

Functional Profiling of *Brevibacterium* in Traditional Bulgarian Green Cheese by Shotgun Metagenomic Sequencing

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Abstract

The distinct sensory properties of artisanal cheeses are defined by unique microbial communities and the key compounds they produce during maturation. Traditional Bulgarian green cheese is produced only in the village of Cherni Vit and was previously shown to contain a high abundance of *Brevibacterium*. To better understand the functional role of this genus, we performed shotgun metagenomic sequencing of a green cheese sample. We find that the dominant microorganisms are various species from the genus *Brevibacterium* (51%), most notably *B. aurantiacum* (29%). Although it has a much lower abundance, the actinobacterial genus *Brachybacterium* (2%) also makes a significant contribution to the community's metabolic activity. Functional profiling suggests that *Brevibacterium* is a major producer of amino acids such as Phe, Arg, and Lys, as well as cofactors and vitamins, including B5 and B6, and lipoic acid. We also found the mold *Aspergillus puulaauensis* (3%) plays a key role in both lipid and amino acid metabolism within the community, despite its low abundance. No pathogens were present, but genes and plasmids encoding antibiotic resistance were detected at low concentrations. We found that green cheese consumption is safe, and that the microorganisms within it have the potential to synthesize valuable secondary metabolites.

Keywords: shotgun metagenomic sequencing, artisanal cheese, functional profiling

Резюме

Отличителните сензорни свойства на занаятчийските сирена се определят от уникални микробни съобщества и ключовите съединения, които те произвеждат по време на зреенето. Традиционното българско зелено сирене се произвежда само в село Черни Вит и преди това е доказано, че съдържа високо количество *Brevibacterium*. За да разберем по-добре функционалната роля на този род, извършихме метагеномно секвениране на проба от зелено сирене. Установихме, че доминиращите микроорганизми са различни видове от рода *Brevibacterium* (51%), най-вече *Brevibacterium aurantiacum* (29%). Въпреки че има много по-ниско разпространение, актинобактериалният род *Brachybacterium* (2%) също допринася значително за метаболитната активност на съобществото. Функционалното профилиране предполага, че *Brevibacterium* е основен продуцент на аминокиселини като Phe, Arg и Lys, както и кофактори и витамини, включително B5 и B6 и липоева киселина. Установихме също, че плесента *Aspergillus puulaauensis* (3%) играе ключова роля както в липидния, така и в аминокиселинния метаболизъм в съобществото, въпреки ниското си разпространение. Не бяха открити патогени, но бяха открити гени и плазмиди, кодиращи антибиотична резистентност, при ниски концентрации. Установихме, че консумацията на зелено сирене е безопасна и че микроорганизмите в него имат потенциала да синтезират ценни вторични метаболити.

Introduction

The diverse microbial communities responsible for cheese maturation (ripening) represent the major factors that determine the attributes of the final product, following the specifics of production (Beresford *et al.*, 2001). These attributes include

flavor, odor, texture, and color, which can represent the signature characteristics of a particular type of cheese. Therefore, to better understand the relationship between microbial community composition and the corresponding cheese characteristics, shotgun

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metagenomic sequencing is one possible approach. This method not only identifies the present species in the sample but also enables complex functional analyses based on their genomic material.

Traditional Bulgarian green cheese, made from raw ewe or goat milk and fermented spontaneously, is produced only in the village of Cherni Vit (near the town of Tetevem, Bulgaria). It was recently subject to amplicon sequencing in a prior study (Dimov *et al.*, 2021). In the current study, we rely on the more detailed approach of short-read shotgun metagenomic sequencing to further elucidate the function and better catalogue the profile of the microbial community within the cheese, as well as to establish the presence of any antibiotic resistance or virulence genes. Amplicon sequencing targets a specific marker gene (most commonly the 16S rRNA for bacteria) and uses the abundance of that marker as a proxy for species abundance, which allows for very fast and detailed microbial profiling; however, the presence of a PCR amplification step, which is required for isolating the marker gene, introduces a level of uncertainty to the final abundance estimates. An alternative approach is shotgun metagenomic sequencing, in which the entire collection of genomes in the sample (the metagenome) is sequenced after random fragmentation, without amplification. Shotgun sequencing also provides additional information, as it covers the entire genome rather than a single marker gene. This additional information can be used to identify the presence of virulence and antibiotic resistance genes, as well as to extract metagenome-assembled genomes (MAGs) and to map out the metabolic pathways present in the community, which, in the case of cheese, are the pathways that ultimately contribute to the product's final attributes.

The process of production of traditional Bulgarian green cheese was recently described in detail in the previous amplicon-based analysis (Dimov *et al.*, 2021). In brief, it is produced from unpasteurized sheep's milk and follows a process of brining and ripening in wooden containers. The final ripening step occurs at 16-20°C for 35-40 days, after which the surface molds are brushed off, and the cheese is conserved for up to 3 years. The previous study also elucidated the genus *Brevibacterium* as one of the dominant bacteria within the cheese core, but no species were reliably classified. To better assess the functional role of this genus, we used shotgun metagenomic sequencing of a cheese sample. We believe the unique production steps and ripening environment set the stage for a specific com-

bination of microorganisms that give the product its sensory properties. These considerations, along with the rarity of the food product and the desire to ensure its consumption is safe, prompted us to conduct the current analysis.

