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ST. KLIMENT OHRIDSKI
FACULTY OF BIOLOGY

SOFIA UNIVERSITY “ST. KLIMENT OHRIDSKI”

FACULTY OF BIOLOGY

DEPARTMENT OF GENETICS

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GENOME TYPING OF PROBIOTIC MICROFLORA ISOLATED FROM NATURAL PRODUCTS

ABSTRACT

of a Dissertation for awarding the educational and scientific degree "Doctor"

Field of higher education: "Natural Sciences, Mathematics and Informatics"

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The Dissertation contains 231 pages, 32 figures and 17 tables.

452 literary sources and six electronic sources are cited.

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The Dissertation defence is scheduled in front of a committee as per below:

Internal Scientific Jury Members:

1. ...

2. ...

External Scientific Jury Members:

1. ...

2. ...

3. ...

The Dissertation defence will take place on.....

All the materials are available at the Department of Genetics, Faculty of Biology, Sofia University "St. Kliment Ohridski".

CONTENTS

LIST OF ABBREVIATIONS

AAI - Amino Acid Identity

AFB - American Foulbrood Disease

ANI - Average Nucleotide Identity

BLAST - Basic Local Alignment Search Tool

bp - base pair

CFU - Colony Forming Unit

DNA – Deoxyribonucleic Acid

EUCAST - European Committee on Antimicrobial Susceptibility Testing

LAB – Lactic Acid Bacteria

MLST - Multilocus Sequence Typing

NGS - Next Generation Sequencing

OTE - Operational Taxonomic Unit

PCR – Polymerase Chain Reaction

RAPD - Random Amplification of Polymorphic DNA

UPGMA - Unweighted Pair Group Method with Arithmetic Mean

INTRODUCTION

Modern people pay more and more attention to the content of the food they consume, trying to avoid chemical additives and flavour enhancers. The application of LAB in developing new probiotics and functional foods is constantly increasing. Increasing efforts worldwide are directed towards developing bioinformatic and molecular methods for analysing and genomic typing of various microorganisms. Particular interest is their differentiation in pathogenicity, antibiotic resistance, and antimicrobial properties.

The increasing number of electronic platforms for performing bioinformatics analysis and the growing number of freely accessible databases provide an opportunity to research and predict options for potential industrial applications of different bacterial strains. Thousands of publicly available genomes are available in databases (Fleischmann et al., 1995) . Many of these genomes were obtained from LAB and used as probiotics or starter cultures in food fermentation (Klaenham er, Altermann, Arigoni, Bolotin, & Breidt, 2002 ; Lkjancenko , Usery, & Wassenaar, 2012) . In addition, the accelerated development in recent years of modern scientific fields such as functional genomics, transcriptomics and proteomics have helped to advance research on the interactions between probiotics and the gastrointestinal tract.

In the current dissertation work, the main goal was to isolate and taxonomically characterise LAB from traditional for Bulgaria naturally fermented products and bee products harvested in different ecological regions of the country. The collection of isolates was tested for antibacterial activity, and the strains with an established activity against pathogenic microorganisms were determined taxonomically by classical and molecular genetic methods. In addition, the most promising isolates were analysed at the genome level by applying Next Generation Sequencing. Finally, the obtained results were processed and further analysed using freely available web-based tools. Thus, we evaluated the potential of LAB to produce bacteriocins that serve as natural agents against bacterial infections.

NGS-based metagenomic study of traditional Bulgarian Green Cheese deserves special attention. It is produced in Cherni Vit village. The current research is the first-ever scientific study of this unique dairy product. Our results show that Bulgarian Green Cheese has an extremely rich microbiome and is the only one Bulgarian noble mould. Its organoleptic characteristics are

determined by the specific region in which it is produced, and its products can be considered a national cultural heritage.

In the assembled genomes of selected isolated from bee products, bacteriocin genes were found, which is a possible explanation for the antimicrobial properties of these isolates. In addition, our studies have shown that some of the strains of *Enterococcus faecium* we have studied possess antibacterial activity against the honey bee pathogen *Paenibacillus larvae* and serve as a natural protection of the hives. The bacterium *Paenibacillus larvae* cause the contagious disease AFD, which is characterised by high mortality in bee colonies and affects many hives worldwide.

The NGS-based approach proved to be a very economical, rapid and efficient way to screen the applicability of new pro- and prebiotic strains. The LAB analysed by us, part of the microbiome of naturally fermented and bee products, have a beneficial effect on human and bee health, exerting a protective effect against pathogenic microorganisms. The obtained data are interesting both from a scientific and a practical point of view, because of the connection between lactic acid fermentation and the longevity of the Bulgarian population. In addition, honey bees are a vital biological resource of great economic importance and the threat to their population can have an impact on the earth's ecosystem as a whole.

GOAL AND OBJECTIVES

The main goal of the present study is the molecular genetic and genomic characterisation of strains of microorganisms isolated from functional foods and bee products, as well as analysis of the microbiota of functional foods and bee products using next-generation sequencing-based methods.

For its implementation, the following experimental tasks were set:

1. Collecting bacterial isolates from fermented functional foods (sour milk, cheeses, sausages, fermented vegetables and sourdough);

2. Collecting bacterial isolates from bee products and from the gastrointestinal tract of honey bees;

3. Screening the collections for protease and peptidase activities against milk proteins;

4. Screening the collections for antibacterial activity due to the synthesis of bacteriocins, including proving the protein nature of the antibacterial agent, determining the spectrum and titer of antibacterial activity, as well as environmental factors influencing its expression;

5. Phenotypic genus and species determination of selected strains showing probiotic potential;

6. Accurate species identification of selected strains showing probiotic potential using molecular genetic methods, including genus-specific PCR, species-specific PCR and sequencing of the genes encoding 16S rRNA on an automatic sequencer;

7. Isolation of high-quality total DNA from selected strains with probiotic potential, with the aim of whole genome sequencing;

8. Isolation of high-quality total DNA from some food and bee products, as well as from the gastrointestinal tract of honey bees, to conduct amplicon-based metagenomic studies of the microbiota composition;

9. Investigation of the degree of phylogenetic relationship between some strains showing probiotic potential using RAPD analysis.

10. Bioinformatics processing of the data from the whole genome next-generation sequencing of the genomes of some strains showing probiotic potential with their assembly;

11. Bioinformatic analysis of the data from the assembled genomes, with the aim of accurate species determination and MLST typing,

12. Bioinformatic analysis of the data from the assembled genomes to screen for the presence of genes determining the expression of bacteriocins;

13. Bioinformatic analysis of the data from the assembled genomes to check for the presence of genes causing antibiotic resistance, virulence factors and pathogenicity factors, to prove the safety of the isolated strains;

14. Bioinformatic processing of the data from the amplicon-based metagenomic next-generation sequencing to assess the quality of the sequencing;

15. Bioinformatic processing of the data from the amplicon-based metagenomic next-generation sequencing of some samples to annotate the operational taxonomic units and to study the microbiota composition;

16. Bioinformatic processing of the data from the amplicon-based metagenomic next-generation sequencing of some samples, with the aim of alpha-diversity analysis;

17. Bioinformatic processing of data from amplicon-based metagenomic next-generation sequencing of some samples, with the aim of beta-diversity analysis.

MATERIALS AND METHODS

1. Bacterial strains, media and culture conditions.

A collection of 368 strains of LAB was isolated from 31 pcs. naturally fermented dairy products (yellow cheese, green cheese and homemade cheeses from cow, goat, buffalo or sheep milk), meat products (sausage, sujuk and dry salami), sourdough bread, sauerkraut, olives and bee products (Bee pollen, Perga and Bee intestinal contents).

1.1. Long-term storage of the strains

Permanent cultures were prepared in 10% dry milk and stored at -70℃.

2. Microbiological methods and techniques

2.1. Methods for LAB strain isolation from different products.

2.2. Cultivation in specific media.

3. Collection screening techniques for isolates showing probiotic potential.

3.1. Screening methods for antibacterial activity.

3.2. Screening methods for proteolytic activity.

4. Species determination of isolates showing probiotic potential

4.1. Physiological and biochemical methods.

4.2. Molecular-genetic methods.

4.2.1. Genus-specific PCR.

Amplification with the enterococci Ent1/Ent2 primer pair.

4.2.2. Species-specific PCR.

Amplification with *Enterococcus faecalis* specific primers (EDAI_FI/EDA I_RI) and *Enterococcus durans* primers (DU1/DU2).

4.2.3. Sequencing of the genes encoding 16S rRNA.

5. Techniques for proving the protein nature of antibacterial agents.

6. Techniques for determining the spectrum of activity of antibacterial agents.

7. Techniques for determining the titer of bacteriocin activity.

8. Techniques for determining factors affecting bacteriocin expression.

9. Methods for protein content study.

10. **Methods for nucleic acids isolation.**
11. **Electrophoretic methods.**
12. **Molecular genetic techniques.**
13. **Sequencing methods.**
14. **Metagenomic studies.**
15. **Multilocus Sequence Typing.**
16. **Tests for the presence of antibiotic resistance and virulence factors.**
17. **Methodshods for the study of phylogenetic relatedness.**
18. **Software products used:**

GeneTools (Syngene), QIIME, Galaxy, LEfSe, R, mothur, WGCNA, FLASH, BAGEL 4, PlasmidFinder 2.1, QUAST, ResFinder 3.2, KmerResistance, VirulenceFinder 2.0, Shovill, MUSCLE, Uchime, UPARSE.

RESULTS

1. Microbial isolates from Bees

1.1. Preparation of a collection of isolates

A collection of 45 strains, mainly belonging to the genus *Enterococcus*, was collected from 15 hives from different apiaries located in the city of Sofia, the city of Vidin, the village of Dushantsi and the village of Momchilovtsi. We worked with freshly collected pollen granules because they are in contact with the bees' saliva, which interacts with the gastrointestinal tract. We used selective medium D-Coccosel for *Enterococcus* species for the isolates obtained from all fifteen hives. The isolates from each bee family were cultured in 15 Petri dishes with the selective D-Coccosel agar solid medium. Total colony counts ranged between 12 and 37 per Petri dish, with black colonies variation between 3 and 17. Isolation of single colonies was performed based on the presence of a black halo around the bacterial colony. Three colonies with a clear halo were randomly selected from each Petri dish. We assigned unique numbers to all isolates encoding the following– the first two digits correspond to the specific beehive from which they originated (from 01 to 15). In contrast, the third digit refers to the sequence of the randomly selected colony. Molecular-genetic studies of the isolates revealed that the same strain was present in four of the six hives, which were designated as *Enterococcus durans* and named EDD2. Representatives of the genus *Enterococcus* were also found in the other two hives, which were stored without further identification.

All strains isolated from bee families and presumed to belong to the *Enterococcus* genus are presented in the table. No. 1.

Table No. 1 - List of isolates from beehives belonging to the genus *Enterococcus* and some of their characteristics

Isolate	Activity against *Peanibacillus larvae*	Specific PCR for the genus *Enterococcus*	Specific PCR for *Enterococcus durans*	Specific PCR for *Enterococcus faecalis*
01-1	No	+	-	+
01-2	No	+	-	+
01-3	No	+	-	-
02-1	Yes	+	+	-

Isolate	Activity against *Peanibacillus larvae*	Specific PCR for the genus *Enterococcus*	Specific PCR for *Enterococcus durans*	Specific PCR for *Enterococcus faecalis*
02-2	Yes	+	+	-
02-3	No	+	-	+
03-1	No	+	-	+
03-2	No	+	-	+
03-3	Yes	+	-	-
04-1	No	+	-	+
04-2	No	+	-	+
04-3	No	+	-	-
05-1	No	+	-	+
05-2	No	+	+	-
05-3	No	+	-	+
06-1	No	+	-	+
06-2	No	+	-	-
06-3	No	+	+	-
07-1	No	+	-	-
07-2	No	+	-	+
07-3	No	+	-	+
08-1	Yes	+	+	-
08-2	Yes	+	+	-
08-3	Yes	+	+	-
09-1	Yes	+	-	-

Isolate	Activity against *Peanibacillus larvae*	Specific PCR for the genus *Enterococcus*	Specific PCR for *Enterococcus durans*	Specific PCR for *Enterococcus faecalis*
09-2	No	+	-	-
09-3	No	+	-	-
10-1	No	+	+	-
10-2	No	+	-	-
10-3	No	+	-	+
11-1	Yes	+	+	-
11-2	No	+	-	-
11-3	No	+	-	+
12-1	No	+	-	-
12-2	No	+	-	-
12-3	No	+	-	+
13-1	No	+	-	+
13-2	No	+	-	+
13-3	No	+	-	-
14-1	No	+	-	+
14-2	No	+	-	-
14-3	Yes	+	+	-
15-1	No	+	-	-
15-2	No	+	-	+
15-3	No	+	+	-

1.2. Perform preliminary species identification

D-Coccosel medium is selective for species of the genus *Enterococcus,* known initially as group D - streptococci. (Facklam & Moody, 1970) . Regardless of this initial selection of isolates, we performed additional taxonomic analyses of the strain collection. For this purpose, we applied various biochemical and physiological methods and molecular tests based on DNA sequences. All forty-five analysed strains demonstrated intensive growth in the presence of 6.5% NaCl, and at low (10ºC) and high (45ºC) temperatures.

Using PCR with genus-specific primers, the isolates were confirmed to belong to the genus *Enterococcus* while using species-specific oligonucleotides, eleven of them were found to belong to the species *Enterococcus durans* (including seven having activity against *Paenibacillus larvae*, later shown to be clonally related and designated as EDD2), and nineteen – to the *Enterococcus faecalis* (none of them showed activity against *Paenibacillus larvae*). The fifteen remaining strains were successfully determined only on the genus level (among them, two was with demonstrated activity against *Paenibacillus larvae*).

1.3. Activity against *Paenibacillus larvae*

Cultures were prepared in a liquid nutrient medium from all studied bee isolates, and neutralised supernatants were obtained from them. These, in turn, were tested for inhibitory activity against the bacterial bee pathogen *Paenibacillus larvae* by applying the well-diffusion method. Thanks to this experiment, we found that eleven isolates secrete into the growth medium components, inhibiting the test microorganism's growth. This was visualised by observing clear inhibition zones around the corresponding wells with dropped neutralised supernatants. Upon treatment with the enzyme proteinase K, the protein nature of the antimicrobial agents was demonstrated with the observated loss of activity. Comparison of the bacteriocin-producing isolates thus identified on taxonomic status in our previous data showed that seven of the nine strains belonged to *Enterococcus durans* and two belong to *Enterococcus faecium*. The remaining two strains, used in our study, did not form products in the species-specific PCRs. Therefore, for future studies, we focused on the nine isolates with elucidated species affiliations from four different beehives.

1.4. Screening for clonal relatedness among bacteriocin-producing strains

Because in two of the four hives, several isolates were characterised as *Enterococcus durans*, we hypothesised that we might have multiple isolates from the same strain. To test this hypothesis, we subjected the RAPD assay with different primers all the seven strains with demonstrated activity against *Paenibacillus larvae.* The results are shown in Fig. 1. Using primer L2, more significant discrimination was achieved (Panels A and C) with a lower lowest similarity coefficient (88%). A denser electrophoretic pattern was performed with primer E1, but with a higher lowest similarity coefficient (95.3%) – panels B and D. However, in both cases, the lowest similarity coefficients were above the officially acceptable for the presence of clonal relatedness, which ranged between 70% and 75% (Grundmann et al., 197 Webster et al., 1996). As a result of these obtained results, we concluded that *Enterococcus durans* belong to the same strain shared between the hives. The strain was named after the first hive from which it was discovered - EDD2 (*Enterococcus durans* from Dushantsi, beehive No. 2).

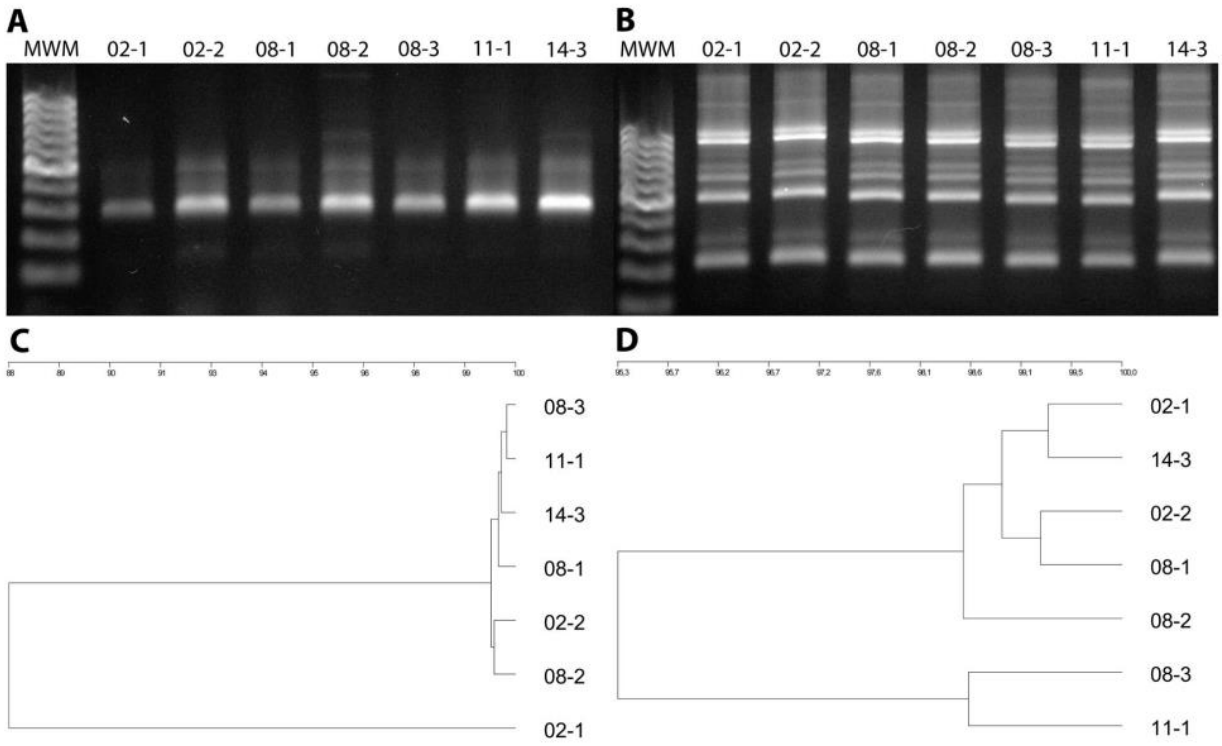


Figure No.1 – RAPD analysis

RAPD analysis was performed with primers L2 (Panels A and C) and E1 (Panels B and D). Panels A and B show the electrophoresis results with primers L2 and E1 (MWM is a DNA marker with a fragment size step of 100 base pairs,

while numbers represent isolates). Panels C and D reflect UPGMA, the dendrograms obtained with primers L2 and E1.

1.5. Molecular and phenotypic characterisation of *Enterococcus durans* strain EDD2

The first step in characterising the EDD2 strain was to confirm its taxonomic status. For this purpose, the 16S ribosomal locus sequence was analysed by Sanger sequencing. The obtained sequences (GenBank ID: MN 865967.1) confirmed the results of the conducted PCR using species-specific primers for *Enterococcus durans* DU1/ DU2, with the isolate confirmed to belong to this species. By performing PCR with primers targeting antibiotic resistance genes, we found that the strain we studied lacked vancomycin resistance genetic determinants *vanA, vanB, vanC1* and *vanC2*. A similar check for virulence factors did not detect the presence of genes encoding hyaluronidase, gelatinase, and collagen-binding protein. Resistance of strain EDD2 against antibiotics was also investigated phenotypically. For this purpose, an antibiogram was prepared using the disc-diffusion method. The reported results demonstrated that strain EDD2 exhibited sensitivity to the following antibiotics: ampicillin (zone of inhibition with a diameter of 30 mm), imipenem (32 mm), gentamicin (25 mm), ciprofloxacin (24 mm), levofloxacin (26 mm.), vancomycin (22 mm.), teicoplanin (21 mm.), linezolid (27 mm.) and tigecycline (20 mm.) (Table 2)., and clearly show the absence of resistance determinants in strain EDD2.

