/100g	0.5	0.2	0.5
salt (from sodium)		g/100g	1.26	1.42	1.96
ash		g/100g	4.1	2.7	4
moisture		g/100g	45.3	49	38.4
sodium		g/100g	0.5	0.57	0.78

[2,2,3,3−$2H_4$] propionate (TSP), and 3 mM NaN_3 dissolved in 1 L of 100% D_2O, pH 7.4). TSP was used as an internal chemical reference standard. A 600 μL portion of solution was then transferred into NMR tubes (5 mm — Wilmad LabGlass, UK), and samples were analyzed using a Bruker Avance III 500 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). Standard 1D spectroscopic data were acquired using a "noesypr1d" pulse sequence with a relaxation delay of 3 s. Water suppression was achieved through presaturation during the relaxation delay and mixing time. ^{1}H NMR spectra were collected with 128 transients (and eight dummy scans) in 32k data points with a spectral width of 14 ppm. Spectra were preprocessed using the built-in software (TopSpin 4.2.0) and referenced to the TSP chemical shift (0 ppm). Data were overlaid in Topspin and also imported into the Chenomx software package (Chenomx Profiler 9.0) for qualitative analysis of metabolite differences between the different cheese samples with reference to the concentration of TSP.

3. RESULTS AND DISCUSSION

3.1. Nutritional Analysis

Results of the comprehensive nutritional analysis of the cheeses are listed in Table 3. The low levels of carbohydrate (below detection) in all three cheeses suggest that lactose was metabolized by LAB during production and maturation. The elevated fat content in Bix (8.8g/100g above Highmoor and 4.6g/100g above Witheridge) is due to the addition of double cream to the milk during the cheese-making process. Bix has a resultant higher fat/protein ratio of 2.89 compared to the relatively similar 1.42 and 1.53 ratios for Witheridge and Highmoor, respectively.

Also of note is the fiber content; Witheridge has only 0.5g/100g of fiber, which is surprising given the presence of hay on the rind of the cheese, especially when compared to the 1.4g/100g of fiber present in Bix, which has no hay or any other fibrous additive. The fungus *P. candidum*, also known as *Penicillium camemberti,* is used during Bix production to form the soft white rind and has been shown to produce chitin, a dietary fiber.[34] It has been shown that chitin can induce type 2

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immune responses in the gut and can alter bacterial composition, giving it potential as a prebiotic.[35] This could imply that the rind on Bix has prebiotic properties and could be beneficial for gut microbiota.

3.2. Changes in Bacterial Populations with Maturation

The three types of cheeses were sampled at different stages of maturation. The DNA extracted was sequenced to identify populations of bacteria present in each. Figure 1 shows the relative abundance of groups of bacteria at the genus and species phylogenetic levels.

3.2.1. Bloomy-Rind Soft Cheese (Bix). The bacterial population in Bix was dominated by *L. lactis* at both stages of maturation; Bix is aged for 9 days before packaging and sale and therefore has the least amount of time among these three cheeses for the development of a more complex microbiota. *L. lactis* and *Leuconostoc pseudomesenteroides*, which are only shown here in very small abundance, are the only bacterial cultures added to the milk at the start of the production procedure; the other starter cultures used are different varieties of yeasts and molds; Bix is a mold-ripened cheese, and fungal DNA was not measured in the sequencing method used in this analysis; therefore, it is possible that Bix has more microbial complexity with yeast and mold populations that were not detected in this study. *L. lactis* is a species with recognized probiotic potential, and therefore, this cheese could be a good candidate for probiotic effects once consumed, depending on the abundance of fungi (namely, *Geotrichum candidum*, *P. candidum*, and *Debaryomyces hansenii*, which are used in the production of Bix) relative to the abundance of *L. lactis*.[1]