Materials and Methods

Sample collection, storage, and preparation

A batch of 12-month-ripened Bulgarian green cheese, prepared from ewe's milk, was collected directly from the dairy plant on the first days of June 2024. The cheese was transported to the laboratory at 4°C, where a 250 mg sample was taken and homogenized using a microcentrifuge tube homogenizer under sterile conditions. The sample included both crust flakes and the interior cheese core. Total DNA from the sample was isolated using the Quick-DNA Fecal/Soil Microbe Miniprep Kit, cat. # D6010 (Zymo Research, USA). DNA concentration was determined by the Quantus fluorimeter (Promega, USA). The obtained DNA was stored at -20°C. Part of the DNA sample was shipped in dry ice for commercial metagenomic sequencing (BGI, China), and the raw sequencing data were downloaded for downstream processing and analyses.

Sample sequencing and quality control

Reads were sequenced using Illumina's NovaSeq 6000 150 bp pair-end sequencing platform. cutadapt (v.5.1) (Martin, 2011) was used to quality filter the reads using a minimum read length of 150 bp, a maximum error rate of 1, and assuring no left-over adapters from sequencing. The reference human genome (GRCh38 - hg38) was used to construct a Kraken2 database, against which the reads were aligned, and human reads were filtered from the data.

Taxonomic profiling

For all software, default settings were used unless specified otherwise. The taxonomy was assigned using Kraken2 (v. 2.1.6) (Wood *et al.*, 2019) against NCBI's complete nt database (accessed 27.09.2025). The reference genomes of prevailing species and subspecies (>0.1% of classified read abundance) were used to create a KrakenUniq (v.1.0.4) database (Breitwieser *et al.*, 2018) to assess the presence of false species positives using the unique k-mer counting approach. At 73 million reads, a threshold of 146,000 unique k-mers was chosen to filter out species false positives, which was based on the inflection point of the cumulative plot of unique k-mers, effectively separating true positives from spurious assignments. False species and subspecies counts were redistributed to the

above taxonomic level, and reads were then redistributed to the species level using Bracken (v.3.0.1) (Lu *et al.*, 2017). To get the final species abundance, read counts were normalized by the genome size of the species reference genomes.

Functional profiling

For protein assignment, forward and reverse reads were independently aligned against NCBI's nr_clust database (accessed 27.09.2025), which contains representative protein sequences grouped at a 90% similarity threshold. To accelerate alignment, the database and reads were divided into prokaryotic and eukaryotic categories and only reads with assigned taxonomy were considered. Diamond (v.2.1.13) (Buchfink *et al.*, 2021) was used for the alignment, and 61.59% of reads were assigned an NCBI protein ID. Hits in the database were then assigned a KEGG ortholog (KO) using kofamscan (v. 1.3.0) (accessed on 25.03.2025), which was able to assign a KO to 34.2% of remaining proteins (Aramaki *et al.*, 2020). The number of hits per gene was normalized to gene length (kb) to obtain reads per kilobase (RPK). KO counts were pooled at the genus or species level, and module counts were calculated for the genera using the module definitions without the use of a one-off strategy. Module counts were then further summed based on KEGG's BRITE hierarchy to the pathways to which they belong.

Metagenome assembled genome (MAG) assembly, binning, and annotation

Metagenomic scaffolds were assembled from the quality-filtered reads using metaSPAdes (v. 4.0.0) (Nurk *et al.*, 2017). Scaffolds with a length <1,500 bp were discarded. Assembled scaffolds were assigned a taxonomy using Kraken2 and the same NCBI nt database. Genomes were then binned using TaxVAMB (v. 4.1.4) (Kutuzova *et al.*, 2024) which incorporates taxonomic data with sequence characteristics for binning. Bin completeness and contamination were assessed using CheckM2 (v.1.0.2).

Bakta (v.1.11.0) (Schwengers *et al.*, 2021) was used to annotate bacterial scaffolds, while metaeuk (7.bba0d80) (Levy Karin *et al.*, 2020) was used for the fungal ones. For annotating resistance and virulence genes, we used NCBI's tool AMRFinder-Plus (v. 4.0.23) (accessed 25.03.2025) (Feldgarden *et al.*, 2021). PlasmidFinder (v.2.1.6) (Carattoli *et al.*, 2014) was used to annotate plasmid origins of replication within the contigs, along with additional origins assigned by bakta.

Results

Sequencing and taxonomic profiling

A total of 73,074,454 forward and reverse 150-bp reads were sequenced on the Illumina platform from the green cheese sample, with an average Phred score of 36. Reads were subjected to quality filtering and removal of the human genome before downstream analysis. Seventy-eight percent of reads were classified to at least the domain level. Twenty bacterial species were confidently assigned using a minimal-unique k-mer approach, along with three fungal species. Species abundance is represented in Figure 1 with a threshold of >1% relative abundance.