Table No. 2 - Antibiogram results

No	**Antibiotic**	**Result (zone of inhibition in mm.)**
1	Ampicillin	S (30)
2	Ciprofloxacin	S (24)
3	Erythromycin	S (23)
4	Gentamicin	S (25)
5	Imipenem	S (32)
6	Levofloxacin	S (26)
7	Teicoplanin	S (21)
8	Vancomycin	S (22)
9	Linezolid	S (27)
10	Norfloxacin	S (21)
11	Tigecycline	S (20)

Legend: S – susceptible, I – intermediate resistant, R – resistant. The results were interpreted according to the recommendations of the European Committee on Antibiotic Susceptibility Testing (EUCAST) 2019.

1.6. Antimicrobial activity of strain EDD2 against *Paenibacillus larvae*

The results of the antibacterial inhibitory activity of *Enterococcus durans* EDD2 against *Paenibacillus larvae* were demonstrated using the agar well-diffusion method (Fig. 2). Antibacterial bacteriocin activity was measured using arbitrary units per ml. (AU/ml) by performing two-fold serial dilutions. One arbitrary unit represents the highest dilution at which an appreciable zone of inhibition is present. The activity was calculated to be 640 AU/ml.

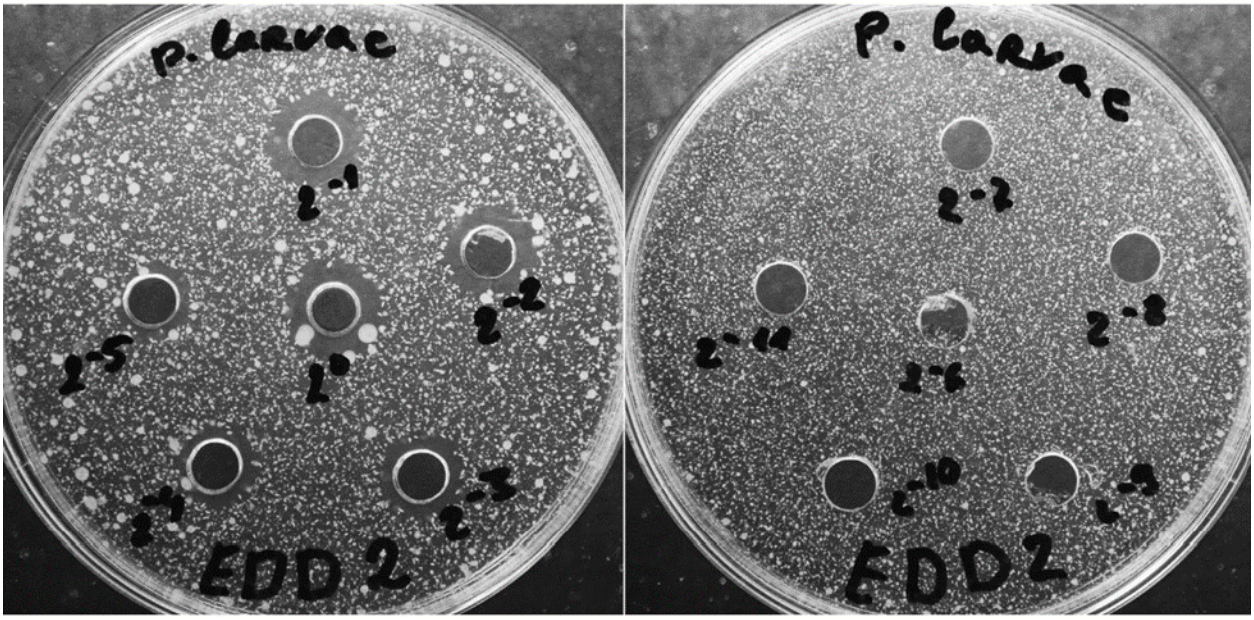


Figure No. 2 - Inhibitory activity of an *Enterococcus durans* EDD 2 against *Paenibacillus larvae*.

To calculate the inhibitory activity of the supernatant in arbitrary units per ml. two-fold serial dilutions were performed.

1.7. Characterization of *Enterococcus durans* EDD2 bacteriocins exhibiting activity against *Paenibacillus larvae*

To study the produced by *Enterococcus durans* EDD2 bacteriocin/s, we chose an approach based on bioinformatic analysis of the isolate genome sequence. Firstly, we sequenced its genome using Illumina's second-generation short-reading frame technology. Then, the sequencing data were analysed with the Galaxy platform. Finally, reads were pre-processed and assembled using

the integrated Shovill software. The resulting output consisted of 139 contigs larger than 500 bp in size (the largest contig was 155,300 bp). The resulting final draft genome sequence of *Enterococcus durans* EDD 2 consisted of 2,961,262 bp and had an average GC content of 37.71% (values typical of the species). We searched in the genome for bacteriocin genetic determinants using the BAGEL platform (de Jong, van H eel, Kok, & Kuipers, 2010) . Through this analysis, we identified two putative enterocin-like clusters, L50A / L50B (Fig. 3) and enterocin P (Fig. 4), localised on contig 48 and contig 49, respectively. As shown in Fig. 10, the open reading frames located on contig 48 are identical to the sequences of L50A and L50B at the amino acid level. In contrast, the bacteriocin-like sequences of contig 79 showed at the amino acid level 93% similarity to the enterocin sequence described in the literature as enterocin P.

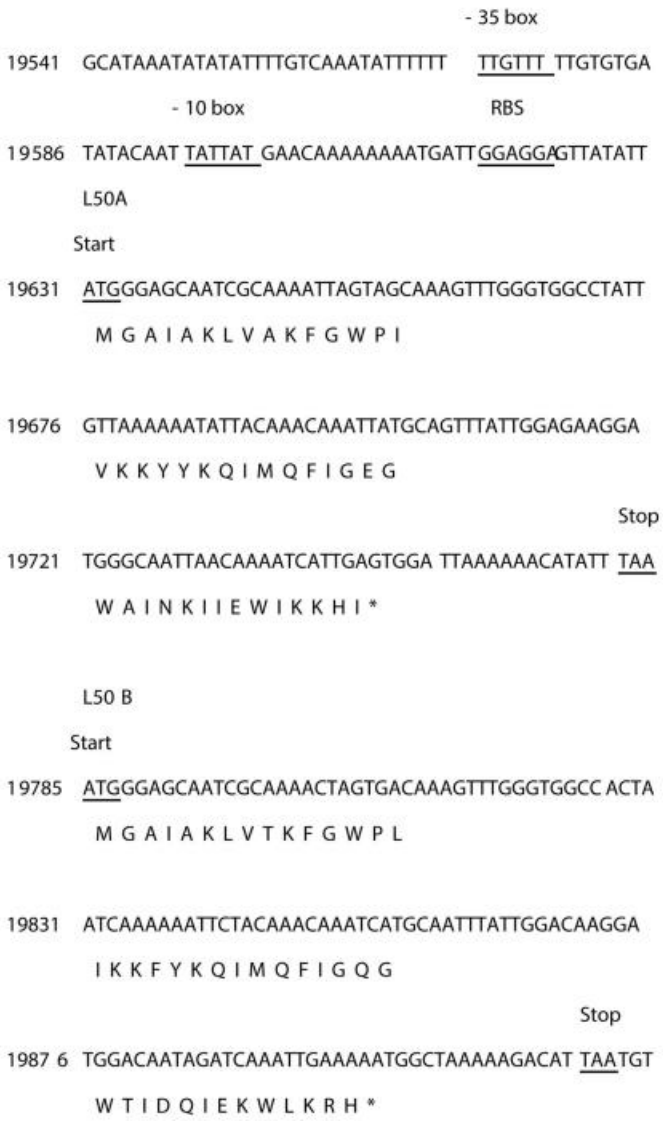


Figure No.3 - Sequences of L50A and L50B, which were found in contig 48.

The figure represents a nucleotide sequence containing structural genes of Ent50A (entL50A) and EntL50B (entL50B). All positions are given corresponding to contig 48. The corresponding amino acid sequence of EntL50A and EntL50B are shown below the DNA sequence. The two putative promoter panels at the -35 and -10 positions and the ribosome binding site are underlined. The two protein sequences are similar to those described by Cintas and co-authors (LM Cintas et al., 1998) .

The most notable difference was the start codon ATT, which we found in the *Enterococcus durans* EDD2 genome. By further manual inspection, we confirmed the presence of putative -10 and -35 promoter sequences and a ribosome binding site at the beginning of the open reading frame (Fig. 4).

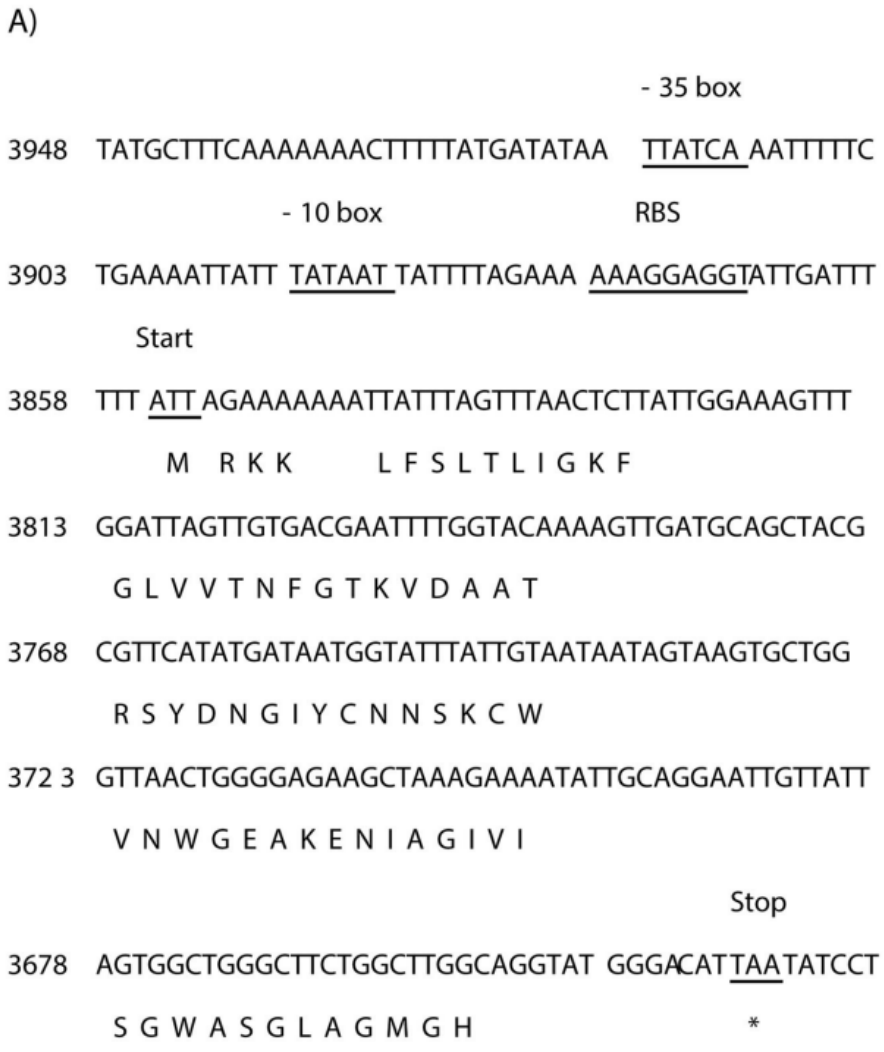


Figure No. 4 - Sequence similar to Enterocin P localised to contig 79.

Panel A: Nucleotide sequence of the Enterocin P structural gene (entP) found in an *Enterococcus durans* isolate EDD2. All positions are given according to contig 79. Encoded by enterocin P amino acid sequences are shown below the DNA sequence. The two putative promoter panels at the -35 and -10 positions and the ribosome binding site are underlined. These regulatory sequences are identical to those described by Cintas and co-authors (LM Cintas et al., 1998) . **Panel C**: Alignment of two enterocin P sequences generated by BLASTp. EntP EDD2 is the new putative bacteriocin discovered in *Enterococcus durans* EDD2. The enterocin P sequence is taken from Cintas and co-authors (Cintas, Casaus, Havarstein, Hernandez, & Nes, 1997) . All non-identical amino acids are underlined.

Subsequently, the assembled genome was analyzed for genetic determinants for bacteriocin production and plasmid origins of replication with the BAGEL4 and PlasmidFinder software tools. On contig 48 of the draft genome sequence, sequences similar to the L50A coding enterocins were found, and L50B (locus tags G 4 V 64_11725 and G 4 V 64_11730). In addition, a sequence analogous to the enterocin coding sequence was found on contig 79 P (locus tag G 4 V 64_13395). The assembled genome is publicly available in GenBank under accession number ASM 1097499 vI. Table 3 summarises leading genomic indicators and metrics obtained using the QUAST software, part of the online-based open-access Galaxy platform (Afgan et al., 2018) . In addition, a plasmid origin of replication was also detected on the same contig. These results are presented in table 4.

Table No.3 – Summary of results obtained from NGS the *Enterococcus durans* EDD2 in terms of genomic metrics

Type of analysis	Value	Tools used	Reference articles
Number of contigs greater than 500 bd	139 pcs.	QUAST software integrated into the Galaxy platform	(Afgan et al., 2018)
Longest contig	155,300 bd	QUAST software integrated into the Galaxy platform	(Afgan et al., 2018)
Final draft genome size	2,961,262 bd	QUAST software integrated into the Galaxy platform	(Afgan et al., 2018)
GC content	37.71%	QUAST software integrated into the Galaxy platform	(Afgan et al., 2018)
Analogues of bacteriocin genetic 3-determinants discovered.	Enterocin L50A/L50B on contig 48 Enterocin P on contig 79	BAGEL4 platform	(de Jong et al., 2010)
Plasmid origins of replication detected	Rep2 on contig 79	PlasmidFinder software	(Carattoli et al., 2014)

Table 4 - Summary of results obtained from NGS the *Enterococcus durans* EDD 2 concerning the bacteriocins and plasmids assays

Type of analysis	Value	Tools used	Reference articles
Analogues of bacteriocin genetic determinants discovered.	Enterocin L50A/L50B on contig 48 Enterocin P on contig 79	BAGEL4 platform	(de Jong et al., 2010)
Plasmid origins of replication detected	Rep2 on contig 79	PlasmidFinder software	(Carattoli et al., 2014)

1.8. Characterization of *Enterococcus faecium* by whole-genome sequencing

Potential *Enterococcus* were selected by initial selection on D-Coccosel medium. Supernatants were prepared from each isolate. Treatment of the supernatant with proteinase K

resulted in a loss of inhibitory activity against the indicator microorganism, demonstrating that the inhibitory agent(s) were a bacteriocin species (Fig. 5). As a result of the BLAST alignment of their 16S rRNA gene sequences, we determined that both isolates belonged to the species *Enterococcus faecium.* To characterise the two isolates, we performed whole genome sequencing. Further analyses of the whole-genome sequencing sequences showed that the isolates were clonally identical. Therefore, both belonged to a single strain, which we named *Enterococcus faecium* EFD. Due to this, we continued our work with only one of the isolates. Using the QUAST software, we evaluated the generated draft assembled genomes' metrics, resulting in the following results: a total length of 2,611,074 bp., N_{50} of 98,462 bp, GC content 38.03% and 69 contigs larger than 1000 bp. These values agree with those of NCBI's publicly available *Enterococcus faecium* genomes. In addition, the generated genomic data allowed us to simultaneously perform the different types of analysis we needed to determine if this strain could be considered as safe. All results are summarised in Table 5.

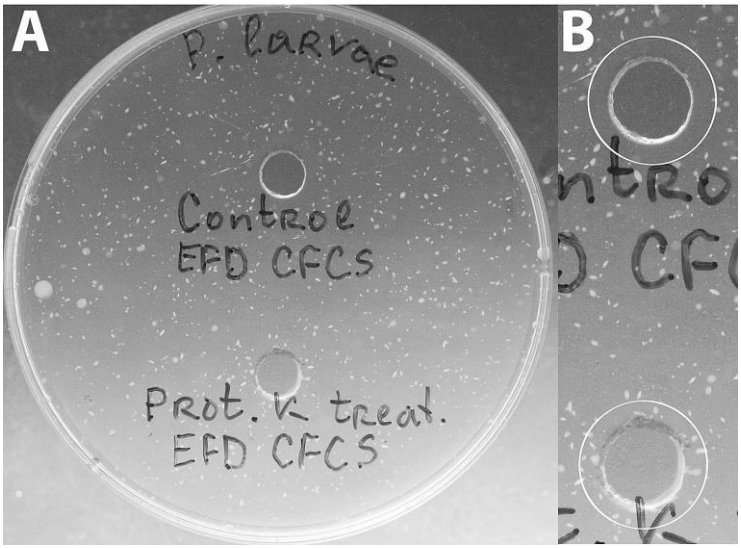


Figure No.5 - The agar-well method demonstrated the inhibitory activity of *Enterococcus faecium* EFD supernatant against *Paenibacillus larvae*. In the upper part, the inhibition of *Paenibacillus larvae* by an untreated supernatant showing a halo of 3 mm is presented. It represents a zone of inhibition without the growth of colonies of *Paenibacillus larvae*. At the bottom, the supernatant was treated with proteinase K, and the development of *Paenibacillus larvae* colonies was observed. Panel A – the entire Petri dish; Panel B – Magnification of the area with the agar wells. The halo with a lack of growth can be observed in the upper part.

Table No. 5 - Genetic characterisation of *Enterococcus faecium* EFD based on the results obtained from next-generation sequencing

Gene	Contig	Product/Function	Database Reference
Bacteriocins and antimicrobial components			
bac32	contig00038	Bacteriocin	NCBI: WP_074398906.1
entA	contig00001	Bacteriocin EntA	GenBank: AAD29132.1
entP	contig00043	Enterocin P	UniProtKB: A0A3F3G1D8
entSE-K4	contig00048	Enterocin SE-K4	UniProtKB: Q8GR39
entlA	contig00022	Enterolysin A	NCBI: WP_002331349.1
Virulence factors			
ac*	contig00001	Collagen adhesin	GenBank: CP003351.1
*efaA**	contig00025	Endocarditis-specific antigen A (adhesin)	GenBank: AF042288.1
Antibiotic resistance			
*aac(6')-Ii***	contig00001	Aminoglycoside 6′-N-acetyltransferase family	NCBI: WP_063978482.1
*msr(C)****	contig00004	ABC-F subfamily protein (MsrC efflux pump)	NCBI: WP_002314314.1
Plasmid origins of replication			
rep1	contig00027	repE(pIP816)	GenBank: AM932524.1
rep18a	contig00038	repA(p200B)	GenBank: AB158402.1

**Probably non-functional due to multiple substitutions, indels and frameshift mutations*

***The chromosomal AAC (6')-It is responsible for the loss of synergism between aminoglycosides (except gentamicin, amikacin and streptomycin) and penicillins or glycopeptides (antimicrobial agents that inhibit cell wall synthesis)*

****Intrinsic resistance to macrolides and low-level resistance (MIC 1–2 μg/ml) to streptogramin B compounds*

As a result of the screening performed for the presence of genetic determinants of bacteriocins, we can say that the antibacterial activity of *Enterococcus faecium* EFD may be the result of the activity of one or more of the five bacteriocins found: bacteriocin 32, enterocin A, enterocin P, enterocin SE -K4 and enterolysin A.

2. Microbial isolates from food products

2.1. Preparation of a collection of isolates

To characterise microbial isolates of food products, we chose 31 pcs. naturally fermented dairy products (cheese and homemade cheeses from cow, goat, buffalo or sheep milk), meat

products (sausage, sujuk and dry salami), sourdough bread, sauerkraut, olives. To cover the full diversity and local variations in the composition, the products used in the experiments were selected from different regions of the country: the city of Ruse, the village of Mokresh, the Montana area, the Iskra village, Parvomai, the village of Kozarsko, Bratsigovo, the city of Sofia, the village of Cherni Vit, as well as from Greece and Italy.