3.2.2. Washed-Rind Semisoft Cheese (Highmoor). Highmoor showed a dramatic increase in diversity between the young sample and the midmaturation sample; this was likely due to growth in rind bacteria that occurred during the maturation process. As might be expected, in the young sample, we saw a dominance of *L. lactis*, *S. thermophilus*, and *L. delbrueckii*, all of which are starter lactic acid bacteria (SLAB) cultures added to the milk at the start of the cheese-making process. They are instrumental in fermenting lactose and decreasing the pH when cheese is made. Once the cheese has passed the initial days of aging and the rind-washing process begins, other species involved in the expression of the key characteristics of this cheese begin to increase in relative abundance: bacteria of the genera *Brevibacterium* and *Corynebacterium* and the species *Staphylococcus equorum* and *Glutamibacter ariliatensis*, as indicated in Figure 1. These cultures are added to the milk to create the desired rind for a washed-rind cheese, which is characterized by a pink-orange color and dry but tacky texture, with distinct tastes and aromas. *Propionibacterium freudenreichii* was also indicated in the species relative abundance plot; this bacterium is another culture specifically added to the cheese for its role in producing nutty and sweet flavors and also in the formation of holes or "eyes" in the cheese as a result of the production of carbon dioxide by heterolactic acid fermentation.[36] *Psychrobacter* sp. and *Pseudoalteromonas* sp. are halotolerant bacteria usually associated with marine environments; however, they have previously been identified in cheeses and specifically isolated from brine baths used to salt these cheeses, which is likely to be the origin of these cultures present in the Highmoor samples.[25] The other identified species present in Highmoor are not recognized as being added intentionally and are therefore likely to be contaminants either from the environment or from humans due to the handmade artisan nature of this cheese.

3.2.3. Mature Semihard Cheese Aged in Hay (Witheridge). The SLABs used for Witheridge are *S. thermophilus*, *L. delbrueckii*, and a mesophilic cocktail of cultures largely consisting of *L. lactis* subspecies and *Leuconostoc*sp., which is reflected in the bacterial profile of the young Witheridge sample. The biggest difference in terms of the cheese-making process between the young and midmaturation samples is the addition of hay to the surface of the cheese at 1 week of age; the cheeses are vacuum-packed in hay at 1 week old and allowed to mature anaerobically with the hay for six months before being opened, rubbed in dry hay, and allowed to finish aging. The hay adds grassy flavor notes, which are enhanced by long anaerobic fermentation. The use of hay also helps with rind development by drying the surface of the cheese to create a thin, dry, and aesthetically pleasing rind. While the hay is treated to remove contaminants, it appears to have a role in the growth of other bacteria, which may be important for flavor formation. This was reflected in Table 4, showing the number of observed species in each sample, where there was a 3.8-fold increase between young and mature Witheridge with the greatest diversity seen across all indices for mature Witheridge. These species are thought to be those represented in the "others" band in Figure 1, which was also seen to triple in size between young and midsamples of Witheridge. As the maturation of cheese with hay is an unusual affinage technique, information as to the nature of the effect of hay on cheese and gut microbiota is scarce. It is known that consumption of hay as opposed to fresh forage can have a sensory and chemical impact on milk and cheese produced by cattle[37−40] and that consumption of alfalfa hay can positively modulate the yak calf and lamb gut microbiota.[41−43] Barley straw can be processed in a way to extract xylooligosaccharides that have been shown to stimulate SCFA production in an in vitro human gut microbiota fermentation, raising the possibility that barley

Table 4. Alpha Diversity Indices Indicating the Number of Total Species Observed in Addition to Shannon, Simpson, Chao1, and ACE Diversity Indices[a]

sample	observed species	Shannon	Simpson	Chao1	ACE
Young Bix	230	0.510	0.095	410.233	470.136
Mature Bix	300	0.540	0.093	559.592	613.321
Young Highmoor	450	1.660	0.408	679.780	767.714
Mid Highmoor	474	3.153	0.808	711.541	703.852
Mature Highmoor	247	2.679	0.696	377.500	427.055
Young Witheridge	309	1.448	0.453	452.000	494.085
Mid Witheridge	1168	4.613	0.787	1338.104	1355.191
Mature Witheridge	1183	5.553	0.901	1390.750	1389.222
Raw Witheridge	1082	4.233	0.699	1283.471	1305.290

[a]Shannon diversity indicating species richness together with evenness to represent the entropy in a sample. Simpson diversity represents the probability of two species in a sample at random to be different. Both Chao1 and ACE calculate species richness with respect to rare species in the sample which are unobserved in sampling.