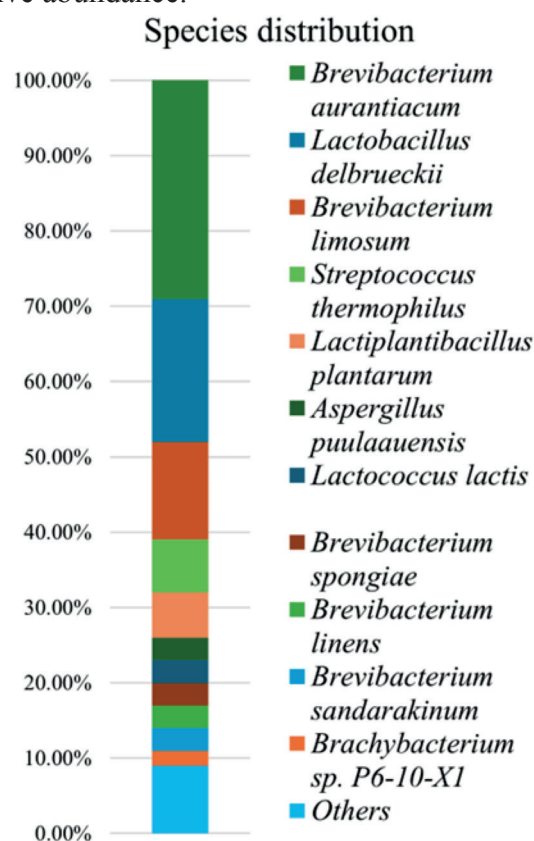


Fig. 1. Microbial species in traditional Bulgarian green cheese. Abundances are based on Kraken2 annotations and corrected using KrakenUniq and Bracken, along with organism genome size.

The dominant genera of bacteria are *Brevibacterium*, followed by *Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Brachybacterium*. These were further stratified into the dominant species *Brevibacterium aurantiacum* and *Brachybacterium sp. P6-10-X1*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactiplantibacillus plantarum*, and *Streptococcus thermophilus*, among other lactic acid bacteria. For the fungi, *Aspergillus puulaauensis* accounted for 95% of fungal reads, while the remaining reads were shared between *A. versicolor* and *A. sydowii*.

Before Bracken read re-distribution, *B. aurantiacum* reads represented 46% of the genus, followed by *B. sandarakinum*, *B. limosum*, *B. spongiae*, *B. BDJS002*, and others, but all with <5% of reads. Additionally, a third of reads in this genus could not be identified to the species level. Ninety-three percent of these genus-level reads successfully mapped back to the *B. aurantiacum* reference genome, indicating that they represent sequences shared among the *Brevibacterium* species and could not be confidently assigned by Kraken2.

A fourth of *L. delbrueckii* reads were identified as the subspecies *bulgaricus*, and 10% as the subspecies *lactis*. Still, the latter did not meet the unique k-mer threshold for being considered a valid assignment. The only identified *Brachybacterium* species that wasn't a false positive was *Brachybacterium* sp. P6-10-X1 but represented only 20% of *Brachybacterium* assignments.

In summary, the dominance of *B. aurantiacum* is consistent with its known role in smear-ripened cheeses (Pham *et al.*, 2017a). *Brachybacteri-*

um has a significant role in carbohydrate and amino acid metabolism during cheese ripening, which is common for cheeses using raw milk (Quigley *et al.*, 2013; Niccum *et al.*, 2020). Lactic acid bacteria are present in significant quantities even during ripening. *A. puulaauensis* appears to be a major player in lipid and amino acid metabolism in green cheese.

Functional profiling

Given that a significant number of reads could not be assigned to a species, especially for the dominant genus *Brevibacterium*, the genus level was chosen to represent the community's metabolic profile, with only the dominant genera considered. At the genus level, a total of 165 KEGG modules were reconstructed across the chosen genera using the module definitions without a gap fill strategy. To better summarize the community's function, the modules were collapsed to their higher brite taxonomy in Figure 2, with a minimal pathway count threshold of 25.

To further interrogate amino acid metabolism, the separate modules of the amino acid metabolism