The strains were isolated from different food products, and a collection of 303 isolates was prepared. An isolate from each food product was cultured in Petri dishes, and the media used to culture the isolates were as follows: Brain Heart Infusion (BHI) broth and agar (*Merck*), De Man, Rogosa and Sharpe (MRS) broth and agar (*Merck, Scharlau Chemie*), M17 broth and agar (*Merck, Scharlau Chemie*), Luria Broth (LB) broth and agar (*Scharlau Chemie*), Elliker broth and agar (*Scharlau Chemistry*). A selective medium used in the cultivation of bacterial morphological and physiological characteristics was D-Coccosel agar (*bioMerieux, Marcy l' Etoile, France).* The total number of colonies varied between 14 and 42 per Petri dish. Isolation of single colonies from D-Coccosel selective medium in the Petri dishes was performed based on the presence of a black halo around the bacterial colony. Three colonies with a distinct halo were randomly selected from each Petri dish. These isolates were assigned with unique numbers encoding the following: a letter symbol at the beginning corresponding to the food product from which the isolate originated and a four-digit character indicating the following: the first digit represents the type of culture medium, the second the type of product and the last two correspond to the sequence number of the respective isolate.

2.2. Initial strain characterisation

In the process of initial characterisation, all tested isolates (including those isolated from bee products) were tested for the presence of bacteriocin and/or proteolytic activity (Fig. 6). In addition, we prepared antibiograms calculated against the EUCAST criteria, presented in Table 2. Futhermore, all strains were also tested for phenotypic antibiotic resistance. The results showed that the isolates we studied were susceptible to all tested antibiotics, and there were no resistant strains according to the quantitative criteria used.

From the total number of analysed strains (348), isolates exhibiting bacteriocin activity were 97, those with proteolytic activity were 23, and simultaneously both types of activity were reported in 9. The remaining 219 strains did not demonstrate activity (Fig. 6).

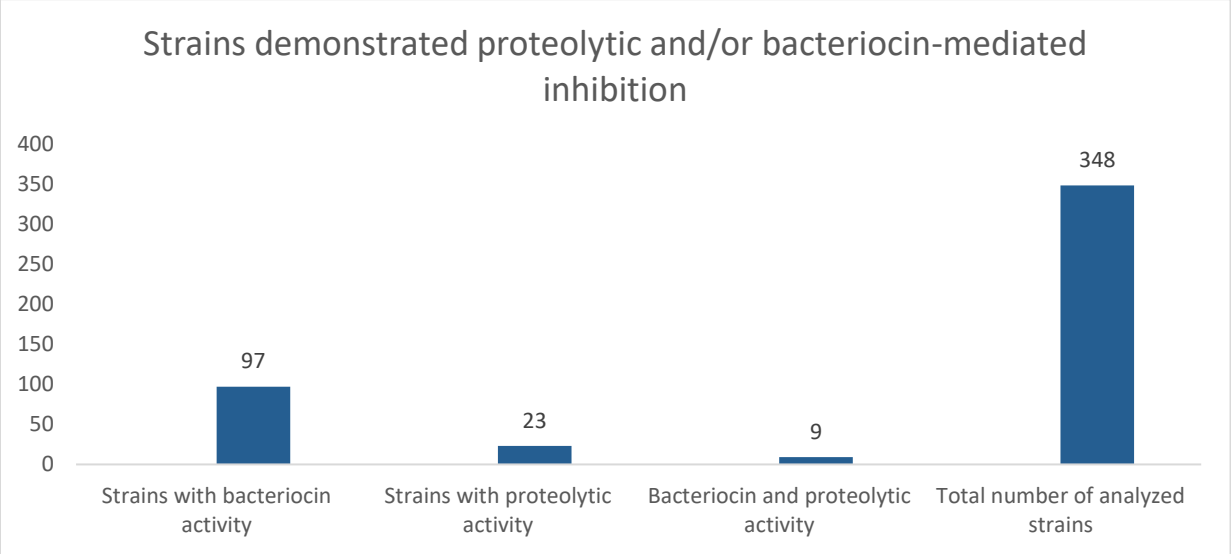


Figure No. 6 – Strains demonstrated proteolytic and/or bacteriocin-mediated inhibition

To establish the full antipathogenic spectrum of action, isolates with pronounced bacteriocin activity were tested against a panel of nine strains. They included representatives of different taxonomic groups with potential pathogens in them. For this purpose, the agar-well method was applied. The strains from the panel, along with the temperatures and culture media, are described in Table 6.

Table No. 6

no	Strain	Cultivation medium	Cultivation temperature
1	*Enterobacter aerogenes* EA	BHI	30°C
2	*Enterococcus durans*	BHI	30°C
3	*Enterococcus faecalis* FL 3915	BHI	30°C
4	*Enterococcus faecium* FM 3587	BHI	30°C
5	*Lactococcus gariveae* LG	BHI	30°C
6	*Listeria monocytogenes* LM	BHI	30°C
7	*Staphylococcus aureus* SA	BHI	30°C
8	*Yersinia ruckeri* YR	BHI	30°C
9	*Paenibacillus larvae*	MPA	30°C

Figures No. 7 - 12 show exemplary results of the tests carried out by the agar-well method for the presence of activity against the following strains: *Enterococcus faecalis* (Fig. 7), *Enterococcus faecium* (Fig. 8), *Yersinia ruckeri* (Fig. 9), *Lactococcus gariveae* (Fig. 10), *Listeria monocytogenes* (Fig. 11), *Paenibacillus larvae* (Fig. 12). Zones of inhibition can be observed as halos around the wells.

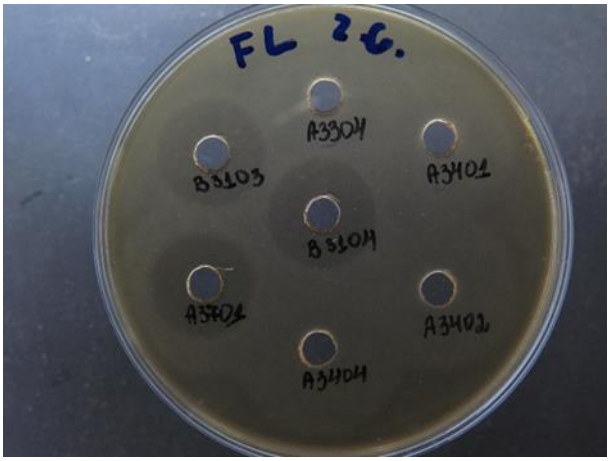


Figure No.7 - Agar-well strain testing against *Enterococcus faecalis*

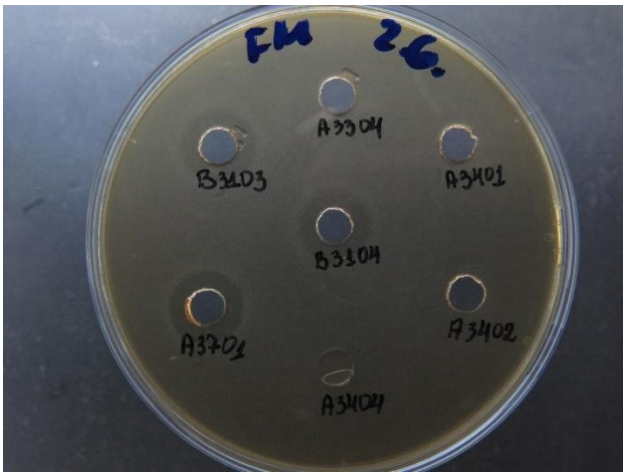


Figure No.8 - Agar-well strain testing against *Enterococcus faecium*

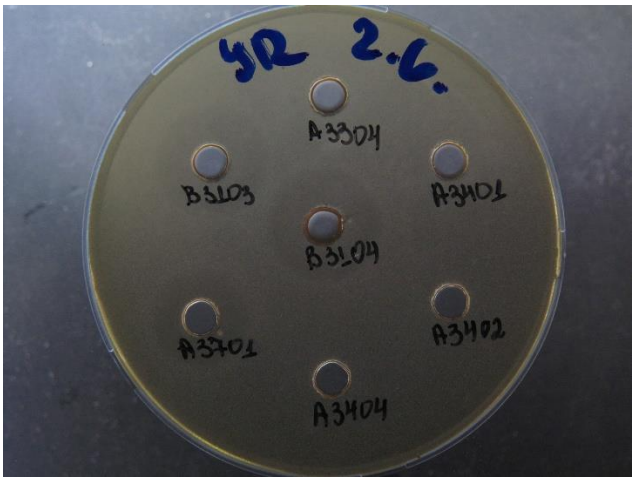


Figure No.9 - Agar-well strain testing against *Yersinia ruckeri*

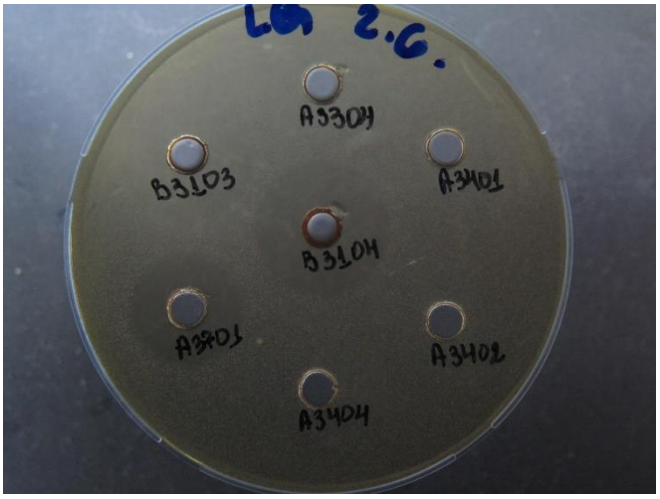


Figure No.10 - Agar-well strain testing against *Lactococcus gariveae*

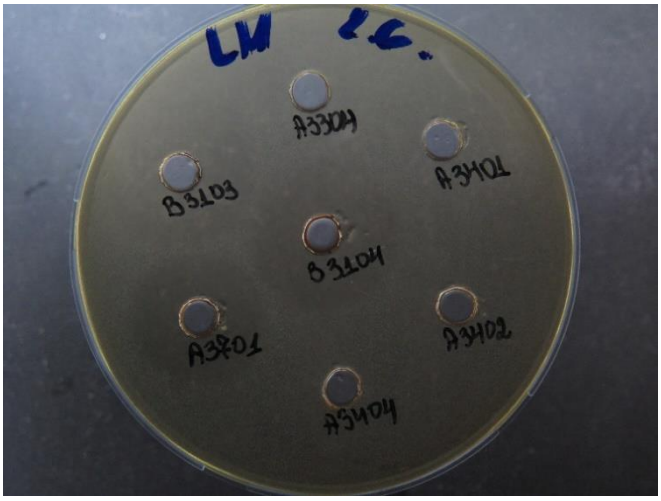


Figure No. 11 - Agar-well strain testing against *Listeria monocytogenes*

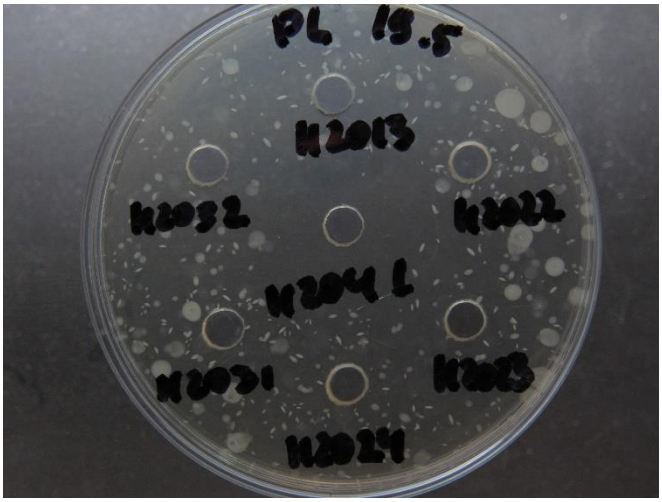


Figure No. 12 - Testing of strains by the agar-well method against *Paenibacillus larvae*

As a result of the tests, the following was found: forty-one isolates showed bacteriocin activity against one strain, twenty-six isolates demonstrated bacteriocin activity against two strains, seven isolates exhibited bacteriocin activity against three strains, eight isolates showed bacteriocin activity against four strains, seven isolates demonstrated bacteriocin activity against five strains, four isolates exhibited bacteriocin activity against six strains, zero strains showed bacteriocin activity against seven strains, three isolates demonstrated bacteriocin activity against eight strains and one isolate showed bacteriocin activity against nine strains (Fig. 13).

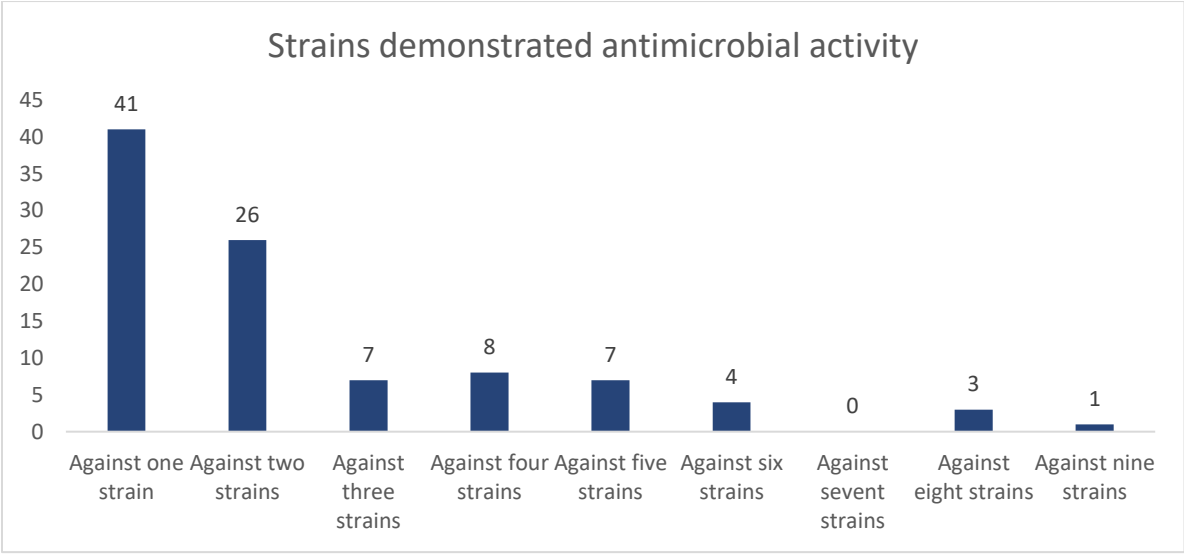


Figure No. 13 – Strains demonstrated antimicrobial activity against different numbers of strains

Inhibition of the development of pathogenic microorganisms was mainly observed in isolates from the following groups of products (Table 7):

Table No. 7 – Products from which the strains were isolated and their activities

Activities	Products from which the strains were isolated
Against 1-2 strains	• Bee hives (Vidin, Sofia, Dushantsi and Momchilovtsi villages); • Italian dry salami; • Green Cheese (Cherni Vit village); • Goat whey, soft goat cheese and sheep yoghurt from Producer No. 1 (Iskra village, Parvomai Municipality).
Against 3-5 strains	• Bee hives (Vidin, Sofia and Dushantsi village); • Sheep yoghurt from Producer No. 1; • Sujuk from Producer No. 2 (Kozarsko village, Bratsigovo Municipality); • Sour cabbage; • Sheep cheese and sheep yoghurt from Producer No. 3 (Mokresh village, Montana region).
Against 6-9 strains	• Sourdough Bread; • Sujuk from Producer No. 2.

2.3. Performing species determination of selected isolates using 16S sequencing

We selected a group of the most interesting strains based on the spectrum and strength of established antimicrobial activity and protease production. From all isolates in the pool, we isolated total DNA and sent aliquots to Macrogen (macrogen.com) to determine the sequences of the 16S regions by Sanger sequencing. The obtained results are described in Table 8.

Table No. 8 - Sequencing results of the 16S regions of the analysed strains

No	Strain	Product from which the strain was isolated	16S sequencing results
1	A1401	Producer No. 2 SRN Cheese "Reserve"	*Lactiplantibacillus plantarum*
2	A1404	Producer No. 2 SRN Cheese "Reserve"	*Lactiplantibacillus plantarum*
3	A1604	Producer No. 2 LN Lukanka	*Pediococcus pentosaceus*
4	A4102	Producer No. 2 PMN Probiotic Milk	*Lactiplantibacillus plantarum*
5	B1103	Sourdough	*Enterococcus faecium*
6	B1104	Sourdough	*Lactiplantibacillus plantarum*
7	B3102	Sourdough	*Lactiplantibacillus brevis*
8	BH111201	Bee strains from: Obelia beehives, Gut, late fall	*Enterococcus faecalis*

9	BH111203	Bee strains from: Obelia beehives, Gut, late fall	*Enterococcus faecalis*
10	BH121206	Bee strains from: Obelia beehives, Pollen, late fall	*Rosenbergia*
11	C2202	SYH - Sheep yoghurt from Producer No.1	*Enterococcus faecalis*
12	D1101	Sour Cabbage	*Lactiplantibacillus plantarum*
13	D1102	Sour Cabbage	*Lactiplantibacillus plantarum*
14	D4103	Sour Cabbage	*Lactiplantibacillus plantarum*
15	F10504	GYCM75 - Goat Cheese from Producer No.3	*Enterococcus faecalis*
16	F10804	GFCM75 - Soft Goat Cheese from Producer No.3	*Leuconostoc mesenteroides*
17	F10902	HMCM75 - Homemade Cheese from Producer No.3	*Enterococcus faecalis*
18	H1041	Green Cheese from the village of Cherni Vit - Sheep's Hard Cheese aged 8 months	*Enterococcus faecalis*
19	H2011	Green Cheese from the village of Cherni Vit - Goat's Cheese aged 5 months	*Serratia sp.*
20	H2013	Green Cheese from the village of Cherni Vit - Goat's Cheese aged 5 months	*Staphylococcus aquorum*
21	H2022	Green Cheese from the village of Cherni Vit - Soft Sheep's Cheese aged 8 months	*Staphylococcus aquorum*
22	H2024	Green Cheese - Soft Sheep Cheese aged 8 months	*Enterococcus faecalis*
23	H2031	Green Cheese from the village of Cherni Vit - Sheep's Hard Cheese aged 7 months	*Staphylococcus gallinarum*
24	H2032	Green Cheese from the village of Cherni Vit - Sheep's Hard Cheese aged 7 months	*Staphylococcus aquorum*
25	H2041	Green Cheese from the village of Cherni Vit - Sheep's Hard Cheese aged 8 months	*Staphylococcus aquorum*
26	H4011	Green Cheese from the village of Cherni Vit - Goat's Cheese aged 5 months	*Serratia species.*
27	H4021	Green Cheese from the village of Cherni Vit - Soft Sheep's Cheese aged 8 months	*Proteus vulgaris*

2.4. Phylogenetic studies of selected isolates by RAPD

To clarify the phylogenetic relationships and the presence of possible clonal connectivity, twenty-one isolates belonging to *Enterococcus faecalis* and twenty-one isolates belonging to *Lactiplantibacillus plantarum* with proteolytic and/or bacteriocin activity were subjected to RAPD analysis. The primers E1 and P2 and E1/P2 in combination were used for this study. The calculation of the similarity matrices based on the gel electrophoresis image and the construction

of the UPGMA dendrograms was performed using the GeneTools software (Syngene) by the Jaccard (1901) algorithm.

The results in the form of UPGMA dendrograms are shown in Fig. 14 and Fig. 15.