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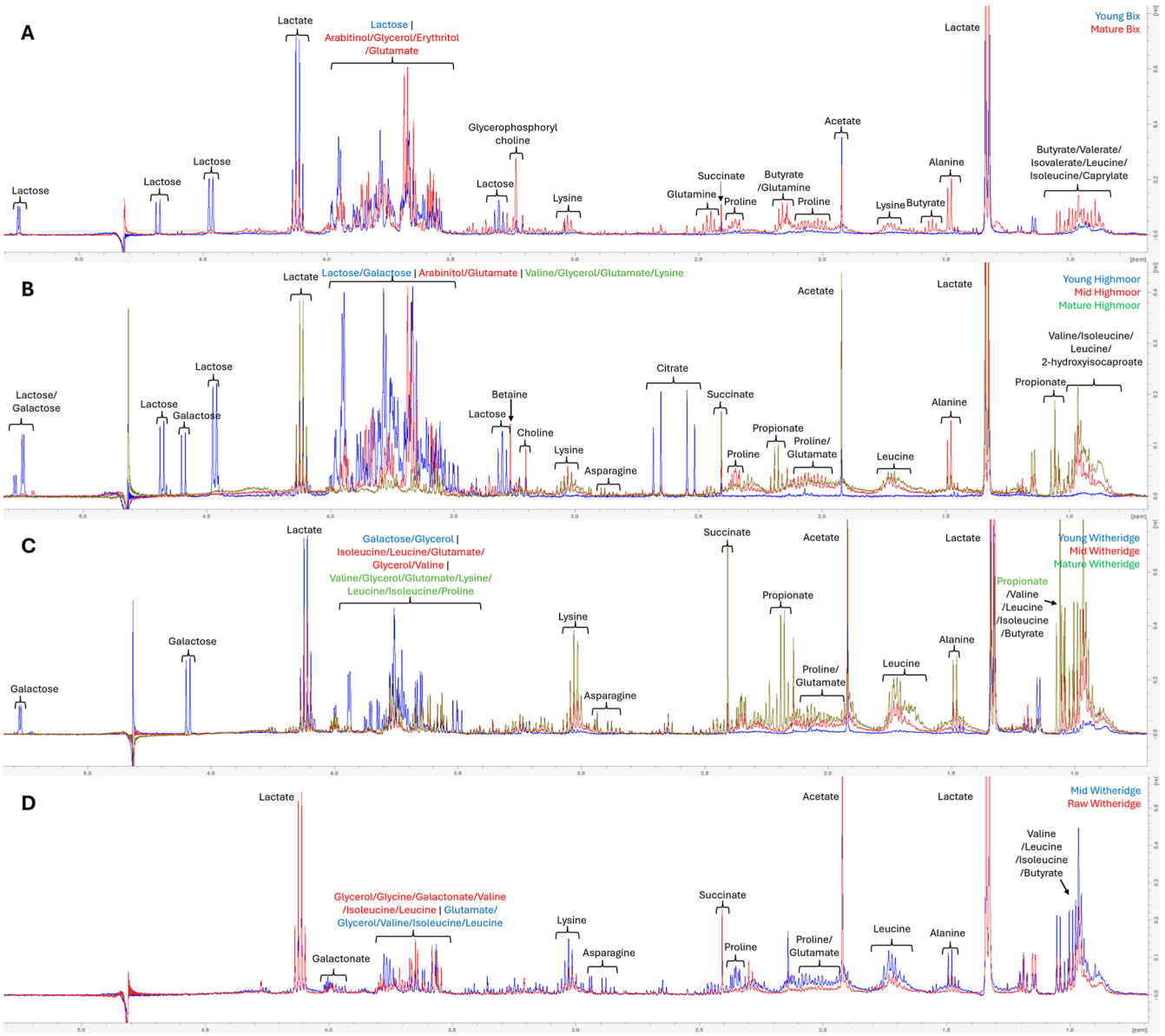

Figure 2. One-dimensional ^{1}H NMR spectroscopic data showing changes in the metabonomic profile at different stages of maturation ("Young", "Mid", and "Mature") of three cheeses: Bix, a soft, mold-ripened cheese (panel A); Highmoor, a semisoft, washed-rind cheese (panel B); and Witheridge, a semihard cheese aged in hay (panel C). The "mid-maturation" sample of Witheridge is also compared with a sample of Witheridge made with unpasteurized milk ("Raw Witheridge") (panel D). A "Mid"-maturation sample of Bix is not included as this cheese only matures for 8 days. Colors of each spectrum are relative to the age of the sample, as indicated by the key on the top right of each panel. In areas of overlapping peaks where distinction between the spectra is unclear, the text labels are colored relative to the color of the spectra they are describing.