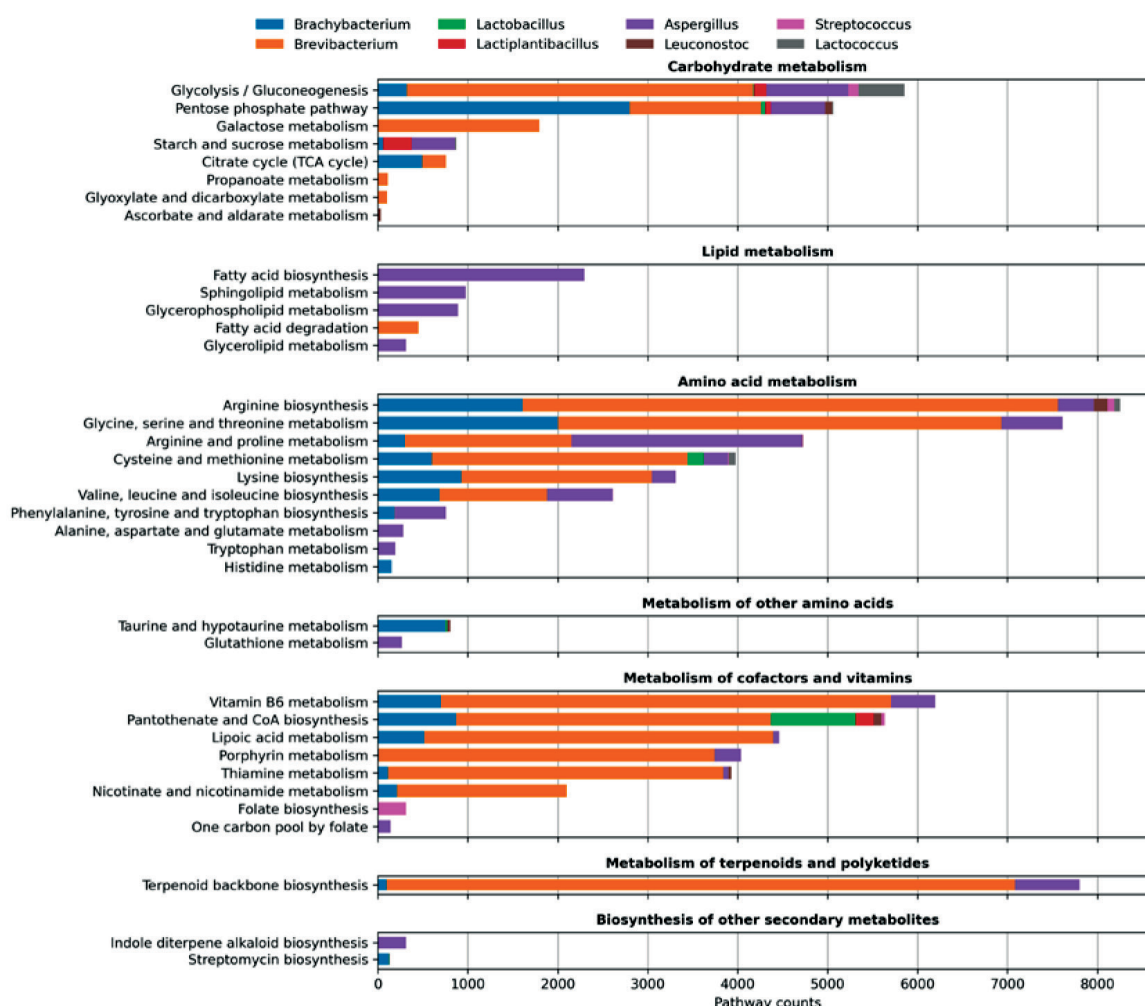


Fig. 2. Metabolic pathway counts at two levels for the green cheese community. The horizontal axis represents pathway counts, which are the sum of KEGG module definition counts within each pathway, based on the RPK of the present KOs in the community. Only pathways with a count greater than 25 are shown for clarity.

pathways are also shown in Figure 3. *Brevibacterium*, *Brachybacterium*, and *Aspergillus* are the genera that contributed most heavily to the metabolic potential of the community, and have all previously been implicated as playing a crucial role in cheese ripening (Quigley et al., 2013; Pham et al., 2017a; Decontardi et al., 2018; Anelli et al., 2019; Kandasamy et al., 2020; Niccum et al., 2020)

Metagenome assembly and binning

MetaSPAdes was used for scaffold assembly, and TaxVAMB was used to group the scaffolds into bins by incorporating taxonomic data from Kraken2 and innate sequence features, with a minimum scaffold length of 1.5 kb. The features of the resulting scaffolds are summarized in the blob plot of Figure 4. The scaffolds with the highest coverage belonged to a species of fungal virus from the Genomoviridae family, which was excluded from the figure for clarity.

At a 50% completeness threshold, as as-

signed by CheckM2, a total of 16 bins were recovered (Table 1). All of them had contamination <3%, except for the bin of *B. aurantiacum*, for which the contamination was nearly 20%, indicating that the assembly is most likely a hybrid from different species of the genus, which again relates to the high level of reads only assigned to the genus level for that group.

Bacterial resistance annotation

Genes that relate to antimicrobial resistance, the taxonomy of the scaffold they're located within, and the presence or absence of a point mutation are summarized in Table 2. While several resistance genes were identified, none of the potential pathogen species to which they belong were detected within confidence thresholds.

Discussion

Role of actinomycetes in green cheese ripening

Brevibacterium species play a crucial role in the ripening of surface-ripened cheeses, owing to