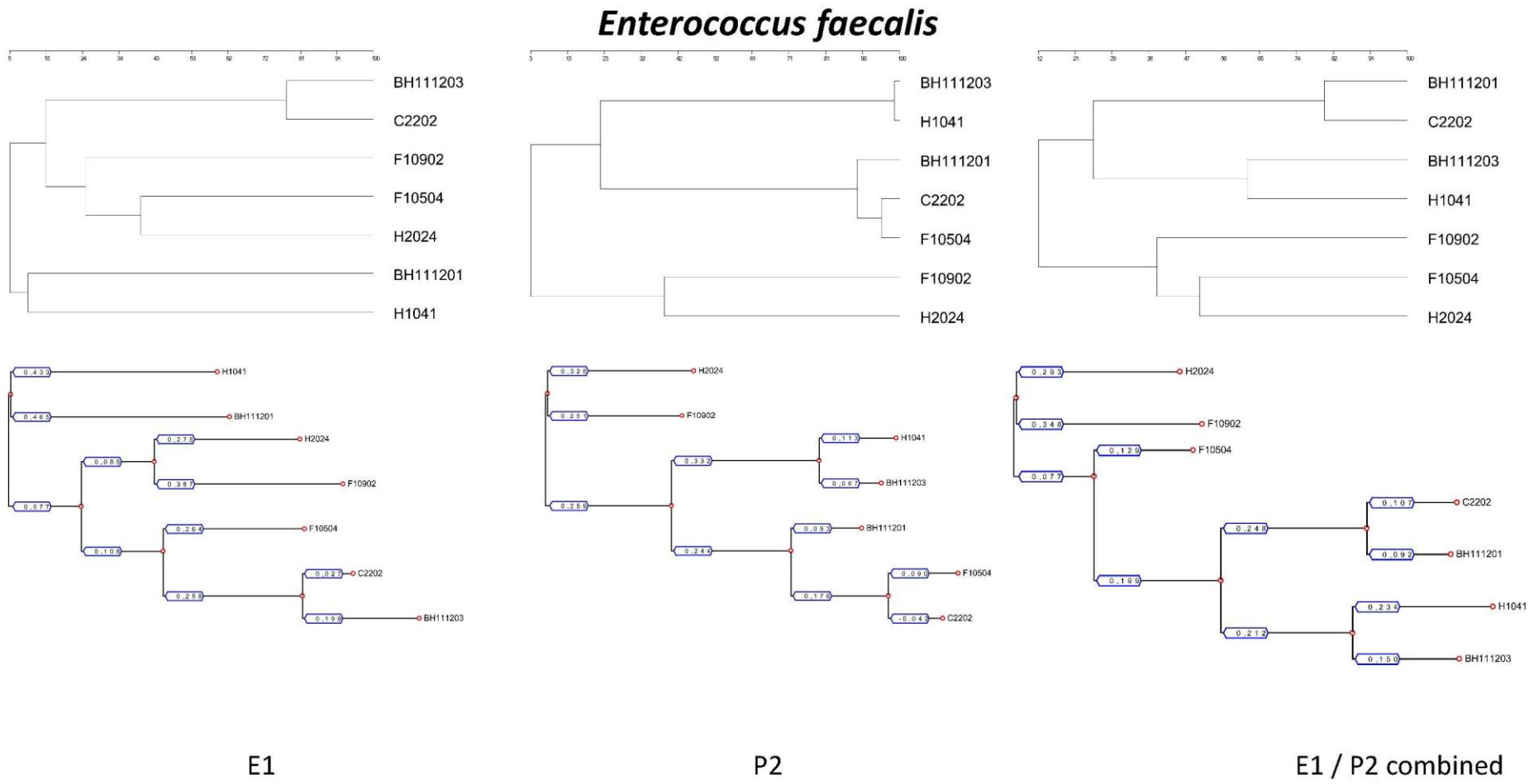


Figure No.14 - UPGMA dendrograms of *Enterococcus faecalis* obtained with primers E1 and P2 after RAPD analysis.

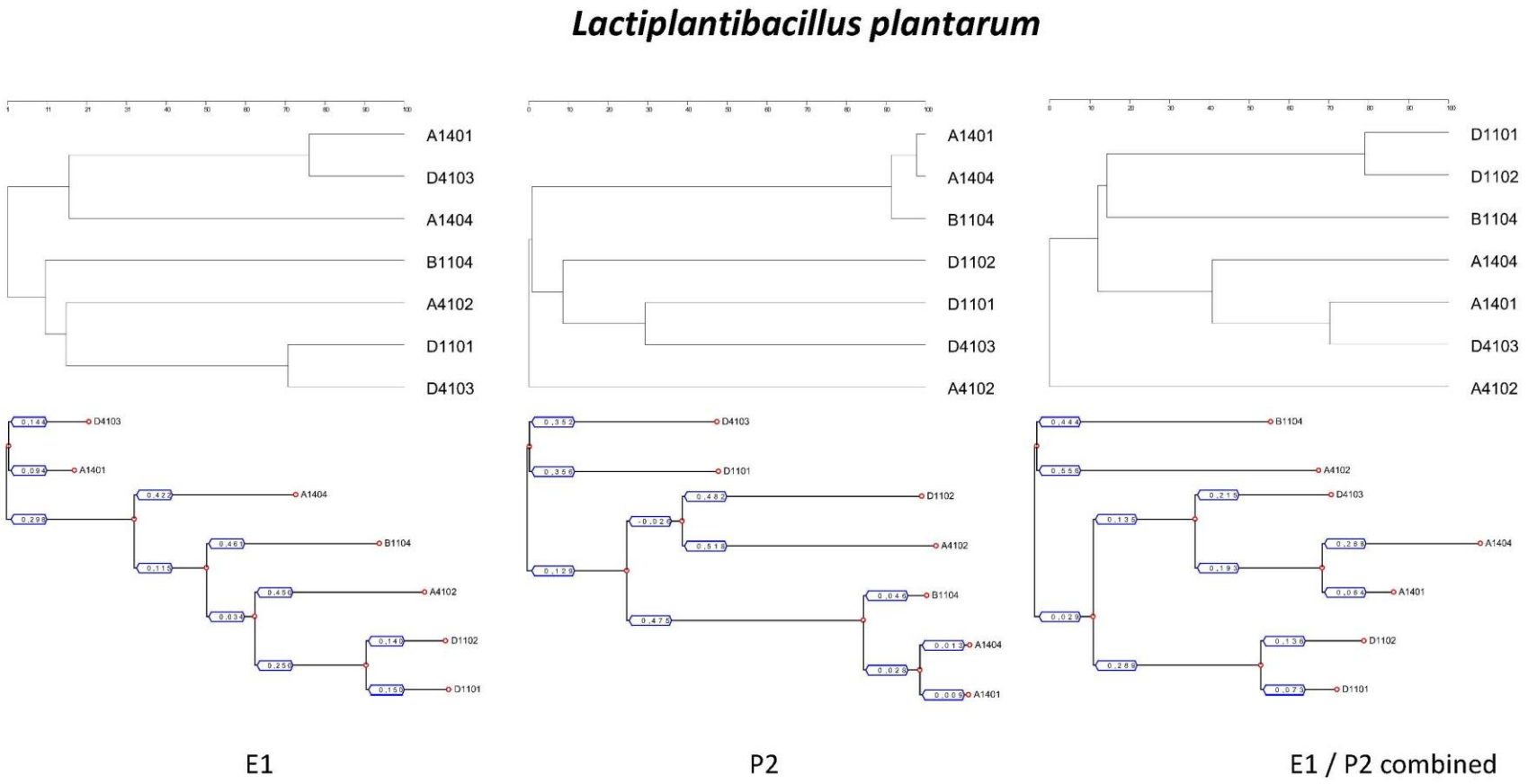


Figure No.15 - UPGMA dendrograms of *Lactiplantibacillus plantarum* obtained with primers E1 and P2 after RAPD analysis.

Prevailing thresholds for grouping clonally related bacterial strains described in scientific publications generally range between 70% and 75% similarity (Grundmann et al., 1997 ; Webster et al., 1996) . With these generally accepted criteria, the formed clusters mean the presence of clonal similarity between the analysed strains.

In the study of phylogenetic relatedness of potentially bacteriocinogenic representatives of *Enterococcus* species, Dimov and co-authors demonstrate that this threshold leads to inconclusive results (S. Dimov, Str a teva, Petkova, Dimitrova, & Atanasova, 2015) , and according to the authors' collective, the threshold from 70% to 75% should be considered as an indication of some clonal relationship to a limited extent. Our results agree with this study for the following reasons: at 70% similarity, the clusters formed in *Enterococcus faecalis* with primer P2 consist of a beehive isolate (BH111203) and a green cheese isolate (H1041). With primers E1/P2 in combination, the cluster is formed of an isolate from a beehive (BH 111201) and an isolate from a dairy product (C2202 - sheep yoghurt) was again observed. The isolates are from different sources and geographical regions of the country. It is observed that as the similarity threshold increased to 95%, unclustered strains were observed.

Cluster formation was similarly observed in *Lactiplantibacillus plantarum* using primers E1, P2 and E1/P2 in combination. When considering clonal similarity with a threshold of 70%, three homogeneous and one heterogeneous cluster by strains D4103 and A1401, isolated from sauerkraut and cow's cheese, respectively, were formed.

The observed heterogeneity in the clusters formed does not suggest segregation of the strains based on their nature or geographic origin. Therefore, in agreement with the results of Dimov and co-authors, we can conclude that using a 70% similarity threshold is inappropriate when examining strains of the clonal relationship of different types and originating from different locations. The higher threshold of 95% required for grouping the isolates could probably indicate the separate evolutionary lineages of the strains we studied, showing greater relatedness between them.

3. Further analysis of microbial isolates from green cheese

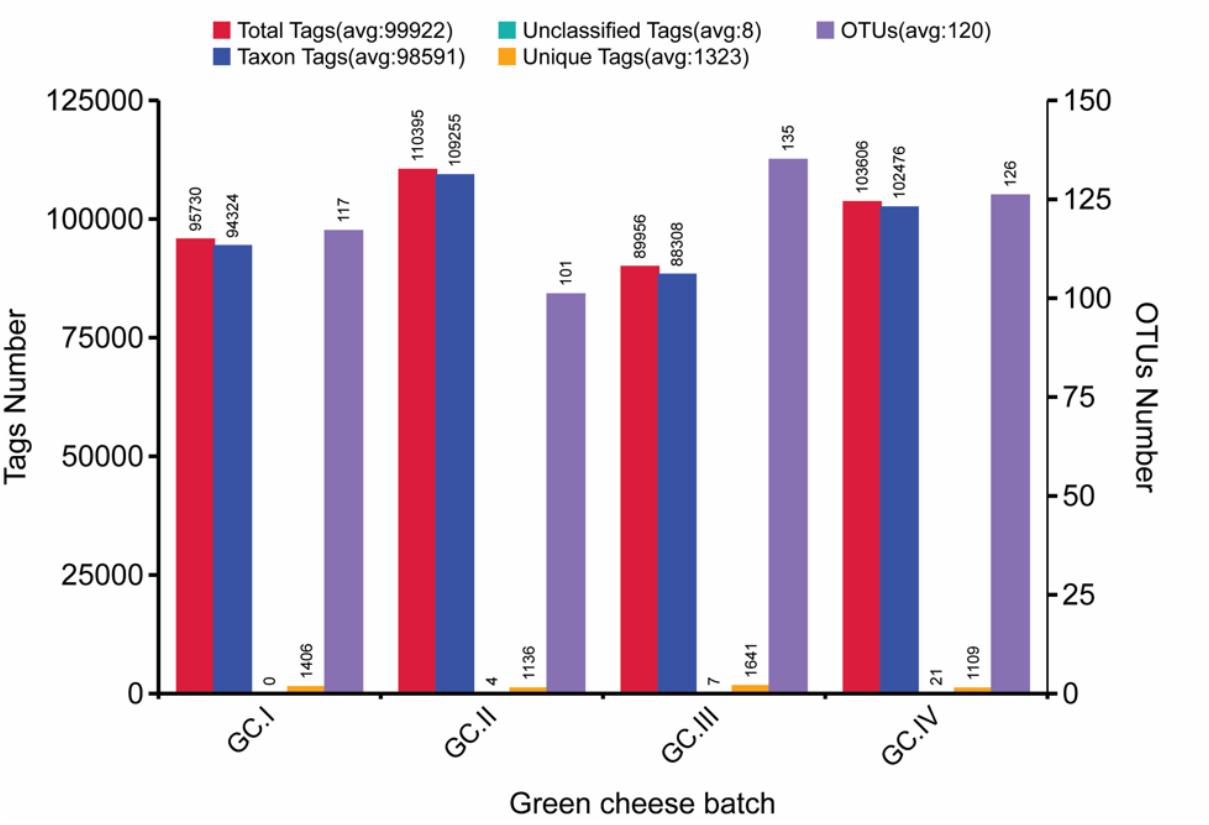
3.1. Results from next-generation amplicon metagenomic sequencing

To determine the species diversity of the microorganisms in the traditional Bulgarian Green Cheese from the Teteven region, we applied the next-generation amplicon metagenomic sequencing method. The regions targeted for amplification and sequencing were the V3 - V4 region of the 16S locus in bacteria and the ITS2 region in fungi. The defined sequencing reads

generated using the Illumina platform HiSeq were of sufficiently good quality for further analyses. The percentage of effective tags obtained varied between 66.20% and 78.98% for the V3-V4 region sequences and between 88.32% and 95.75% for the ITS2 region. The higher percentages for ITS2 are partly explained by its shorter length (386 bp) compared to the 16S target region (466 bp), despite the relative values of the Q20 and Q30 parameters indicating the sequencing quality (about 98% and respectively 94%). The resulting average lengths of both sequence types are close to expected.

3.2. Determination of the number of operational taxonomic units (OTUs)

We determined the number of operational taxonomic units - the results are shown in Fig. 16 and summarised in the Table 9. The average values of the various indicators for the bacterial 16S sequences of the four batches of Green Cheese were: effective tags - 99922, annotated tags - 98591, unclassified tags - 8 and unique tags - 1323. For fungal species, in the analysis of the ITS2 regions, the corresponding values were: effective tags - 93984, annotated tags - 93793, unclassified tags - 0 and unique tags - 190. These were merged into OTEs, totalling 179 for bacterial species and 23 for fungal species. The average OTEs were 120 for bacterial and 16 for fungal species. Of the bacterial species, 47 OTE counts consisted of more than 100 sequences (approximately more than 0.1% of the total), while the number of similarly composed OTEs of fungal species is 11. As a result of the comparison of OTEs obtained based on the effective tags with ≥97% similarity (Table 9, Fig. 16), in the samples of the analysed four lots of Green Cheese, from 117 to 135 were identified (average of 120) bacterial species and from 12 to 20 (average 16) fungal species.



Graph A: Eubacterial OTEs based on performed 16S analysis

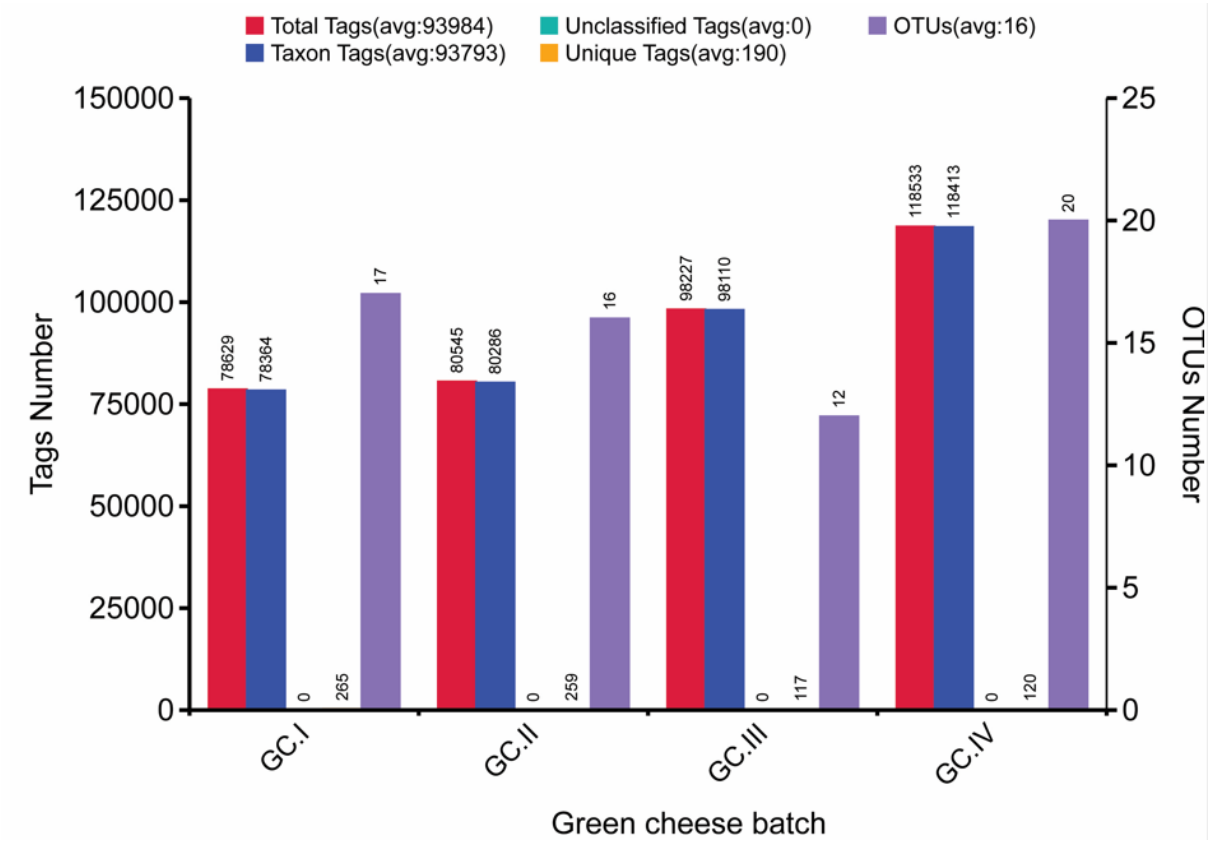


Chart B – OTE in fungal species based on ITS2 analysis

Figure 16 - Summary of the number of tags and the number of operational taxonomic units in each sample.

In bacterial species (Chart A) and fungal species (Chart B).

Table No. 9 - OTE analysis

A batch of green cheese	Number of effective tags	Number of annotated tags	Number of unclassified tags	Number of unique tags	Number of OTE
Section A – Bacterial 16S Assay					
GC I	95730	94324	0	1406	117
GC II	110395	109255	4	1136	101
GC III	89956	88308	7	1641	135
GC IV	103606	102476	21	1109	126
Average	*99922*	*98591*	*8*	*1323*	*120*
Section B – analysis of the ITS2 region in fungi					
GC I	78629	78364	0	265	17
GC II	80545	80286	0	259	16
GC III	98227	98110	0	117	12
GC IV	118533	118413	0	120	20
Average	*93984*	*93793*	*0*	*190*	*16*

3.3. Taxonomic annotation of OTE

Using the Qiime software v.1.7.0, we obtained information for the number of operational taxonomic units in each sample. Based on the gained results, we built taxonomic trees of groups composed of the first ten genera in bacterial (Fig. 17) and fungal species (Fig. 18) - twenty bacterial species and ten fungal species. Among bacterial OTEs, the most predominant phylum was Firmicutes (50.61%), represented by the genera *Streptococcus*, *Staphylococcus*, *Lactobacillus,* and *Lactococcus*, which was followed by Actinobacteria (42.96%), represented by the genera *Brevibacterium*, *Corynebacterium,* and *Kocuria*, and finally, the Proteobacteria (6.43%) represented by the genera *Cobetia*, *Psychrobacter* and *Halomonas*. The most significant percentage (99.99%) of the examined species of fungi belonged to Ascomycota, represented by *Penicillium roqueforti, Scopulariopsis flava, Debaryomyces hansenii, Pichia membranifaciens, Candida zeylanoides, Kluyveromyces lactis, Torulaspora delbrueckii, Fusarium oxysporcium* and *Trichothecium roseum*, while Zygomycota (0.01%) is mainly represented by *Circinella muscae*. The results of the relative abundance of taxon types are presented in Fig. 19, where three bacterial phyla (Firmicutes, Actinobacteria and Proteobacteria) and two fungal phyla (Ascomycota and Zygomycota) together form almost 100%. We compiled a taxonomic cluster heatmap according to the information on the species diversity we received. It is based on the predominant 35 genera from all samples, and the similarities and differences between them are presented in Fig. 20 (the fungal heatmap includes only 11 genera due to the smaller number of observed genera).