grasses may confer benefits in the human gut, although this study used steam explosion (autohydrolysis), which is, of course, not physiologically relevant when considering the consumption of hay or straw.[44] As hay is a source of protein and fiber, we hypothesize that it stimulates the growth of bacteria that do not otherwise thrive in the cheese environment, particularly during the period where the hay was fermenting on the surface of the cheese anaerobically. Therefore, despite being expressed as a low fiber content (0.5g/100g, Table 3), hay increases the diversity of bacteria present in Witheridge, with possible resultant effects from consumption of the cheese on the gut microbiota.

Another difference to consider is between the midmaturation Witheridge and the unpasteurized (raw) Witheridge sample; though similar in age, the Raw Witheridge sample had no hay added, and therefore, the large number of observed species (Table 4) and significant proportion of bacteria labeled as "other" in the relative abundance chart (Figure 1) were likely due to the presence of populations of bacteria present in the milk that survive, in this case, due to a lack of pasteurization.

3.3. Changes in Metabolite Composition

Cheese samples were analyzed using ^{1}H NMR spectroscopy for the assessment of biochemical composition. Figure 2 illustrates the distinct differences among young, mid-, and mature cheeses of the same type. There were profound changes in metabolic profiles as all cheeses transitioned from young to later stages; the presence of lactose was very prominent in young Bix and Highmoor samples and yet was not present at all in the mid and mature samples. This is likely

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due to the fermentation of lactose into lactate by the glycolytic pathway undertaken by LAB. The concentration of lactate reduced by 0.32 and 0.49 mM as Bix and Witheridge matured; this was not the case for Highmoor, where lactate increased by 0.22 mM in the mature sample compared to the young sample (Table 5). Lactose can be fermented into lactate by homolactic acid fermentation, but when fermented by heterolactic acid fermentation, acetate is also produced.[45] Hence, we saw increases in acetate as the cheeses aged; however, this was not seen as clearly in Highmoor. A systems biology approach modeling lactose fermentation by three different starter cultures in a cheese has demonstrated how different conditions in the cheesemaking and aging process can influence the lactose metabolic pathway;[46] thus, conditions during the making and aging of Highmoor may favor homolactic acid fermentation, resulting in higher lactate concentrations but lower acetate concentrations.

Table 5. Concentrations of Lactate in Each of the Samples, Calculated from NMR Spectra[a]

cheese	lactate concentration (mM)		
	young	mid	mature
Bix	0.52		0.20
Highmoor	0.15	0.12	0.36
Witheridge	0.56	0.35	0.07
Raw Witheridge	0.39		

[a]Shown to 2 decimal places.

Butyrate increased in concentration in both Bix and Witheridge; however, this was not seen in Highmoor. Butyrate can be produced by clostridia in cheese, which is a common contaminant derived from silage consumption by cows, and the spores can survive pasteurization; this can result in "late-blowing" or spoilage of cheese[47] and is possibly the source of the butyrate observed here.

In Highmoor cheese, *P. freudenreichii* is used as a starter culture (and, as shown in Figure 1, was detected using microbial profiling). This bacterium ferments lactate into propionate as well as acetate;[51] hence, we also observed the presence of the former (as shown in Figure 2) in our study. Propionate was also found in the mature Witheridge samples, which, despite *P. freudenreichii* not being a starter culture for this cheese, is not surprising given its presence in the sequencing results for this sample, likely due to cross-contamination during the production process. Relative levels of propionate in the mature Witheridge sample were greater than that in the mature Highmoor (Table 6), which could be due to a longer maturation time of Witheridge, giving the propionibacteria more time for lactate fermentation and propionate production, which has been shown to continue through to the end of maturation.[51]

Succinate is a product of lactose fermentation by *P. freudenreichii*,[46] alongside propionate, which explains a higher concentration of succinate in the Witheridge sample relative to the other cheeses (Table 6). Succinate is also a product of the metabolism of dietary fiber,[52] and its presence in this cheese could be a result of the fermentation of the hay on the rind of Witheridge by the cultures within.