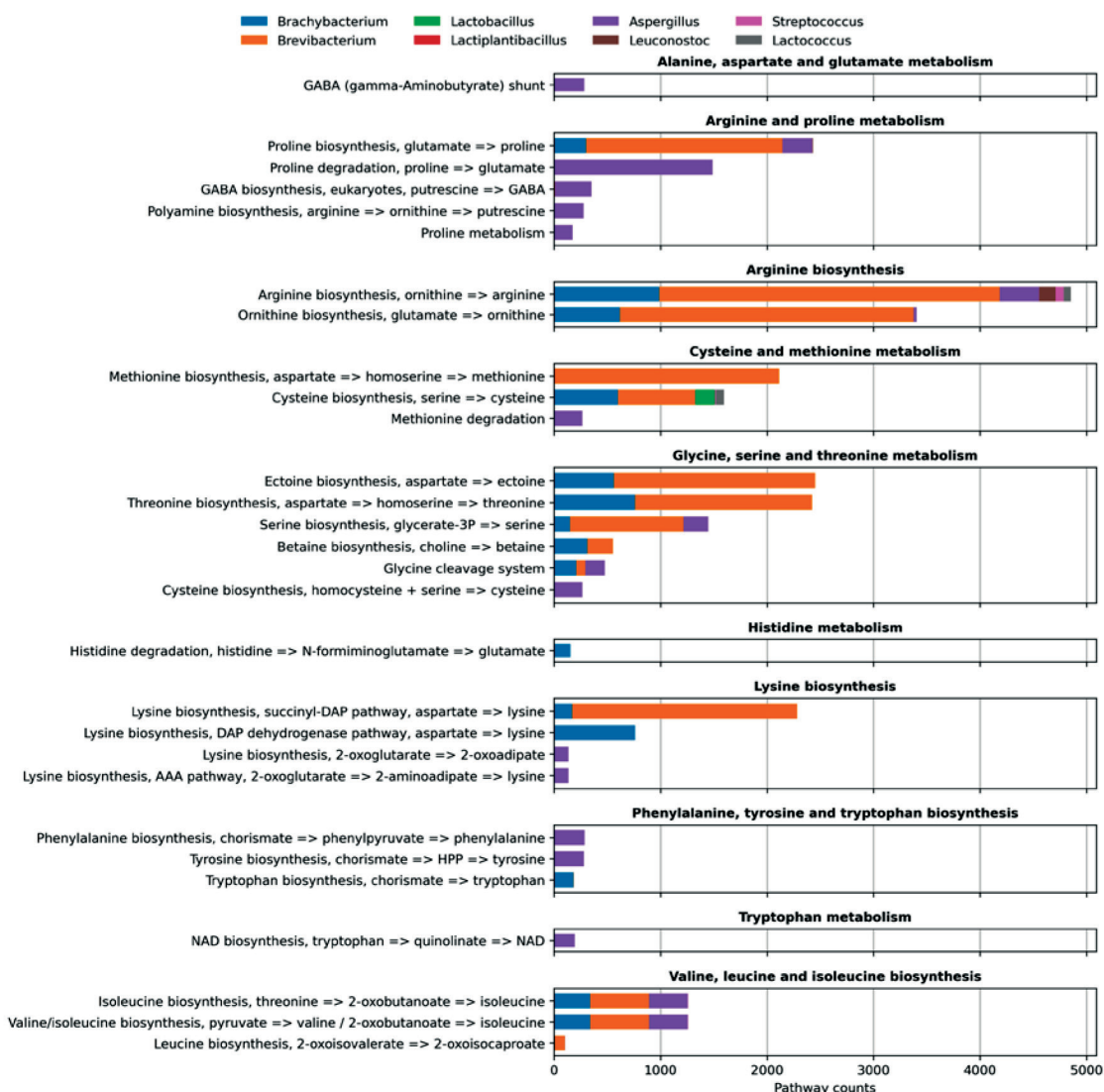


Fig. 3. Module counts for the pathways of amino acid metabolism of the community. The horizontal axis shows module counts based on the RPK of the present KOs in the community. Only modules with a count greater than 25 are shown for clarity.