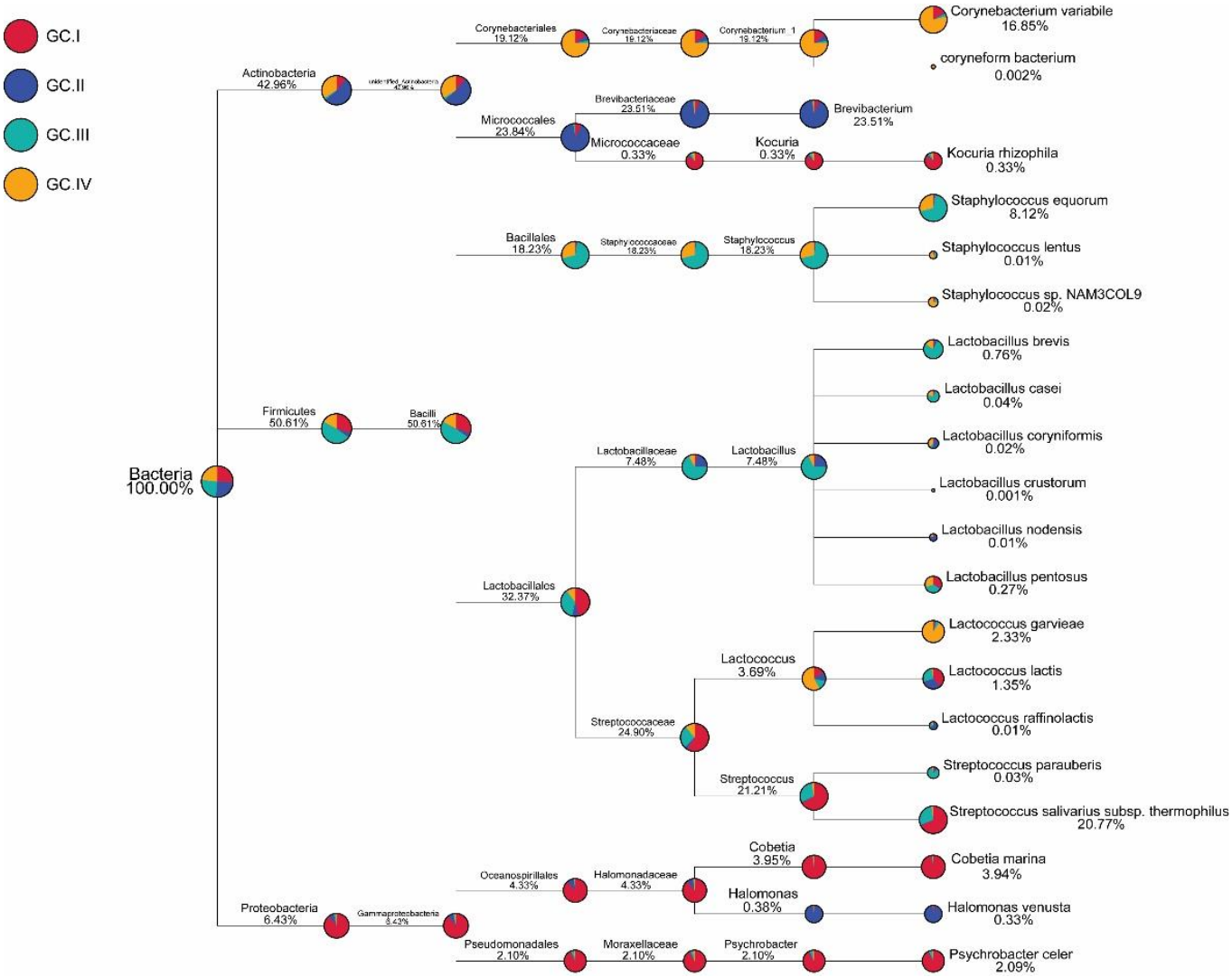


Fig . 17 - Taxonomic tree of the bacterial group.

The size of the circles represents the relative species abundance. Percentages are presented in taxa.

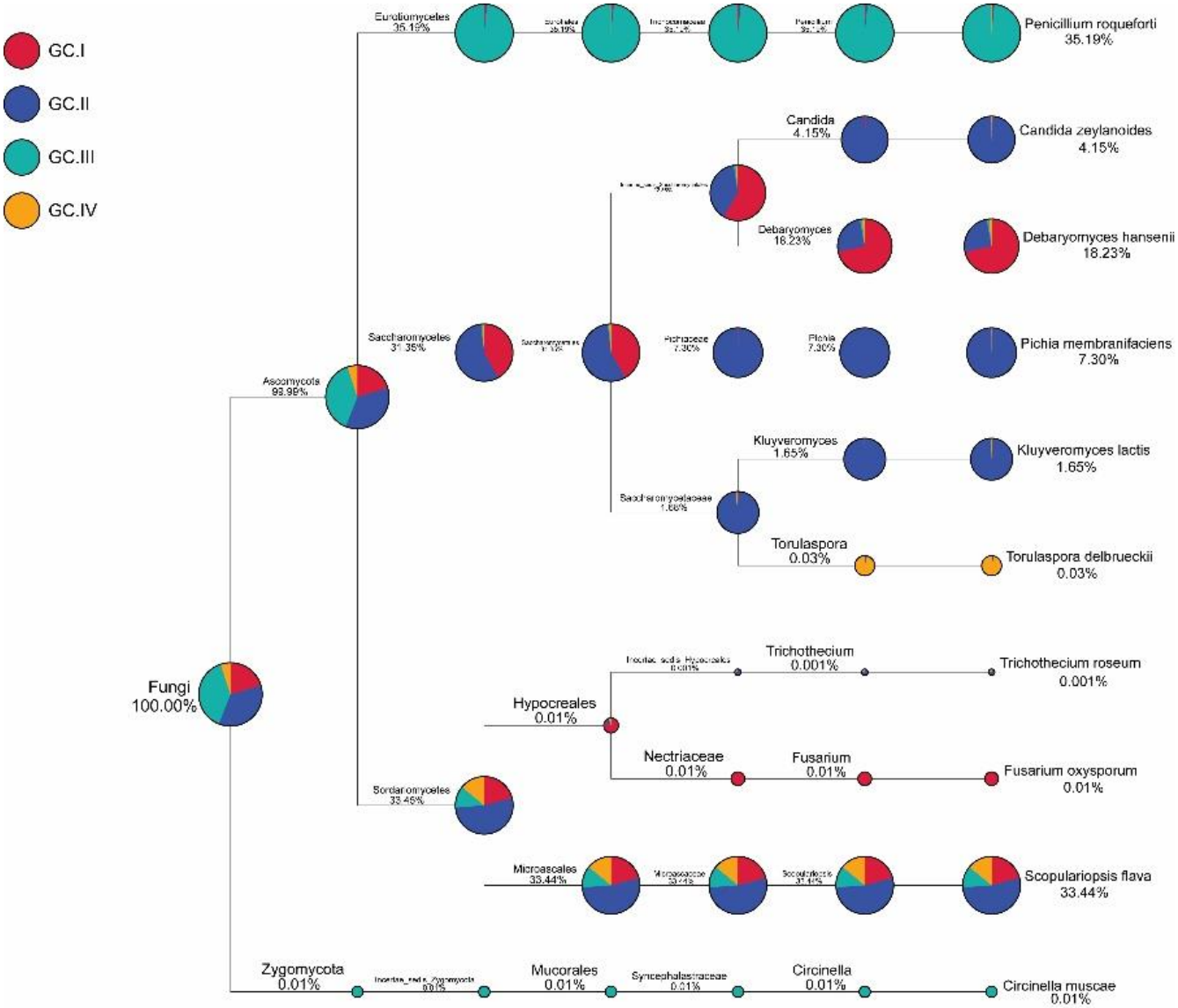
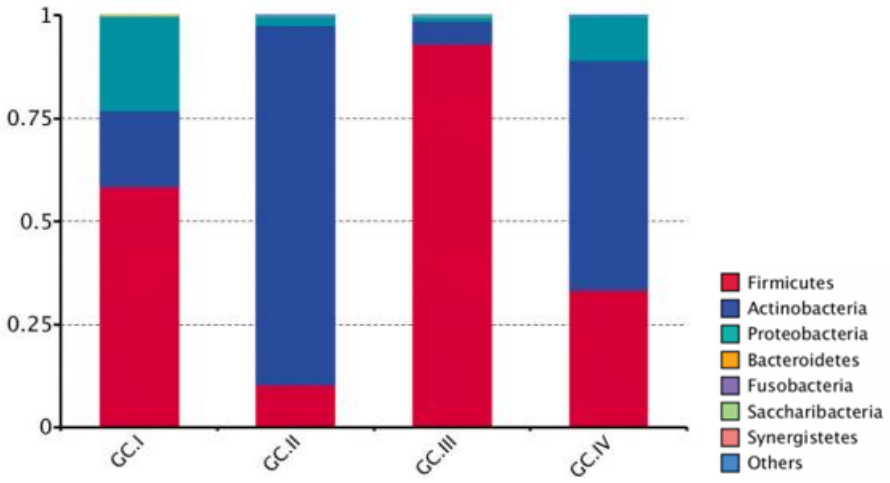
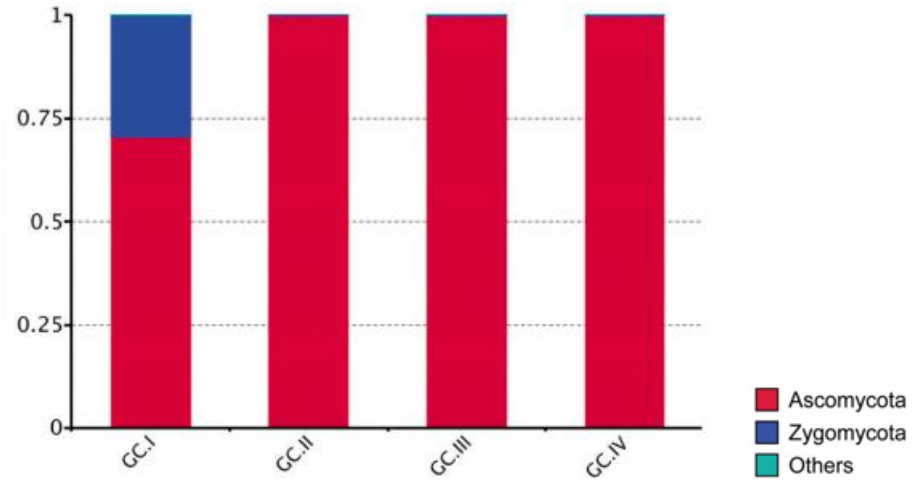


Fig. 18 - Taxonomic tree of a fungal group.

The size of the circles represents the relative species abundance. Percentages are presented in taxa.



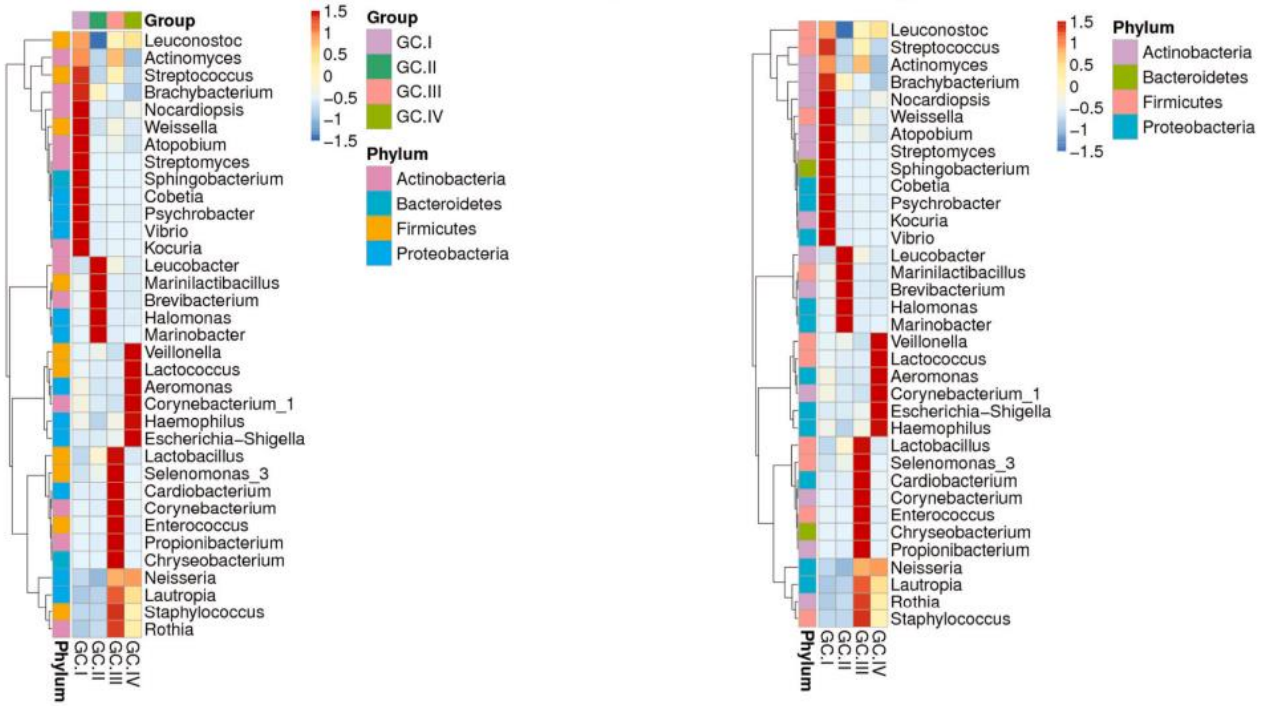
Graph A: Comparative diversity of eubacterial phyla



Graph B: Relative diversity of fungal types

Fig. 19 - Relative abundance of observed types.

Graph A – eubacterial types; Chart B - types of fungi.



Graph A: Heat map indicating the taxonomic abundance of genera in eubacteria

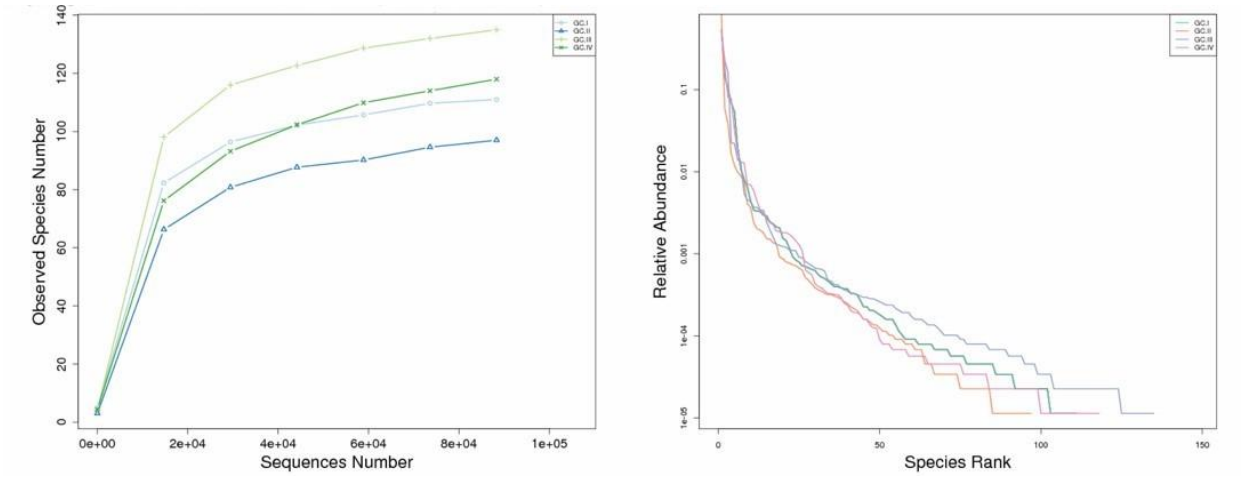
Graph B: Heat map indicating the taxonomic abundance of fungal genera

Fig. 20 - Heat map indicating the taxonomic abundance of genera.

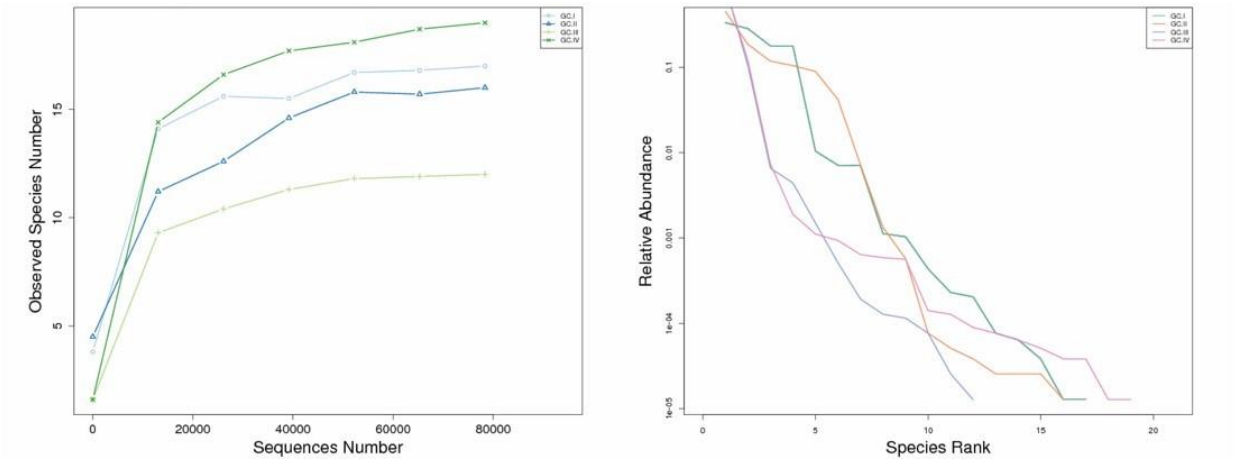
In bacteria (Chart A) and fungi (Chart B). The sample name is plotted on the X-axis, while the Y-axis indicates the genus. The absolute value represents the standard deviation distance between the raw result and the average value - it is negative when the raw result is below the average value and vice versa.

3.4. Analysis of Alpha diversity

Dilution curves and species diversity of bacterial and fungal species of the Green Cheese batches were calculated based on the Alpha diversity analyses. They are shown in Fig. 21, Panel A, and the information therein allows for a primary microbiome assessment. In this study, all batches showed similar curves for bacterial and fungal species. The curves characterising the biodiversity of bacteria and fungi in the batches of Green Cheese are shown in Fig. 21 Panel B.



Panel A: Curves characterising the biodiversity of bacterial species



Panel B: Biodiversity curves for fungal species

Figure No. 21 – Curves characterising biodiversity.

Bacterial species (Panel A) and fungal species (Panel B) were calculated from the Alpha diversity analyzes of the Green Cheese samples.

Chao1 and ACE Alpha diversity indices were used to estimate the expected species richness within the four cheese lots (Kim et al., 2017) . The lowest values in bacterial species for both indices were observed in GC II, but they were much closer to the values of the other three batches. A similar pattern was observed in the analysis of the diversity of the fungal species. The calculated values of the Chao1 and ACE indices are very close to the observed number of bacterial and fungal species, which is not surprising since the calculated Good coverage index in all samples was 1.000, meaning that all samples were representative of all species present in samples (Chao , Lee , & Chen, 1988 ; Zhang et al., 2018) . As a final step, we calculated total phylogenetic diversity indices, which provide information on the phylogenetic realisation of species richness but do not account for species abundance (Faith , 1992) . For the fungal species analyses, the index values showed the same trend as the Chao1 and ACE indices, but no such correlation was observed in the bacterial analyses, probably due to the much larger number of species compared to fungi.

Venn colour diagrams are presented in Fig. 22, based on the common and unique content analysis from the different OTE batches. The numbers of the corresponding types of OTE found in the different batches are also indicated in the figure.