Galactose was present in the Young Highmoor and Witheridge samples (Figure 2). This is a residual sugar present in cheese similar to lactose; it is also an intermediate sugar in the breakdown of lactose by LAB.[53,54] As the young Witheridge sample was 5 days old at the point of sampling, as opposed to one and zero days old for Bix and Highmoor, respectively, a lack of lactose present in the young Witheridge sample, concurrent with the presence of galactose, could indicate that lactose fermentation was already taking place.

Table 6. Comparison of Concentrations (Shown in mM) of Notable Short-Chain Fatty Acids and Amino Acids Present in the Mature Samples of Each Cheese, Calculated from NMR Spectra[a]

metabolite	Bix	Highmoor	Witheridge	Raw Witheridge
acetate	0.0167	0.374	0.1276	0.0831
butyrate	0.0152	0	0.0082	0
formate	0.0011	0.0121	0.0034	0.0053
propionate	0	0.0317	0.1082	0
succinate	0.0049	0.0092	0.029	0.0096
lactate	0.2009	0.3626	0.073	0.3914
asparagine	0	0.0149	0.0232	0.0091
glutamate	0.0385	0.0117	0.1201	0.0065
glutamine	0.0524	0.0068	0.267	0.0047
isoleucine	0.0073	0.042	0.029	0.0037
leucine	0.0168	0.0305	0.0846	0.0291
lysine	0.0203	0.0121	0.0868	0.0108
proline	0.0397	0.007	0.0291	0.0089
valine	0.109	0.0095	0.0481	0.0138

[a]Shown to four decimal places.

Citrate, which is naturally present in milk, was only seen in the young Highmoor sample; its absence in the mid and mature samples of Highmoor was likely due to utilization of citrate by *L. lactis*. Citrate utilization is most efficient at pH values below 6,[55] and as Highmoor has the highest starting pH of all three cheeses (5.8 as opposed to 5.6 for Witheridge and 4.8 for Bix), it is possible that the initial utilization of citrate was slower, which would explain the absence of citrate in the young samples for Bix and Witheridge. A reduction in citrate throughout cheese maturation has previously been reported and linked to citrate-fermenting organisms, such as *L. lactis* subsp. *lactis* and *Leuconostocpseudomesenteroides*, as well as to the citric acid cycle.[56]

The presence of amino acids leucine, isoleucine, asparagine, valine, glutamate, proline, and lysine was negligible in all of the young cheese samples but could be seen to increase with age; this has previously been seen in other studies and is a key part of cheese maturation.[57] Primary proteolysis of casein in milk is known to take place due to the pepsin and chymosin present in rennet, and secondary proteolysis occurs with the production of peptidases by bacteria present during cheesemaking and ripening. LAB, particularly *L. lactis* but also *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, produce peptidases to break down casein peptides into amino acids for their own catabolism.[58] In almost every amino acid measured, Witheridge had the highest concentration (Table 6); this could be due to the reduced moisture content of this cheese, which will increase the relative concentration of protein.

Glycerophosphoryl choline was found in the mature sample of Bix. This was not found in any of the other cheeses; however, choline and betaine, which is a choline derivative, were found in Highmoor. Choline is an essential nutrient for lipid transport and brain function and is common in dairy products.[59]

Glycerol, which was found in six of the nine samples, is a product of lipolysis by LAB. Arabinitol, also known as arabitol,

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was found in both mature Bix and mid-Highmoor samples. Arabinitol is usually known for its production by *Candida* species, but it is also produced by a number of other yeasts including *D. hansenii*,[60] which is used as a starter culture for Bix to aid in rind formation. The presence of arabinitol in mid-Highmoor could indicate growth of *D. hansenii* on the rind.