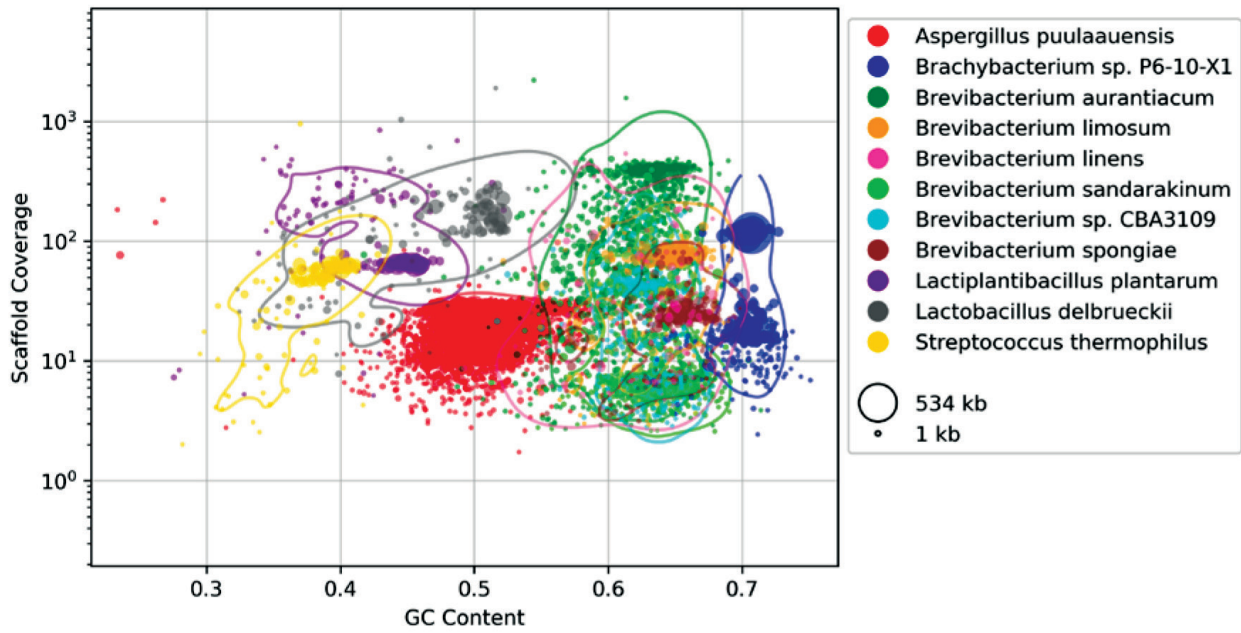


Fig. 4. Blob plot of the assembled scaffolds. Blob plots represented by their taxonomic annotation, size, GC content and coverage. Scaffolds below 0.01 coverage and length below 1.5 kb are not shown. Kraken2 was used to assign taxonomic annotations to the scaffolds.

their ability to break down lipids and proteins and produce volatile sulfur compounds, which are essential for the cheese's aroma profile. Additionally, they provide a typically red-orange pigment to the cheese surface (Pham *et al.*, 2017a). The genus is also implicated in utilizing histamine as a carbon source (Anast *et al.*, 2019). The primary species of *Brevibacterium* involved in cheese ripening, and most often found on the cheese surface, are *B. linens*, *B. aurantiacum*, and *B. antiquum* (Gavriš *et*

al., 2004). The source of *Brevibacterium* in cheese is often from the use of raw milk, which serves as the inoculant for the starting culture of microorganisms in the cheese (Quigley *et al.*, 2013; Niccum *et al.*, 2020).

Similarly, our study identifies *B. aurantiacum* as the dominant species, but distinguishing other species in the genus is difficult. While taxonomic annotation reads show a relatively even distribution of a few reads between other species in the genus,

Table 1. Bins formed by the grouping of scaffolds

Bin Taxonomy	Completeness	Contamination	N50	L50
<i>Brachybacterium</i> sp. P6-10-X1	100	2.5	120479	8
<i>Lb. plantarum</i>	99.31	1.06	50434	20
<i>Lc. lactis</i>	100	0.88	133251	6
<i>S. simulans</i>	100	0.13	50608	14
<i>L. brevis</i>	100	0.36	52054	18
<i>S. thermophilus</i>	100	2.76	35987	15
<i>L. mesenteroides</i>	100	0.43	34786	15
<i>Corynebacterium glyciniphilum</i>	100	0.67	39133	26
<i>Staphylococcus equorum</i>	99.28	1.82	14552	52
<i>Mammaliicoccus lentus</i>	100	2.75	11713	65
<i>L. falkenbergense</i>	100	0.12	53532	10
<i>Enterococcus faecium</i>	100	2.88	11855	59
<i>L. delbrueckii</i>	100	0.63	52959	9
<i>B. aurantiacum</i>	99.83	19.28	14652	95
<i>Brevibacterium limosum</i>	100	2.52	24147	35
<i>Brevibacterium spongiae</i>	100	1.06	16330	43

Scaffolds were assigned to bins using TaxVAMB, and completeness and contamination were assessed using CheckM2. All scaffolds with completeness >50% are shown. Taxonomic annotation is based on Kraken2.

Table 2. AMRFinderPlus and Bakta results for resistance annotation

Scaffold taxonomy	Plasmid origin in contig	Gene	Resistance	Point mutation
<i>E. faecium</i>	-	aph(3')-IIIa	Amikacin/Kanamycin	+
<i>E. faecium</i>	rep	eat(A)_T450I	Pleuromutilin	-
<i>E. faecium</i> Com15	-	msr(C)	Azithromycin, Erythromycin, Streptogramin B, Tylosin	-
<i>E. faecium</i> Com15	-	liaR_E75K	Daptomycin	+
<i>E. faecium</i> DO	-	aac(6')-I	Aminoglycoside	-
<i>L. formosensis</i>	-	tet(S)	Tetracycline	-
<i>M. lentus</i>	-	sal(B)	Lincosamide, Pleuromutilin, Streptogramin	-
<i>M. lentus</i>	-	mph(C)	Erythromycin, Spiramycin, Telithromycin	-
<i>S. aureus</i>	-	blaI	Beta-lactam	-
<i>S. aureus</i>	-	blaR1	Beta-lactam	-
<i>S. aureus</i>	pS194	str	Streptomycin	-
<i>S. aureus</i>	pS0385p1	tet(K)	Tetracycline	-
<i>S. equorum</i>	-	mph(C)	Erythromycin, Spiramycin, Telithromycin	-

Only genes with an identity to the reference sequence of >90% are shown. Pfinder and Bakta assessed the presence of plasmid origins of replication. Whether the resistance is the result of a point mutation or not is indicated by a '+' or a '-' sign.

only the genomes of *B. limosum* and *B. spongiae* could be binned with a completeness above 50%, which makes them the most likely candidates for other species present in the sample.

Since low-contamination and high-completeness genomes could not be obtained for every prevalent species, quality-controlled reads were used for functional annotation rather than assembled scaffolds. To reduce ambiguity arising from Kraken2's genus- and species-level read assignments, functional profiling was also performed at the genus level.