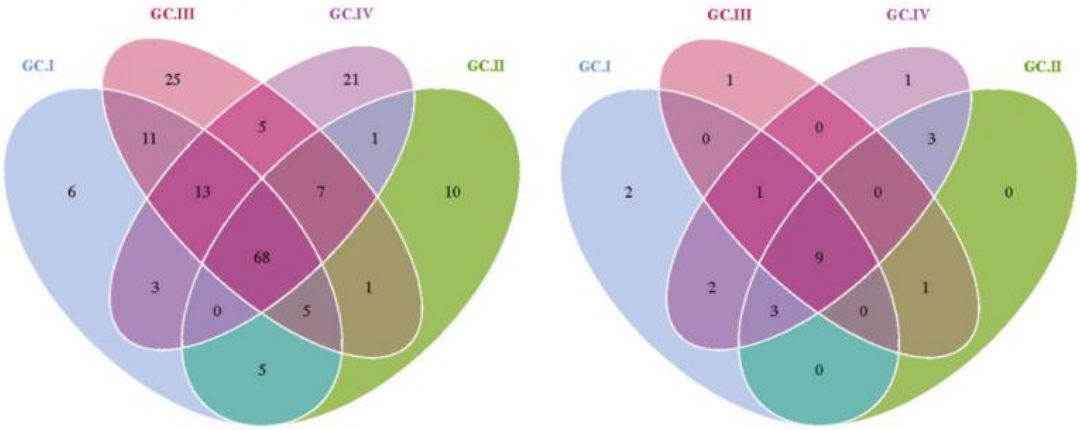


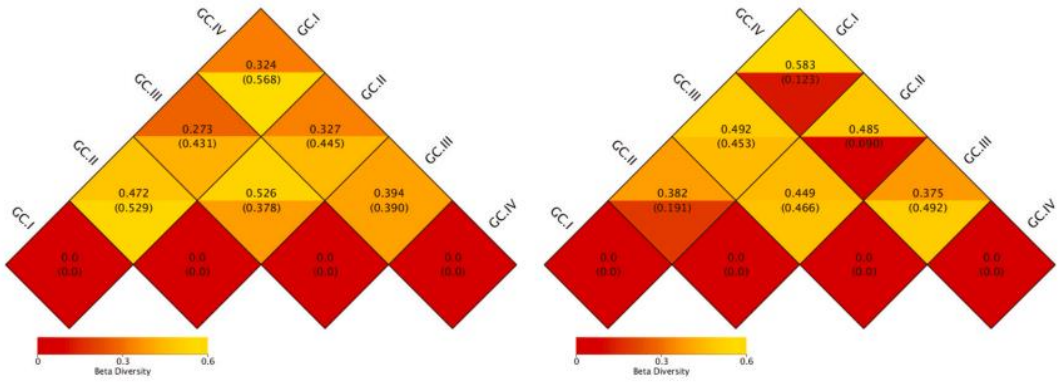
Chart A: Eubacterial Species *Chart B: Fungal Species*

Figure No. 22 - Colour charts for bacteria and fungi

For bacteria (Chart A) and fungi (Chart B) based on analysis of the common and unique OTE information in the different batches of Green Cheese. Each ellipse represents one batch sample. Values in overlapping parts represent common OTE. The rest are specific OTE in each sample.

3.5. Beta diversity analysis.

Heatmaps based on both weighted (quantitative) and unweighted (qualitative) distance metrics used in performing the comparison of biological communities (Unifrac) are shown in Fig. 23.



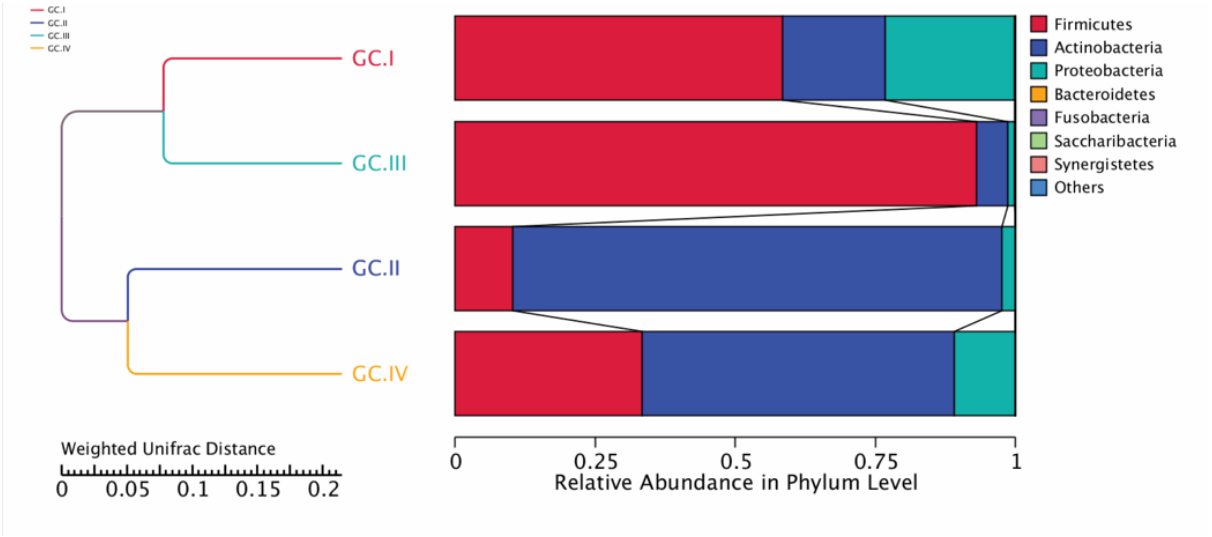
Panel A: Beta heatmap eubacterial diversity communities

Panel B: Beta heatmap fungal diversity communities

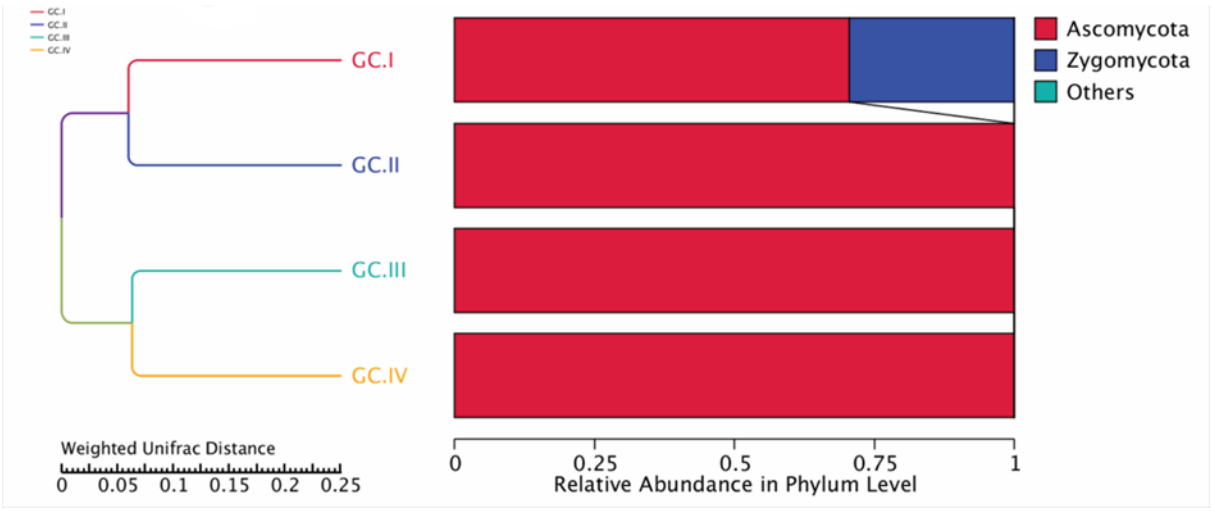
Figure No. 23 - Heat map of Beta diversity

It was constructed based on the weighted Unifrac and unweighted Unifrac distances of the microbial communities in bacteria (Panel A) and fungi (Panel B). Each grid represents a pairwise difference coefficient between paired samples, in which the weighted Unifrac distance is shown above and the unweighted Unifrac distance below.

The cluster trees calculated by the unweighted pair method with arithmetic means (UPGMA) and constructed based on the calculated distance matrices are shown in Fig. 24 for the weighted Unifrac distances and in Fig. 25 for the unweighted Unifrac distances.



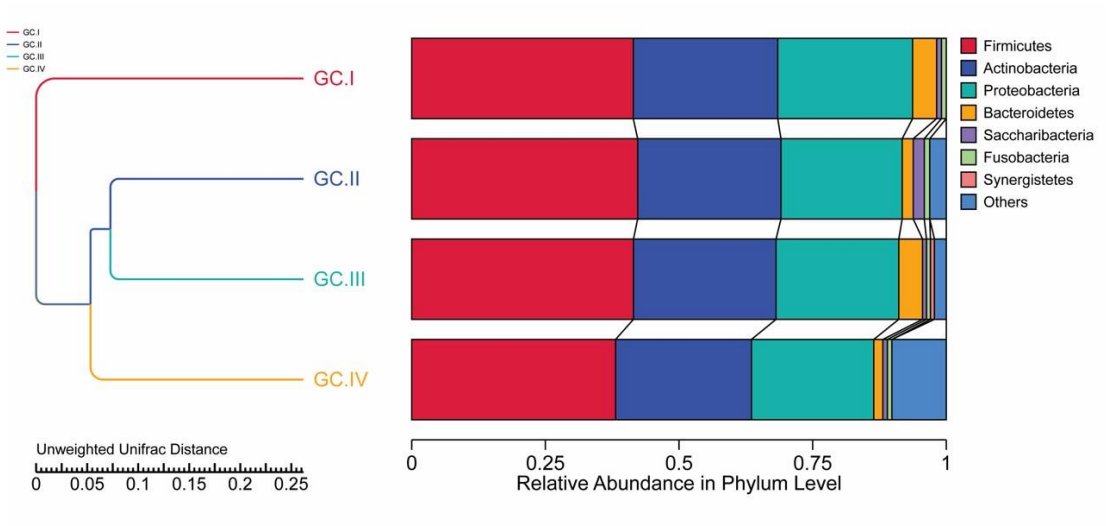
Graph A: UPGMA cluster tree drawn based on weighted Unifrac distance in bacteria



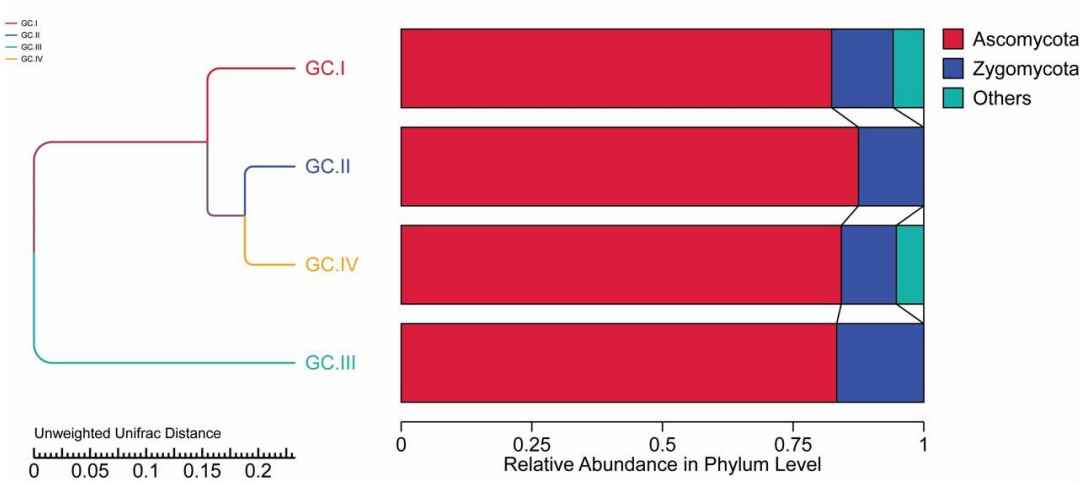
Graph B: UPGMA cluster tree drawn based on weighted Unifrac distance in fungi

Figure No. 24 – UPGMA cluster tree based on weighted Unifrac distance

In bacteria (Chart A) and in fungi (Chart B)



Graph A – UPGMA cluster tree in eubacteria



Graph B - UPGMA cluster tree in fungi

Figure No. 25 - UPGMA cluster tree based on unweighted Unifrac distance

In bacteria (Graph A) and in fungi (Chart B).

DISCUSSION

In our study, we collected and examined a collection of 348 isolates isolated from various food products, as well as from bees and bee products, for the presence of antimicrobial activity. The focus of our study was the identification of bacteriocin-producing enterococcal strains possessing the potential to inhibit the growth of the causative agent of the American disease foulbrood, as well as strains possessing probiotic potential.

Our experiments with bee products were part of a larger project aimed at characterising the bee and hive microbiome in different areas of the country. After establishing contact with a local beekeeper, samples were taken from all of his hives. According to the information received from the producer of bee products, his hives were not affected by the AFD disease. This information was of great interest to us, as his neighbours reported that beehives from neighbouring farms had repeatedly fallen victim to *Paenibacillus larvae* - as a result it was necessary to burn the affected inventory. The beekeeper also categorised his hives regarding their productivity as prosperous.

LAB are commonly found in the bee intestinal tract and are known to protect their hosts through antimicrobial metabolites such as organic acids, hydrogen peroxide and antimicrobial peptides, as well as by modulating the host's immune response. |In addition, many beneficial bacteria have been isolated from adult and larval honey bees and bee products such as honey and pollen (Alo n so-Salces et al., 2017 ; Jaouani et al., 2014) .

Studies of the potential of the bee lactic acid microbiome in terms of its benefits for the health status enhancement date back more than a decade. Olofsson and Vasquez (Olofsson & Vasquez, 2008) demonstrated that bees and the bee microbiome co-evolve. Audisio et al. (item-Ahrendts, 2011) were among the pioneers who demonstrated how a probiotic lactic acid strain isolated from a honey bee’s intestinal tract impacts the productivity of the entire hive. The autor's detailed research pinpointed the lactic acid producer, *Lactobacillus johnsonii* CRL 1647, as responsible for the observed effect. In recent years, pieces of evidence has been accumulated regarding the probiotic potential of isolated LAB from bees and bee products. The studies mainly focused on the production of bacteriocins, inhibiting pathogenic microorganisms and *Paenibacillus larvae* . Most of these strains belong to the genera *Bacillus, Lactobacillus, Bifidus* and *Brevibacillus* (Bartel, Abrahamovich, Mori, López, & Alippi, 2018 ; Minnaard & Alippi, 2016) . In this regard, the literature have recently announced strains *Fructobacillus fructose, Proteus mirabilis, Enterobacter kobei* and *Morganella morganii* with

similar activity (Al-Gham d i et al., 2020) . However, the data concerning representatives of the genus *Enterococcus* isolated from bees and bee products are pretty scarce, despite the widespread distribution of this genus in various ecological niches. The ambivalent role of enterococci can partly explain the lack of scientific data. They bring together resistant pathogens to obligate participants in different starter cultures in the fermentation of various food products such as cheese, raw meat, etc., including explaining their probiotic action (Ben Braiek & Smaoui, 2019 ; Graham, Stack, & Rea, 2020) .

We aimed to collect different isolates and to identify and analyse the genus *Enterococcus* representatives. Using the agar-diffusion method, we studied proteolytic and bacteriocin activity in selected isolates. As a result, we found that 90 pcs showed bacteriocin activity and 21 pcs. Showed proteolytic activity against nine indicator microorganisms (*Paenibacillus larvae, Lactococcus garvieae, Staphylococcus aureus, Enterobacter aerogenes, Yersinia ruckeri, Enterococcus faecium, Listeria monocytogenes, Enterococcus faecalis, Enterococcus durans*). Nine strains showed both proteolytic and bacteriocin activity at the same time - these strains were isolated from beehives (3 pcs.), Green Cheese (3 pcs.), sheep yoghurt (1 pc.) and sourdough bread (2 pcs.). Our result is similar to other studies studying bacteriocinogenic enterococci from different sources (Birri, Brede, Tessema, & Nes, 2013 ; SG Dimov, 2007 ; SG Dimov et al., 2010 ; Klibi et al., 2012 ; Ozdemir, Oryaşın, Bıyık, Ozteber, & Bozdoğan, 2011) .

We were surprised that isolates from six hives possessed inhibitory activity against *Paenibacillus larvae* (Table 1) . In other words, it can be said that 40% of the colonies had some "protection" against the AFD. To establish antimicrobial activity, we used the agar diffusion method. Using the same method, Yoshiyama et al. found that bacterial isolates (among which *Enterococcus, Lactobacillus* and *Weissella*), isolated from the midgut of *Apis japonica* also showed an inhibitory effect against *Paenibacillus larvae* (Yoshiyama & Kimura, 2009 ; Yoshiyama et al., 2013) . Inhibitory activity against *Paenibacillus larvae*, which we found, is also in agreement with the study of Janashia and co-authors (Janashia, Choiset, et al., 2016), who found that bacteriocin-producing exogenous and bacterial strains *Enterococcus durans A5-11, Enterococcus faecalis* KT2W2G and *Lactobacillus lactis* subspecies *lactis* KT2W2L exhibit inhibitory action against two strains of *Paenibacillus larvae*.

After the initial analysis regarding antibacterial activity against indicator strains, we continued our work with selected strains (27 pcs.). The selection was based on their demonstrated proteolytic and bacteriocin activity. Upon neutralising the supernatants by

treatment with NaOH, these strains were found to possess a slightly acidic pH in the range of 5 to 5.5. It can be assumed that in the strains analyzed by us, there is the presence of bacteriocin-like protein substances possessing inhibitory activity against the indicator strains. In support of this hypothesis is the presence of antimicrobial activity exhibited by *Enterococcus* isolates, which was lost after treatment with proteinase K, thus confirming that the protein nature of the substance is responsible for the inhibition (Dal Bello et al., 2010) .

We continued our work with 27 strains based on their demonstrated activity against test microorganisms, and we did the subsequent species determination by 16S RNA gene sequencing. After successful isolates identification, we found that they mainly belong to *Lactiplantibacillus plantarum*, *Enterococcus faecium*, *Enterococcus faecalis, Pediococcus pentosaceus*, *Levilactobacillus brevis, Rosenbergia*, *Leuconostoc mesenteroides, Serratia* species, *Staphylococcus equorum* and *Staphylococcus saprophyticus* (Table 8). There are a several studies in the literature reporting antibacterial activity for these species as well as others (eg, *Enterococcus columbae* and *Enterococcus casseliflavus*) that were not identified in our study (Butaye, Baele, Devriese, & Haesebrouck, 2002 ; Poeta et al., 2007, Sabia, Messi, de Niederhausern, Manicardi, & Bondi, 2004) . Among the 335 strains, 9 pcs. showed activity against *Listeria monocytogenes*. It is important to emphasize that five isolates showed a large zone of inhibition (> 10 mm). Most enterococcal bacteriocins can inhibit the growth of Listeria (Cocolin , Foschino, Comi, & Grazia Fortina, 2007 ; Pinto et al., 2009 ; Rivas, Castro, Vallejo, Marguet, & Campos, 2012) , as this ability is related to the close phylogenetic relationship of enterococci and Listeriae. The strains of the *Enterococcus* genus, which possess antilisterial activity due to the production of bacteriocin are essential for the food and dairy industry (De Vuyst, 2003) . Thirty-three isolates demonstrated activity against *Enterococcus faecalis*, with a sizeable observed zone of inhibition occurring at nine of enterococcal isolates. Regarding *Staphylococcus aureus*, seventeen isolates demonstrated an inhibitory activity. Rivas and co-authors reported that these two species were sensitive to enterococcal bacteriocins (Rivas et al., 2012) . It is generally accepted that the inhibitory activity of enterococcal bacteriocins covers Gram-positive bacteria, including *Listeria monocytogenes*, but shows limited or no activity against Gram-negative bacteria. (Banwo, Sann i , & Tan, 2013) , due to their outer membrane.

In our study, 13 isolates demonstrated inhibitory activity against *Paenibacillus larvae*. These results have a possible biotechnological application, especially considering that the genus *Enterococcus* is part of the microbiome of the digestive tract of the honey bee (Carina

Audisio , Torres, Sabate, Ibarguren, & Apella, 2011) . Furthermore, bacteriocin-producing isolates have been suggested to stimulate an innate immune response in honey bees, which may be beneficial in preventing bacterial diseases in honey bees. Among the isolates from bee products, we focused our attention on three isolates that showed wider zones of inhibitory activity against *Paenibacillus larvae* (>10 mm), in which, as a result of the BLAST alignment of their 16S rRNA gene sequences, we found that two of them belong to *Enterococcus faecium* and one to *Enterococcus durans.* The strains originate from a small bee farm in the village of Dushantsi - a foothill region characterized by well-developed agriculture and preserved natural regions. These ideal conditions for the development of beekeeping favour the prosperity of several small bee farms in the vicinity. Unfortunately, some have had documented cases of AFD in the recent years.