The results from this study provide new insight into the bacterial and biochemical profiles of three types of British cheeses and how they change as they mature. We observed that populations of starter cultures added to the cheeses were sustained throughout aging; however, relative abundances changed as the cheeses matured to reflect developing characteristics. By understanding profiles of the cheeses at the point of consumption, we can also start to hypothesize potential effects on the human gut microbiota. *L. lactis* is a bacterial species with recognized probiotic potential, and while it does persist throughout aging of all three cheeses, its relative abundance in mature Highmoor and Witheridge was low (<10% in mature Highmoor and <5% in Witheridge). This was not true for *S. thermophilus*, which remains dominant until the point of maturation; this bacterium, along with *L. delbrueckii*, is routinely used as a yogurt starter culture and also has recognized probiotic potential with beneficial effects on the consumer including utilization of carbohydrates and folate synthesis, as well as exerting anti-inflammatory and antioxidant properties.[61] *P. freudenreichii* also has potential as a probiotic bacterium, and the environment that the cheese matrix provides has been shown to support its growth and protect it from digestive stresses.[51,62] Propionic acid has also been shown to have antimicrobial and anti-inflammatory properties, suggesting that its presence in Highmoor and Witheridge could be of benefit.[63] Propionate also has benefits due to its role in reducing lipogenesis and cholesterol synthesis as well as regulating appetite.[64,65] The presence of succinate, especially in comparatively high concentrations in Witheridge, is possibly a cause for concern, as high succinate concentrations in the gut have been associated with dysregulated inflammation and the promotion of the growth of succinate-consuming pathogens such as *Clostridium difficile* and *Salmonella typhimurium*.[52] However, succinate can also be used in cross-feeding and promote the growth of other succinate consumers, such as *Phascolarctobacterium succinatutens*, that metabolize succinate into propionate,[66] which has health benefits as described above. It is also thought that succinate has an important role in immune regulation, which in moderate quantities can have a protective effect via increased elimination of pathogens. It is only when accumulation of succinate becomes excessive that this is a pathological issue.[52]

This study utilized 16S amplicon sequencing, which has been previously used extensively for bacterial profiling of cheeses,[18,67−69] and while this is an effective method, it can allow for the detection of DNA from dead bacterial cells. This could mean that any probiotic bacteria that we observed in the mature cheeses may not impact the gut microbiota as they may not be alive. However, these findings provide a basis for understanding these cheeses and allow further study into effects that these cheeses may have when consumed, such as the role of the cultures present as probiotics in the gut microbiota and that of the metabolites in modulating the gut environment.

What was not explored in this study is the differences between the bacterial and metabolic profiles of different parts of the cheeses (e.g., rind, under-rind, and core segments), as previous studies have addressed.[18,25,68,70] In this study, the whole cheese was used in each part of the process so that results reflected the bacterial and biochemical profiles of these cheeses as a whole and were proportional to the nature of the rind/core distribution. This is a possible avenue for future research in terms of the ripening process of these cheeses. However, in terms of consumption of cheeses such as these with a natural rind, the entire cheese will be eaten, and thus, there is less of a requirement to distinguish possible effects. Additionally, it has previously been noted that differences in the season of production result in vast differences in the quality of the milk, which can impact the microbial and chemical profiles of cheeses;[23] this study did not have the scope to consider this, and a larger-scale study could better compare mature cheeses that had been made at different points in a year.

AUTHOR INFORMATION

Corresponding Author

Sabrina Longley − *Food Microbial Sciences Unit, Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6LA, U.K.;* orcid.org/0009-0006-4293-4698; Phone: +44 7587 142910; Email: s.i.c.longley@pgr.reading.ac.uk

Authors

Glenn Gibson − *Food Microbial Sciences Unit, Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6LA, U.K.*

Anisha Wijeyesekera − *Food Microbial Sciences Unit, Department of Food and Nutritional Sciences, University of Reading, Reading RG6 6LA, U.K.;* orcid.org/0000-0001-6151-5065

Complete contact information is available at: https://pubs.acs.org/10.1021/acsfoodscitech.5c01243

Author Contributions

Sabrina Longley (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing—original draft, Writing—review and editing), Anisha Wijeyesekera (Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing—review and editing), and Glenn Gibson (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing—review and editing).

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ABBREVIATIONS USED

^{1}H NMR, proton nuclear magnetic resonance spectroscopy
BMI, body mass index
DNA, deoxyribonucleic acid
HR-MAS NMR, high-resolution magic angle spinning nuclear magnetic resonance spectroscopy
LAB, lactic acid bacteria
PCR, polymerase chain reaction
PDO, protected designation of origin
SCFA, short-chain fatty acid
SESI-MS, secondary electrospray ionization mass spectrometry
SLAB, starter lactic acid bacteria

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