In our study, *Brevibacterium* species seem to be the main group capable of metabolizing the present galactose. Galactose is produced in cheese as a result of the activity of *S. thermophilus*, and can thus remain in the ripening phase of cheese production (Michel Martley, 2001). Additionally, fatty acid degradation pathways were detected primarily in this genus, suggesting that the remaining fats in the milk are metabolized mainly by these organisms.

Multiple pathways for the secondary metabolism of cofactors and vitamins were also assigned to *Brevibacterium*. These include lipoic acid biosynthesis, pyridoxalphosphate (vitamin B6) biosynthesis, pantothenate biosynthesis (vitamin B5), the thiamine salvage pathway, porphyrin (heme) biosynthesis, and others. Lipoic acid is crucial for anaerobic respiration in mammals, acting as an antioxidant (Packer *et al.*, 1995). It has also been

proposed as a treatment for various human diseases due to its ability to quench free radicals (Goręca *et al.*, 2011). Another beneficial secondary metabolite with a high pathway count within the microbial community is pyridoxal phosphate (vitamin B6), which is indispensable for numerous biological functions in the human body, since its deficiency can lead to diabetes, neuropathy, immune disorders, and other health complications (Stach *et al.*, 2021). Pantothenate (Vitamin B5) was also detected and is important for energy generation, hormone synthesis, and other regular bodily functions (Sanvictores Chauhan, 2024). While it is tempting to speculate on the absolute presence of these secondary metabolites in the cheese, caution should be taken when correlating the presence of a pathway with its end metabolites, since the pathway's actual activity cannot be inferred from genomic data alone.

The functional profiling also showed significant metabolic contributions by the genus *Brachybacterium*, despite a lower overall abundance. *Brachybacterium* is commonly found in cheese rinds, and its origins can be attributed to the use of raw milk, in which different *Brachybacterium* species are identified (Quigley *et al.*, 2013; Niccum *et al.*, 2020). We speculate that the prevalence of this genus in the cheese rind, rather than the core, partially explains the lower abundance. We found that this genus has a metabolic profile similar to that of *Brevibacterium* during cheese ripening.

The unique roles *Brachybacterium* plays in

the community may be related to pathways for taurine, histidine, and phenylalanine metabolism, as well as its very high activity in the pentose phosphate pathway. PPP is an alternative pathway for glycose that ultimately serves cell anabolism. Upon closer inspection, the pathway for the oxidative phase of the PPP is missing in the *Brevibacterium* KOs. This observation suggests that glycolysis is a significant source of NADH for the cell, which would explain the disparity in carbohydrate metabolism between the two actinomycete genera. Our study also showed that porphyrin (more specifically, heme) biosynthesis was detected in *Brevibacterium* at high levels, suggesting that the genus is capable of efficient aerobic respiration.

We found that the *Brevibacterium* genus possesses a high number of pathways for amino acid biosynthesis, which contribute significantly to cheese flavor. This observation encompasses the biosynthesis of Pro, Arg, Met, Cys, Thr, Ser, Lys, Ile, and Val, as well as ornithine and ectoine. Interestingly, no pathways for amino acid degradation were detected in our study. Catabolism of amino acids during ripening is very significant to the flavor profile of cheese (McSweeney, 2004). It's shown that the potential for amino acid catabolism varies within the genus *Brevibacterium* (Pham *et al.*, 2017b). It's most likely that the pathways used by *B. aurantiacum* for amino acid degradation include the synthesis of aromatic sulfur compounds, which may be missing from the KEGG module definitions, and are thus not accounted for in our study.

Despite the lower abundance of *Brachybacterium*, the genus still appears to play a significant role in amino acid metabolism, primarily through the synthesis of Pro, Arg, Cys, Thr, Ile, Ser, and Gly, as well as ornithine, ectoine, and betaine. The genus also produces Lys, which is lacking from the amino acid repertoire of *Brevibacterium*. His degradation pathway was also identified in this group.

Ectoine was produced in significant quantities by both *Brevibacterium* and *Brachybacterium* and typically functions as an osmoprotectant for bacterial cells (Hahn *et al.*, 2017). This process could be crucial for the survival of actinomycetes in the highly saline cheese environment, since it is not observed among lactic acid bacteria.

Another amino acid that was produced in great quantity in this study by actinomycetes was ornithine, which is found to be present in cheese due to the activity of lactic acid bacteria; however, we demonstrate that other bacteria involved in ripening also make a significant contribution (Di-

ana *et al.*, 2014). Positive health effects have been shown for the consumption of ornithine (Kurata *et al.*, 2011). Ornithine can also be transformed into putrescine and other polyamines by bacteria, which could also impact the flavor profile of the cheese (Li *et al.*, 2025).

Role of LAB in green cheese ripening

Lactic acid bacteria in the sample were represented by *L. delbrueckii*, *S. thermophilus*, *L. plantarum*, and *Lactococcus lactis*. The bacteria are responsible for metabolizing lactose in milk into lactic acid during the acidification step of cheese production (McSweeney *et al.*, 2017). This process can continue into the cheese's ripening phase. Taken together, these species represent a significant portion of the microbial community in our study, accounting for 40% of the species abundance. Even though the counts of lactic acid bacteria are high, their contributions to the community's metabolic functions during the ripening stage appear low in our study, with only *Lactobacillus* showing a high number of pathways for cys and CoA biosynthesis.