We performed whole-genome sequencing on both *Enterococcus faecium* isolates to better characterize them. Additional sequence analyzes of the resulting data showed that the isolates were clonally identical, i.e. both belong to the same strain, which we named *Enterococcus faecium* EFD. Therefore, we performed our subsequent studies only on one of the isolates. Using the QUAST software, we evaluated the metrics of the generated draft assembled genomes, which resulted in the following results: a total length of 2,611,074 bp., N_{50} of 98,462 bp, GC content 38.03% and 69 contigs larger than 1000 bp. These values agree with the NCBI 's publicly available *Enterococcus faecium* genomes.

The generated genomic data allowed us to simultaneously perform different types of analysis to determine its safe status. All data are summarized in the Table. 5. As a result of screening for the presence of bacteriocin genetic determinants, we can say that the antibacterial activity of *Enterococcus faecium* EFD may be result of the activity of one or more of the five bacteriocins found: bacteriocin 32, enterocin A, enterocin P, enterocin SE-K4 and enterolysin A. Through whole-genome sequencing, it is not possible to say with certainty which one of them is responsible for the inhibitory activity against the tested strains, especially against *Paenibacillus larvae*. A research group that performed a mass screening of 300 bacteriocinogenic enterococcal strains reported that enterocin A and enterocin P in combination or alone inhibited the growth of *Paenibacillus larvae* (Jaouan i et al., 2014) . Their findings highlight the presence of enterocin A and enterocin P (in combination or not) as most likely explanation for the observed antibacterial activity. Since *Enterococcus faecium* species are known for their dualistic nature, we investigated whether *Enterococcus faecium* EFD could fall into the GRAS (generally regarded as safe) category. The first step was to screen its genome

for antibiotic resistance genes. Using Res-Finder 3.2 software, we determined the presence of a *msr(C)* gene (the gene confers resistance to erythromycin and other macrolides and antibiotics), as well as the aac *(6')-Ii* gene (conferring resistance to some aminoglycosides). However, both resistance determinants have no clinical significance because they are considered intrinsic to the *Enterococcus faecium* species. In addition, two genes have known chromosomal locations, which significantly reduce the risk of horizontal gene transfer to other bacteria through conjugation. Given this fact, these determinants should not be considered as a potential reason for withdrawing the safety status. In order to verify the results obtained by us through *in silico* analysis, we performed an additional *in vitro* assay to verify its strain resistance against all classes of clinically important antibiotics. The results showed that *Enterococcus faecium* EFD was susceptible to ampicillin, imipenem, ciprofloxacin, levofloxacin, gentamicin, teicoplanin, vancomycin, linezolid and tigecycline, and resistant only to erythromycin, the latter being intrinsic primarily to the species (Hollen Beck & Rice, 2012) .

Using the VirulenceFinder tool 2.0 we performed additional bioinformatic analysis and we found the presence of two genes that can be considered as virulence determinants – *acm* and *efaA*, encoding respectively Acm collagen-binding protein and endocarditis-specific antigen A. However, detailed sequence analysis showed that within the *acm* CDS there is a frameshift mutation at position 507 (out of a total of 1865 bp), and an additional 18 base substitutions. A frameshift mutation suggests only a non-functional protein. The number of nucleotide substitutions found in efaA was 53, together with four indels affecting the protein product by adding two pieces of 23 and 9 amino acids at positions 45 and 726 of the gene. After BLAST analysis of the protein translation, we did not find the presence of clinical strains, suggesting altered protein functionality. Further analysis with the PlasmidFinder tool showed that only one of the contigs with bacteriocin genes (encoding bacteriocin 32) was associated with a sequence associated with plasmid replication. This leads to the assumption that the genetic determinants of the other four bacteriocins can be chromosomally located and stably inherited.

The overall conclusion of the virulence software analysis was that the *Enterococcus faecium* EFD isolate did not have functional pathogenic traits and therefore, should not be considered a potential pathogen. However, the presence of *acm* and *efaA* gene suggests that the isolate may have originated from animal contamination, as over time, within the hive environment and in the absence of specific selective pressure to maintain its pathogenic

properties, it becomes non-pathogenic by accumulated mutations in these determinants. This agrees with our hypothesis about the long-term persistence of the isolate in the beehive as a part of its normal microbiome.

We continued our study with a comprehensive molecular and phenotypic characterization of an *Enterococcus durans* EDD2, originated from freshly collected pollen granules, possessing probiotic characteristics and exhibiting inhibitory activity against *Paenibacillus larvae*. As far as we know, this is the first study on a bacterial isolate isolated from beehives, possessing beneficial properties, and further subjected to whole-genome sequencing.

Interestingly, the hives from which EDD 2 was isolated were characterized by the bee farm owner as good and prosperous. The results showed that *Enterococcus durans* isolates are not numerous. In general, in gut samples from honey bees, *Enterococcus* is significantly less common than lactobacilli (Janashia, Carmin a ti, et al., 2016) . However, the fact that the strain was predominant in at least half of the hives from which was isolated (two out of three and three out of three isolates, respectively) was the first indicator of selective maintenance in the hive by the strain.

The humoral response is the most important defence system in the honey bee and is governed by the production of antimicrobial peptides such as apidaecin, abaecin, hymenopthecin and defensin. These compounds act by limiting enzymes necessary for the growth and reproduction of pathogens by forming channels in the cell membranes of pathogenic microorganisms. (Iorizzo et al . , 2022) . In a study on the inhibition of *Paenibacillus larvae* by LAB isolated from fermented products, Yoshiyama and co-authors reported that with oral intake of several species belonging to the genera *Enterococcus, Weissella,* and *Lactobacillus* from larval and adult bees, the transcription levels of antimicrobial peptides such as abaecin, defensin and hymenopthecin are significantly increased. This suggests that selected LABs stimulate the innate immune response of honey bees, which may be beneficial in preventing bacterial diseases (Yoshiyama et al., 2013) .

It can be assumed that the four hives from which *Enterococcus durans* EDD2 was isolated can be considered protected against AFD. The other two hives in which were found the undetermined *Enterococcus* isolates can also be included in this hypothesis, keeping in mind their activity against *Paenibacillus larvae*. However, since they are fewer in number, there is no solid basis for speculation regarding selective protection. The confirmation of the

same strain in our study suggests sharing protective strains in neighbouring hives in the bee farm, which would facilitate the treatment of compact bee colonies protection against AFD and similar diseases. In support of this hypothesis is the absence of diseases in the analysed hives, in contrast to the rest of the neighbouring ones. The sharing of protective strains in honey bees may be due to interactions between individuals from the same colony providing opportunities for the transfer of non-gut bacteria – some of the most distinct and consistent gut microbiomes owning protective functions have been found in these insects (Ramos, Basu a ldo, Libonatti, & Vega, 2020) . The bee microbiome is vertically acquired and transmitted from various environment sources. LAB communities and their diversity vary depending on bee activity (Vásquez, Olofsson , & Sammataro , 2008) . Therefore, it can be assumed that some LABs associated with honey bees (*Apis mellifera*) are transmitted from the environment, while others are inherited vertically through the maternal line (McFr e derick et al., 2012) . Functional studies of the gut microbiome of *Apis mellifera* through metagenomic analysis show a possible link of LAB with the protection of bees against pathogens (Mohammad, Mahmud-Ab-Rashid, & Zawi , 2021) .

The presence of *Enterococcus durans* EDD2 could easily be committed to human and animal faecal pollution given to the bees. In a study about the presence of multidrug-resistant enterococci in animal meat and faeces and the possibility of transferring the resistance, Pasquaroli et al. provide further evidence for the frequent occurrence of multidrug-resistant enterococci in farm animals and products. The authors emphasize that intestinal enterococci, including *Enterococcus durans*, of animal origin are more prone, compared to enterococci of food origin, for the transfer of antibiotic resistance to human strains (Vignaroli , Zandri , Aquilanti, Pasquaroli, & Biavasco, 2011) . Antibiotics have also been detected in honey samples because they are used in beekeeping to treat bacterial diseases. Antibiotic residues mainly originate from the environment and are a consequence of incorrect beekeeping practices. In samples of honey residues of oxytetracycline and chloramphenicol were above regulatory standards (Al- Waili, Salom, Al-Ghamdi, & Ansari, 2012 ; Saridaki-Papakonsta d inou , Andredakis, Burriel, & Tsachev, 2006) . Oxytetracycline is commonly used to treat European and American FD caused by *Paenibacilus larvae* and *Streptococcus pluton*. Resistance to tetracycline has also been reported. Other antibiotics, such as erythromycin, lincomycin, monensin, streptomycin and enrofloxacin are also used (Al-Waili et al., 2012) .

To test the hypothesis of microbial contamination, the strain was subjected to several tests for the presence of virulence factors and antibiotic resistance. All performed tests were

negative, and *Enterococcus durans* EDD2 was classified as safe. However, these findings, combined with its activity against *Paenibacillus larvae*, are in conflict with the probability of accidental introduction of the strain into the hives. Therefore, the strain should be considered as selectively maintained and possess probiotic properties.

Many LABs produce antibacterial peptides, including bacteriocins, whose mechanism of action is aimed at destroying target cells through pore formation and/or inhibition of cell wall synthesis. Bacteriocins exhibit antimicrobial activity with a variable spectrum depending on the peptide, which may target several bacteria. There is scientific evidence that bacteriocins are effective against some bacterial infections in honey bees (Iorizzo et al., 2022) . The presence of bacteriocin-like inhibitory substances has been reported in some strains of *Enterococcus* isolated from the honey bee gut (Janas h ia, Choiset, et al., 2016) .

We subjected the *Enterococcus durans* EDD2 strain to whole-genome sequencing to detect the presence of bacteriocins and their genetic determinants. Genome sequencing of *Enterococcus durans* EDD2 was performed by BGI Genomics Company (BGI, China) by 2 × 150 bp Illumina HiSeq paired-end genome sequencing. As a result, we received 4,370,827 pcs. and we processed these raw paired reads using the Galaxy online platform (Afgan et al., 2018) . Next, we checked the base pairs with FastQC software, and finally, we used the Trimmomatic tool (Bolg e r, Lohse, & Usadel, 2014) to remove the adapters and the sequences with a low Phred score. The original *de novo* assembly of the processed reads was performed using the Shovill platform with default settings. Before the assembly, reads were randomly sampled to approximately 100× coverage. The assembled genome consisted of 139 contigs with sizes larger than 500 bp. (the largest contig was 155,300 bp). Through QUAST analysis (Gurevich, Saveliev, Vyahhi , & Tesler, 2013) we found that the genome is composed of 2,961,262 bp. with an average GC content of 37.71% and an N_{50} value of 57,760 bd. We aligned the assembled contigs to the *Enterococcus durans* KLDS6.0930 genome (GCA_001267865.1) using the progressive Mauve algorithm (Darling, Mau, Blattner, & Perna, 2004) . We chose the *Enterococcus durans* KLDS6.0930 genome as a reference due to its probiotic properties and the availability of the complete genome sequence of this strain (Liu et al ., 2016) . The alignment of the two genomes (using Mauve) showed an overall relationship between these two sequences. Additionally, we performed MiGA analysis (Rodrigu e z et al., 2018) and we found that the selected *Enterococcus durans* KLDS6.0930 genome was the most closely related representative in the database of this tool. The two genomes have an average nucleotide identity (ANI) of 99.71 % and share 91.6% of their proteins with an average amino acid identity (AAI)

of 99.76%. The final draft genome of *Enterococcus durans* was annotated using NCBI's Prokaryotic Genome Annotation Tool (PGAP) (Tatuso v a et al., 2016) , which contains 2648 protein-coding genes, 3 complete pRNA gene clusters (5S, 16S, 23S) and 62 mRNA genes. In addition, we identified sequences related to plasmid replication with the PlasmidFinder tool (Carat toli et al., 2014) . Given the fact that enterococci are considered as opportunistic human pathogens (Kirschner et al., 2001) , we evaluated the resistance and virulence of *Enterococcus durans* EDD2 using ResFinder 3.2 and VirulenceFinder 2.0 (Clausen , A restrup , & Lund, 2018) . We identified the bacteriocins genetic determinants using the BAGEL4 platform (van H eel et al., 2018) , which revealed two putative clusters for the production of enterocin L50A and enterocinP variants.

Subsequent analysis showed two putative enterocin clusters L50A/L50B (Fig. 3) and enterocinP (Fig.4), located on contig 48 and contig 49, respectively. Using the BLAST tool, we determined the degree of sequence similarity between the new putative bacteriocins found in *Enterococcus durans* EDD2 and their reference analogues (enterocin L50A/L50B and enterocinP) (LM Cintas et al., 1998 ; Cintas et al., 1997) . The presence of enterocin L50A/L50B and enterocinP is relatively common in bacteriocinogenic enterococci. Audisio and co-authors (Audisio & Benitez-Ahrendts, 2011) reported an *Enterococcus faecium* strain isolated from the gastrointestinal tract of worker bees, which contains enterocinA , enterocinB and enterocinP genetic determinants determined using PCR. Actually, *in vitro* strain inhibited the growth of *Listeria monocytogenes*, but not *Paenibacillus larvae*. In another study, for several strains of *Enterococcus faecalis* and *Enterococcus faecium* have been reported to possess genetic determinants for enterocin L50A/L50B, enterocinP and enterocinA, individually or in combination, which inhibit the growth of *Paenibacillus larvae* (Jaouani et al., 2014) . The same study indicated that one *Enterococcus durans* strain possesses genetic determinants for enterocinP and enterocinA, and inhibits the growth of *Paenibacillus larvae*. In Jaouani et al.'s study, none of the enterococcal strains were isolated from bees or bee products. In the two studies cited above, the presence of genetic determinants was only determined using PCR. Since no sequence definition or expression experiments were performed, it is impossible to say which bacteriocins are involved in the inhibition of *Paenibacillus larvae*. Both research teams, in their experiments, amplified only structural genes with different combinations of bacteriocins. Therefore, the most logical conclusion would be that all bacteriocins could be potential growth inhibitors of *Paenibacillus larvae*, but it would

not be possible to establish with certainty which of them has a role in this. Additionally, there is a high probability that some genes are in mutant form or not expressed.

Thanks to our choice to work with new-generation technologies, we can state that the gene clusters for enterocins L50A/L50B and P found in *Enterococcus durans* EDD2 are complete and contain all the necessary genes for their expression. Therefore, the observed activity against *Paenibacillus larvae* can be the result of one of them, both simultaneously or synergistically. However, the exciting discovery regarding the start codon (ATT) in the structural gene for enterocinP is unusual, as it is the substitution of three amino acids, a finding that allows for a wide range of hypotheses regarding expression as well as its activity against *Paenibacillus larvae*, which remain the subject of future studies.

To our knowledge, *Enterococcus durans* EDD2 is the first bacteriocin-producing isolate associated with a beehive active against *Paenibacillus larvae*. A previous study reported the isolation of a strain of *Enterococcus durans* from the gut of adult worker bees, but further studies showed a lack of inhibitory activity *in vitro* against Paenibacillus *larvae* (Janashia, Choiset, et al., 2016) . In contrast, our study indicates that the EDD2 strain may play a role in the defence against the invasion of bacterial pathogens, especially considering that our analysis did not detect in its genome the presence of virulence factors, or resistance determinants, to clinically important antibiotics. A strain needs other essential qualities to be considered as probiotic, such as being non-pathogenic, genetically stable and viable in high populations. In addition, the strain must survive and proliferate in the highly aggressive environment of the intestinal tract. Other vital features required intestinal cell adhesion and colonization, as well as possessing potential therapeutic benefits. The draft genome sequence we described will allow further genome-scale analyzes of these properties to help us better understand the biological role of enterococci in honey bees and their colonies.

This draft genome has been deposited in DDBJ/ENA/GenBank under accession number JAAILD000000000.

Our study showed that several isolates from Green Cheese also possess proteolytic and bacteriocin activities. Because until now no studies have been carried out regarding the microbiome of this exceptional Bulgarian product, we subjected these isolates to a metagenomic study to identify and analyze the types of microorganisms contributing to its distinctive characteristics, including sensory and organoleptic properties.

The unique green cheese has a fascinating history - it owes its name to the colour of its rind and is produced according to a traditional Bulgarian recipe. The noble moulds that make up the crust of the product are not intentionally inoculated but develop independently. Although the Green Cheese is produced only in one single settlement but it is known throughout the country thanks to its specific taste and aroma.

In the middle of the 20th century, there was a decline in cheese production. On the one hand, this is due to the forced collectivization under the pressure of the then active communist regime. On the other hand, the ban on commercialised dairy products consisted of raw milk, limited only to domestic use, also played a role in this process. These circumstances explain the lack of scientific research on Green Cheese from the village of Cherni Vit.

Production was minimal, given the hard times in the middle of the last century. However, there is an increased interest, especially from "gourmet" restaurants, where the Green Cheese is served as a delicacy. These circumstances explain the need to conduct the present study. Its necessity is further motivated by the fact that this traditional product can be considered as a national cultural heritage.

The Green Cheese is produced only in Cherni Vit village, located in the northern foothill region of Stara Planina. Its specificity is thought to be due to two factors: the first one is the unique microclimate. The village is located in the narrow gorge of the Cherni Vit river, which springs from the top of the mountain. This makes the air relatively constantly moist throughout the year. As a result, winters are pretty cold, while summers are cool, despite the continental climate. The second factor is that in the past, some of the main economic activities in the region relied on sheep farming for wool and milk production.

Bulgarian Green Cheese is a hard type, with a rind of noble moulds. It is produced from raw milk - mostly sheep, sometimes goat. It is generally not made from cow's milk, as it has a low fat and protein content. Its primary specifics are that unpasteurized milk is used as raw material immediately after milking, which explains the local microbiome’s role in obtaining the final product’s distinctive characteristics. Also, a key step in the process is adding a small amount of traditional Bulgarian yoghurt during maturation.

In this study aimed at characterizing Bulgarian green cheese, as a first step, we undertook a next-generation sequencing-based metagenomic study. The study covered V3-V4 regions of the 16 S RNA gene (Claesson et al., 2010) in bacterial species and ITS2 region in fungi (Alanagreh, Pegg, Harikumar, & Buchheim, 2017) . The used methodology is standard, and in

recent years, many scientific studies have been published concerning artisanal and industrially produced cheese varieties that are typical for a given region (Ianni et al., 2020 ; Marino et al., 2019 ; Papademas et al. ., 2019) . To date, there are still no publications in the scientific field about a metagenomic or other similar microbiome analysis of Bulgarian Green Cheese.

Despite the established unique labels and taxonomically assigned OTEs, we found no significant differences in the number of species among the four batches. This relative abundance of species in the substance is typical for cheeses made from raw milk without starter cultures (Dugat-Bony et al., 2016) .

Fig. 19A shows the relative abundance of eubacterial types in the four batches of Green Cheese. Fig. 17 shows a combined phylogenetic tree with the relative distribution of the 20 predominant bacterial species depicted. Fig. 20A represents the heatmap containing the taxonomic diversity. These values cannot be assumed to be completely accurate since next-generation sequencing technology uses a fragment amplification step by PCR that is not amenable to accurate quantification. However, they give a good idea of the specific bacterial composition. Firmicutes occupy the first place with more than 50% presence, followed by Actinobacteria with slightly more than 40% and Proteobacteria with about 6% in third place. The remaining bacterial types are represented in insignificant quantities. These findings are consistent with others described in the literature (Dugat-Bony et al., 2016), particularly with studies concerning surface-ripened hard cheeses (Escobar-Zepeda, Sanchez-Flores, & Quirasco Baruch, 2016) . In a study on the relationship between the microbiome and metabolites in soft-ripened cheese using an integrated omics approach, Unno and co-authors found that almost all cheese samples were dominated by Firmicutes, Proteobacteria and Actinobacteria (Unno, Suzuki, Matsutani, & Ishikawa, 2021) . In Poro, an artisanal type, Mazorra-Manzano et al. reported that the phyla Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes predominated. With fermentation, the phylum Firmicutes was found to dominate, followed by Proteobacteria and Actinobacteria (Aldrete-Tapia, Escobar-Ramirez, Tamplin, & Hernandez-Iturriaga, 2014 ; Mazorra-Manzano et al., 2022) . Lusk et al. reported that in a study of different brands of Latin-type cheeses (eg queso fresco), Firmicutes dominated all cheese samples, followed by Proteobacteria (Lusk et al., 2012) .