Role of mold in green cheese ripening

One of the dominant microorganisms on the cheese was the filamentous fungus *A. puulaauensis*, accounting for 16% of classified reads and 3% of the organism's abundance. This mold is shown to grow on a variety of milk and goat cheeses, including Grana and some artisan Italian cheeses (DeContardi *et al.*, 2018; Anelli *et al.*, 2019; Kandasamy *et al.*, 2020). It's also shown to produce the mycotoxin sterigmatocystin; still, its concentration on cheeses is low, and does not pose a health risk (Finoli *et al.*, 1999; Muñoz-Tebar *et al.*, 2021).

In this study, we found that fatty acid biosynthesis pathways in the cheese are almost entirely from *A. puulaauensis*, which could shape the cheese's final flavor profile. Additionally, this species plays a significant role in carbohydrate and amino acid metabolism, particularly through proline degradation pathways.

Interestingly, the dominant organism in this study, by sheer abundance, was a virus from the family Genomoviridae. This family contains viruses that infect fungi, and it is certainly plausible that one of these viruses infects the *Aspergillus* species identified in this sample.

While no typical pathways for amino acid degradation were detected in bacteria, the filamentous fungi *A. puulaauensis* was shown to have pathways for the degradation of Pro, GABA, and Met, as well as pathways for the synthesis of putrescin

from ornithine. Our findings suggest *A. puulaauensis* can metabolize compounds produced by the actinomycetes community, rather than directly participating in the proteolysis of casein and other peptides in the cheese.

Antibiotic resistance and virulence genes in green cheese

Upon assembling the sequencing reads, several scaffolds were identified as containing genes encoding resistance to antibiotics, including kanamycin, tetracycline, and streptomycin. The resistances to tetracycline and streptomycin, in particular, were found on the same contigs that contain *S. aureus* plasmid origins of replication, strongly implicating them as part of a plasmid. Reads identified as *S. equorum* represented approximately 0.02% of all reads, which is too few to consider the species present in the sample. We speculate this represents either environmental DNA, contamination during handling, or the plasmid being carried by another host. Similarly, *E. faecium* was found with only 0.02% of reads.

Antimicrobial resistance has been identified in several species of lactic acid bacteria in cheese, including some of the resistance genes identified in this study (Herreros *et al.*, 2005; Belletti *et al.*, 2009). *E. faecium* resistance is also identified in French cheeses (Jamet *et al.*, 2012). While our study reinforces the presence of antimicrobial resistance genes in cheese products, their origins and environmental impact fall outside the scope of this work. We believe this trend is of public importance and warrants further inquiry.

Comparison between taxonomic profile results from shotgun sequencing and amplicon sequencing

A previous study on traditional Bulgarian green cheese employed amplicon metagenomic sequencing using several targeted regions of both the 16S and ITS marker genes (Dimov *et al.*, 2021). While *Brevibacteria* was present in most samples, it was only dominant in one of them and could not be classified to the species level using any 16S region. The current shotgun approach unequivocally identifies *B. aurantiacum* and, most likely, additional species *B. limosum*, *B. spongiae*, and *B. sandarakinum*. We speculate that the genetic distance between the species in this genus is small, making the unambiguous assignment of the many sequences shared among them difficult.

Discrepancies between the taxonomic profiles of the two studies might be attributed to sam-

pling location on the cheese, the length of the maturation period, and the particular 16S region chosen for sequencing, since different regions are known to differ in the outcome of taxonomic annotation (Zhang *et al.*, 2024).

One striking difference between the amplicon sequencing of the cheese and the previous study is the dominant fungus, which was *Debaryomyces hansenii* in most samples. Considering that the fungi grow on the cheese rind, it is most likely that multiple fungi inhabit the cheese surface, and these differences are sample-dependent. This also supports the idea that multiple sub-communities of organisms could form distinct clusters within the cheese and on the rind. An essential limitation of the current study is the limited number of samples, which makes it difficult to draw firm conclusions about the role of other microorganisms in the cheese.

Conclusions

Our study found that several species of the genus *Brevibacterium* are present in traditional Bulgarian green cheese, along with other actinomycetes, LAB, and mold, all of which contribute to the sensory properties of the food product. We identified multiple pathways for the synthesis of useful secondary metabolites, suggesting potential benefits from consuming the cheese. However, it's important to note that the presence of these pathways does not guarantee the presence of their end products; in this regard, our study provides a starting point for potential biochemical quantification of their products. We were also able to confidently rule out the presence of any harmful microorganisms within the cheese, yet found traces of plasmids carrying microbial resistance genes, the source of which remains speculative. We were also able to provide a potential framework for the interactions of microorganism groups within the cheese sample and how those interactions shape the quality of the end product during ripening. We believe this study helps elucidate the microbial structure and the role of the genus *Brevibacterium* on the surface of traditional Bulgarian green cheese.

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