Within the phylum Firmicutes, at the genus and species level, gram-positive LAB from the genera *Streptococcus*, *Lactobacillus* and *Lactococcus* rank first, with more than 30%. They are followed by representatives of the genus *Staphylococcus* (about 18%). Although Green Cheese is a product without a starter culture, its production includes the addition of traditional

Bulgarian yoghurt. This step could explain the presence of *Streptococcus salivarius* in the first-place (about 21%). However, the absence of the other yoghurt "starter" strain *Lactobacillus delbrueckii* is quite surprising given that among the lactic acid bacteria, lactobacilli come in second place with almost 7.5% and are represented by 6 different species. *Lactobacillus delbrueckii* subspecies *bulgaricus* is one of the predominant lactic acid bacterial species used as starter cultures in the industrial production of fermented milk products (Dan et al., 2019) . Investigation of food waste by pyrosequencing revealed that Lactobacillus species are dominant in the studied microbiomes and have a key role in fermentation (Tsapekos, Alvarado-Morales, Baladi, Bosma, & Angelidaki, 2020) . Given this, it can be said that this is a feature of Bulgarian Green Cheese.

Lactococcus species are the third main group with 3.7% presence. Less represented genera are *Leuconostoc, Weissella*, *Marinilactibacillus* and *Enterococcus.* Among Gram-positive bacteria, the genus *Staphylococcus* is also well represented, especially by the species *Staphylococcus equorum*. The Gram-negative bacteria are in insignificant amounts; among them the most represented genera are *Veillonella* and *Selenomonas*. While the various species of *Lactobacillus, Leuconostoc* and *Enterococcus*, as well as *Lactococcus lactis* and *Streptococcus salivarius* have a major role in the ripening of the cheese and their presence is expected, the presence of some species of *Staphylococcus*, as well as the presence of *Lactococcus garvieae* causes certain concerns. It is important to note that the species *Staphylococcus equorum* is coagulase-negative. Therefore the bacterium is considered benign and there is also no evidence available in the literature for its possible pathogenicity (Jeong, Heo, Ryu, Blom, & Lee, 2017) . However, there is information about a strain isolated from cheese brine producing anti-listerial bacteriocins (Bockelmann et al., 2017) . This observation indicates that *Staphylococcus equorum* could also be counted as an advantage. For the other observed coagulase-negative species, such as *Staphylococcus lentus,* there is also available literature data that they are typical microorganisms in some Turkish cheeses (Kurekci, 2016) . Another found species is *Lactococcus garvieae*, a well-known fish pathogen. Recent studies have shown that this species is part of the typical microflora of some Spanish cheeses made from raw milk and without the use of a starter culture (Fern á ndez , Alegr í a , Delgado , & Mayo , 2010) . *Lactococcus garvieae* also predominates in some artisanal Italian cheeses and contributes to their characteristics (Fortina et al., 2007) . Some authors attribute probiotic properties to some strains, because they can inhibit the growth of pathogens such as *Staphylococcus aureus* (Abdelfatah & Mahboub, 2018) . Among the Gram-negative species

of Firmicutes, *Veillonella* is known to be part of the mammals' normal intestinal and oral mucosa. Genus was discovered in artisanal Italian Alpine cheese 'Plaisentif' using metagenomic studies (Dalmasso et al., 2016) , where its role is associated with lactate fermentation. At this stage, reasonable assumptions about the positive role of the other representative of the genus *Selenomonas,* namely *Veillonellaceae,* could not be made. Nevertheless, it is a typical bacterium that plays a role in the digestion of ruminants, and its presence should be considered as a dangerous contamination.

Actinobacteria ranks second, represented mainly with the genera *Brevibacterium* and *Corynebacterium* , as described for other cheeses (Dugat-Bony et al., 2016) . There is evidence in the literature that *Brevibacterium* species can be found in abundance in the rind of hard cheeses. Also, they can play a significant role in the degradation of histamines that can be formed during fermentation, using them as a carbon source (Anast et al., 2019) . A similar role for *Corynebacterium* species was reported by Tittarelli and co-authors (Tittarelli, Perpetuini, Di Gianvito, & Tofalo, 2019) , while Gori and colleagues found that they are usually the predominant surface bacteria during the ripening of several types of cheese and contribute to the structure, taste and color of the cheese (Gori, Ryssel, Arneborg, & Jespersen, 2013) . A small but noticeable amount of *Kocuria rhizophila* was observed, and some authors reported that this species could enhance the formation of volatile compounds and thus contribute to the specific flavour of the cheese (Centeno, Garabal, Docampo, Lorenzo, & Carballo, 2017) . The other representatives of the type are insignificantly represented, so they could not play a significant role in the quality of the cheese.

The third bacterial phylum that was significantly represented was Proteobacteria, with two families, *Halomonadaceae* and *Moraxellaceae*. The former was represented mainly by *Cobetia marina* and a small but noticeable number of *Halomonas venusta*, while the latter was represented by *Psychrobacter celer*. The role of *Cobetia marina* is quite unclear, but its presence has been established in dairy plants (Schön et al., 2016) . According to literature data, *Psychrobacter celer* is associated with some French and Belgian types of cheese, but to date there are no clinical infections associated with this gram-negative bacterium (Delcenserie et al., 2014 ; Imran et al., 2019) . There is information that *Halomonas venusta,* together with *Psychrobacter celer* and *Vibrio* (which was also discovered by several OTE readings) are part of the microbiome of some well-known worldwide ripened types of cheese (Mounier, Coton, Irlinger, Landaud, & Bonnarme, 2017) . However, their role in cheese ripening remains unclear. Of the remaining minor Proteobacteria, the presence of *Marinobacter* was found in

some Belgian rind cheeses together with Dabaryomices fungi (Vermote, Verce, De Vuyst, & Weckx, 2018) , analogous to green cheese. Two other genera *Haemophilus* and *Neisseria* containing human pathogens were detected with few OTE reads, but there is evidence of their presence in aged Gouda cheese (Salazar et al., 2018) . The presence of the remaining three Gram-negative pathogens found in low numbers - *Escherichia-Shigella*, *Cardiobacterium* and *Lautropia* observed in the heat map in Fig. 27A, can be most easily explained by the contamination because the Green Cheese is made from raw milk. There is literature data that in artisanal cheeses produced similarly from raw milk, the remaining microorganisms inhibit the growth of pathogenic species (Aldrete-Tapia, Escobar-Ramirez, Tamplin, & Hernandez-Iturriaga, 2018) .

Chryseobacterium is the only member of the genus Bacteroides to rank among the 35 genera included in the heatmap in Fig. 20A. There is no literature data for the presence of clinical infections caused by any of the members of the genus (Imran et al., 2019) . Due to its token presence it is not possible to make assumptions about its involvement in the maturation process.

Almost all (99.99%) of the discovered fungal species belong to the Ascomycota, represented almost equally by three classes: *Eurotiomyces*, *Saccharomycetes* and *Sordariomycetes* (Fig. 17). Among them, the first class is represented by one genus and one species - *Penicillium roqueforti*, which is the most prevalent fungal species. These fungi are well known in the production of various blue-colored cheeses around the world. *Penicillium roqueforti* has been shown to contribute to the specific characteristics of cheese through the production of many secondary metabolites, and through its efficient lipolysis and proteolysis. Through them, water activity is reduced and thus the growth of bacteria that spoils the product is limited (Caron et al., 2020) . These species also contributes to the cheese's aroma and texture (Garcia-Estrada & Martin, 2016) . In the case of the Bulgarian Green Cheese, the strain is not deliberately introduced into the production process, but is self-evolving, similar to the Spanish Cabrales cheese (M. Nunez, 1978 ; M. Nunez, Medina, Gaya, & Dias-Amado, 1981) , thereby distinguishing it from other blue cheeses such as Roquefort, Danish blue, Gorgonzola, etc. This leads to the assumption that the strain observed in our case belongs to the recently cultivated ones (Dumas et al., 2020) .

Sordariomycetes is the second most abundant class of Ascomycota, represented almost entirely by *Scopulariopsis flava*, and minimal amounts of *Fusarium oxysporum* and *Trichothecium roseum*. There are data in the literature that *Scopulariopsis flava* and *Fusarium*

oxysporum are part of the microbiome of cheeses with rich fat and protein content (Ropars, Cruaud, Lacoste, & Dupont, 2012) . *Trichothecium roseum* has also been reported to aid mould formation and ripening in some hard-surface cheeses (Zamberi et al., 2016) .

Among the class Saccharomycetes, *Debaryomyces hansenii* was the most abundant, followed in descending order by *Pichia membranifaciens, Candida zeylanoides, Kluyveromyces lactis* and *Torulaspora delbrueckii*. The non-pathogenic species *Debaryomyces, Candida, Kluyveromyces* and *Torulaspora* have been reported in the scientific literature as fungal species supporting the ripening process in several French kinds of cheese (Dugat-Bony et al., 2016) . There is also information about *Pichia membranifaciens* in some fermented foods, for example, olive brines (Carota et al ., 2017) , and in Egyptian soft cheese (Moharram, A. Abd El Haleem, & RS Refaie, 2018) .

The phylum Zygomycota represents only 0.01% of the reported operational taxonomic units in fungi, with the only genera *Circinella* and *Mucor* (mostly *Circinella muscae*) found. The role of these yeasts in the cheese-ripening process is debatable and most likely insignificant. However, there is literature data for the presence of this type of yeast in other fermented foods (Walther et al., 2013) . A summary of the distribution of the different bacterial and fungal species observed is illustrated in Fig. 22 in the form of a Venn diagram.

Based on the information obtained, the following conclusions could be drawn: 1) there are 68 bacterial and 9 major fungal species that are necessary for the ripening of all of the investigated cheese types; 2) there are 25 bacterial and 4 fungal species necessary for the ripening of at least three of the cheese types; 3) there are 26 bacterial species and 6 fungal species necessary for the ripening of at least two types of cheese; and finally 4) each batch of cheese was characterized by 5 to 25 specific bacterial species, while only GC II, GC III and GC IV were characterized explicitly by 1 to 2 fungal species, and no GC II-specific fungal species were observed.

The rich microbiome of the Bulgarian Green Cheese was further characterized by examining its Alpha diversity. Shannon and Simpson's indices were used to estimate diversity based on observed OTEs , with the former giving more weight to species richness and the latter to species evenness (Kim et al., 2017) . Regarding the indices in the bacterial species, both indices had relative values, except for the semi-hard Green Cheese GC II, which values were significantly lower. This circumstance is related to fewer species being observed within this batch. No such dependence was observed for the values of the Simpson and Shannon indices

for the types of fungi - the 8-month-old sheep's hard Green Cheese contained the most significant number of the observed species, while the values of the corresponding indices were in third place. The two fungal species indices were about 4 times higher for GC I and GC II compared to GC III and GC IV batches. Nevertheless, the discrepancy in fungal species may be partly explained by the smaller number of different OTE, and the lower observed species diversity, leading to more significant error in the calculations.

In order to explore and compare the composite communities of the samples in the four batches of cheese, beta-diversity indices were analyzed using the QIIME software using UniFrac distances. The calculated pairwise dissimilarity coefficients for the weighted and unweighted Unifrac distance measures, were used to construct beta diversity heatmaps (Fig. 23). The highest coefficient of dissimilarity for the weighted Unifrac distances was observed between batches GC II and GC III for the bacterial analysis and between batches GC I and GC IV for the fungal species analysis. Conversely, the lowest coefficient values were reported between batches GC I and GC III and GC III and GC IV, for bacteria and fungi, respectively. For the unweighted Unifrac distances, the highest dissimilarity coefficients were observed between GC I and GC II (bacteria) and between GC III and GC IV (fungi). At the same time, while the lowest was found between GC II and GCIII and between GC II and GC IV for bacteria and fungi, respectively.

Discrepancies between weighted and unweighted coefficients of dissimilarity within the four batches on the one hand and between bacteria and fungi on the other were visualized by conducting principal coordinate analysis (PCoA) and principal component analysis (PCA). Regardless of whether weighted or unweighted Unifrac distances were applied, no clustering was observed for at least some of the samples (results not shown).

Likewise, UPGMA trees constructed for the weighted Unifrac (Fig. 24) and unweighted Unifrac (Fig. 25) distances in bacteria and fungi did not provide conclusive information on the microbiome connectivity of the four cheese batches analyzed. One of the most plausible explanations is that Bulgarian Green Cheese is produced without a starter culture. In the ripening process, the primary role is played by microorganisms from the local environment. The four batches of cheese are produced from different types of milk (sheep and goat) and at different times of the year, meaning that different climatic conditions also affect the environmental microbiome. It is important to note that, in general, the coefficients of difference in beta diversity are not very high, which means that this factor also affected the grouping and, in general, it shows us that the four types of cheeses analyzed are not very different, despite

observing some specific types for each of them. Such a complex microbiome of cheese produced from raw milk and relying solely on the environment has already been described in the literature, especially regarding the milk microbiome (Tilocca et al., 2020) , the environmental microbiome and the continuity of microbial communities during the ripening process (Ceugniez et al., 2017 ; Escobar-Zepeda et al., 2016 ; Quijada et al., 2018) .

CONCLUSION

In conclusion, we established the ability of isolates from the genus *Enterococcus*, isolated from various food products subjected to natural fermentation, to produce antibacterial substances to inhibit the growth of pathogenic microorganisms, incl. *Paenibacillus larvae*. This result is very auspicious and may open a vast horizon for the scientific community working on controlling several diseases, including the AFD in honey bees. Further studies must confirm the bacteriocin nature of the substances inhibiting the indicator strains. Of course, *in vivo* experiments are also needed to establish the applicability of this finding.

It was proved that *Enterococcus durans* EDD2 possesses solid inhibitory activity against *Paenibacillus larvae*, due to the production of bacteriocins. Although some details regarding bacteriocin expression remain unclear, the strain has proven probiotic potential. It can be used as an alternative to antibiotics, which are currently the only way to treat beehives. The effects of bacteriocin-producing isolates from the genus *Enterococcus* in case of infection with the AFD *in vivo* should be further studied to understand their full applicability to protect honey bees.

We found that whole-genome sequencing by next-generation technologies saves time and resources in characterizing new isolates possessing interesting probiotic properties. Furthermore, with the help of these technologies, a tremendous amount of information can be collected, allowing not only to identify the presence of structural genes (beneficial or causing virulence and pathogenicity), but also providing an opportunity to gather information regarding their genetic clusters and operons.

We performed the first metagenomic study of the traditional Bulgarian Green Cheese produced in Cherni Vit village. This unique dairy product is the only Bulgarian cheese with noble mould. As a result of the studies carried out, we found that Green Cheese possesses a rich microbiome, containing mainly microorganisms that are not starter cultures by nature and which in turn contribute to its distinctive characteristics, including olfactory and organoleptic

properties. We also found that the presence of potential pathogens, typical of all fermented raw milk products, is barely noticeable and most likely poses no risk for consumption - because of the extremely low OTE count and because they are suppressed and non-viable, as described for other world-famous cheeses (Salazar et al., 2018) . However, the results obtained in this first study raise new questions and ideas for further analyses—the most important of which is the genomic characterization of the predominant species, especially of *Staphylococcus equorum, Lactococcus garvieae, Corynebacterium variabile, Scopulariopsis flava*, the various observed yeasts and most importantly of *Penicillium roqueforti*, which is the only cultured strain of this species found in our country. The eventual sequencing of whole microbiomes would allow a more precise quantitative characterization of the microbiome communities, and the performance of some metabolomic analyses necessary to characterize the final product.

SUMMARY

As a result of our work, the following was achieved:

- A collection of 348 strains was created;
- It was developed a system for storing strains at low temperatures;
- It was developed a system for the taxonomic determination of the isolated strains based on a combination of microbiological, molecular biological and genomic approaches;
- It was made and identification of the genetic determinants of protein molecules with antimicrobial activity, as well as their expression;
- Tests for the presence of antibiotic-resistance genes were performed;
- The bacteriocin-producing activity was found in some of the strains;
- Activity against *Paenibacillus larvae* was found in beehives in some of the strains;
- A check for clonal relatedness between the bacteriocin-producing strains was performed;
- Analysis of data obtained from whole genome sequencing of selected bacteriocin-producing strains was performed.

CONCLUSIONS

Based on the performed work and analysis, the **following conclusions were formulated**:

1. Fermented functional foods have a significant potential to be a source of probiotic strains of bacteria of various genera showing bacteriocin activity against pathogens (about 28% of all isolates) and protease and peptidase activity (about 7% of all isolates), such as some show both bacteriocin and protease and peptidase activity (about 3% of all isolates);

2. The antibacterial activity of the strains isolated from fermented functional foods varies widely: against 1 pathogenic species (about 42% of the isolates), against two pathogenic species (about 27% of the isolates), against three pathogenic species (about 7% of isolates), against four pathogenic species (about 8% of isolates), against five pathogenic species (about 7% of isolates), against six pathogenic species (about 4% of isolates), against eight pathogenic species (about 3% of isolates) and against all nine pathogenic species (about 1% of isolates);

3. The bacterial microbiota of the Green Cheese consists almost entirely of Firmicutes (mainly representatives of the genera *Streptococcus, Lactobacillus and Lactococcus*), Actinobacteria (mainly representatives of the genera *Brevibacterium, Corynebacterium*) and in smaller quantities Proteobacteria (mainly representatives of the genera *Cobetia, Psychrobacter and Halomonas*);

4. The fungal microbiota of the Green Cheese consists almost entirely of Ascomycota (mainly representatives of the genera *Penicillium, Scopulariopsis and Debaryomyces*) and in smaller quantities Zygomycota (mainly representatives of the genera *Circinella* and *Mucor*);

5. The composition of the microflora of Green Cheese varies among the different samples studied, which can be explained by the fact that the fermentation of the cheese depends almost entirely on microorganisms coming from the environment, as well as the type of the milk used (sheep or goat);

6. Bee colonies in "good health" have significant potential to be a source of probiotic strains of bacteria for honey bees, including those that inhibit pathogenic bacteria;

7. An exchange of probiotic strains is observed between spatially close bee families;

8. Based on a complete genome analysis of bacteriocin-producing probiotic strains from the *Enterococcus* strains with activity against the causative agent of AFD, it was found that they possess genetic determinants for the synthesis of more than one type of bacteriocin, and most likely the activity is due to synergism and/or of specific variants of enterocins P and A;

9. Based on a whole genome analysis of bacteriocin-producing probiotic strains of the *Enterococcus* species with activity against the causative agent of AFD, it was found that they are most likely the product of intra-hive evolution since they have lost most of the factors of virulence and pathogenicity;

10. Next-generation whole genome sequencing combined with bioinformatic analysis on free-access, online-based platforms are a convenient, cost-effective, reliable, and rapid way for detailed analysis and characterization of probiotic strains;

11. Amplicon-based metagenomic sequencing of samples containing complex microbiota, combined with bioinformatic analysis on free-access online-based platforms, appears to be a convenient, cost-effective, reliable, and rapid way for detailed analysis and characterization of fermented functional foods and some other natural products possessing their microbiomes.

CONTRIBUTIONS

The scientific contributions in this thesis are the following:

1. For the first time in Bulgaria, an amplicon-based metagenomic analysis of a fermented dairy food product (Green Cheese from Tcherni Vit) was performed;

2. It has been proven that the Green Cheese has a specific microbiota such as its ripening depends on the specific conditions in the area of the villages of Cherni Vit;

3. For the first time in Bulgaria, genomes of probiotic *Enterococcus* strains from bees were sequenced;

4. The isolated probiotic strains *Enterococcus durans* EDD2 and *Enterococcus faecalis* EFDs can be used as a completely natural alternative to treat bee colonies that are infected with the causative agent of the AFD – *Paenibacillus larvae*;

5. Based on the available genetic determinants in *Enterococcus durans* EDD2 and *Enterococcus faecalis* EFD, they were identified as the most likely candidates for bacteriocins with activity against *Paenibacillus larvae*.

PUBLICATIONS

Gyurova , **A.** Vladimirova, A., Peykov, S., Dimitrov, M., Strateva T. & Dimov , SG (2021) Characterization of *Enterococcus durans* EDD2, a strain from beehives with inhibitory activity against *Paenibacillus larvae* , Journal of Apicultural Research, doi : 10.1080/00218839.2021.1936915

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