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Functional and Safety Characterization of *Weissella parameseteroides* Strains Isolated from Dairy Products through Whole-Genome Sequencing and Comparative Genomics

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Abstract: Strains belonging to the *Weissella* genus are frequently recovered from spontaneously fermented foods. Their functional, microbial-modulating, and probiotic traits enhance not only the sensorial properties but also the nutritional value, beneficial effects, and safety of fermented products. Sporadic cases of opportunistic pathogenicity and antibiotic resistance have deprived safety status from all *Weissella* species, which thus remain understudied. Our study increased the number of available high-quality and taxonomically accurate *W. parameseteroides* genomes by 25% (9 genomes reported, leading to a total of 36 genomes). We conducted a phylogenetic and comparative genomic analysis of the most dominant *Weissella* species (*W. cibaria*, *W. parameseteroides*, *W. viridescens*, *W. soli*, *W. koreensis*, *W. hellenica* and *W. thailadensis*). The phylogenetic tree corroborated species assignment but also revealed phylogenetic diversity within the *Weissella* species, which is likely related to the adaptation of *Weissella* in different niches. Using robust alignment criteria, we showed the overall absence of resistance and virulence genes in *Weissella* spp., except for one *W. cibaria* isolate carrying $bla_{\text{TEM-181}}$. Enrichment analysis showed the association of *Weissella* species several CAZymes, which are essential for biotechnological applications. Additionally, the combination of CAZyme metabolites with probiotics can potentially lead to beneficial effects for hosts, such as the inhibition of inflammatory processes and the reduction of cholesterol levels. Bacteriocins and mobile genetic elements MGEs (*Inc11* plasmid and *ISS1N* insertion sequence) were less abundant, however *W. thailadensis* and *W. viridescens* showed significant association with specific bacteriocin-encoding genes. Lastly, an analysis of phenotypic traits underlined the need to carefully evaluate *W. cibaria* strains before use as food additives and suggested the possibility of employing *W. parameseteroides* and *W. hellenica* in the fermentation process of vegetable products. More studies providing high-resolution characterization of *Weissella* strains from various sources are necessary to elucidate the safety of *Weissella* spp. and exploit their beneficial characteristics.

Keywords: bioinformatics; fermented products; molecular microbiology; Next-Generation Sequencing; starter cultures; *Weissella* spp.

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1. Introduction

Weissella species are non-spore forming, catalase-negative, and Gram-positive bacteria. They are facultative anaerobes and are found in the gastrointestinal tract of humans and animals [1]. They are also found in the environment, including soil, water, and plants. Numerous *Weissella* strains have been isolated from foods, including dairy products, meat, and vegetables. *Weissella cibaria*, *W. parameseteroides*, and *W. hellenica* are the most frequently isolated species from fermented foods [2]. *Weissella cibaria* is most frequently isolated from fermented meat products, whereas *W. parameseteroides* is from fermented dairy products. *Weissella hellenica* is often isolated from fermented vegetables. Furthermore, *W. koreensis* is

the most frequently isolated species from kimchi, a traditional fermented vegetable food in Korea [3,4].

The use of starters is recommended compared to spontaneous food fermentation, as it is a more controlled process, and the use of selected strains can improve the quality and organoleptic characteristics of the final product [5]. Bacterial starters are used for dairy products, sourdough, meat, and other fermented foodstuff. Among lactic acid bacteria (LAB), the predominant strains employed as starters belong to the former-*Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Leuconostoc* genera [6]. *Weissella* spp. strains are often retrieved from various spontaneously fermented foodstuff, indicating their ability to adapt and survive in different niches and environmental conditions. They also appear to have a large repertoire of functional traits and probiotic properties, which can promote the safety aspects, nutritional value, and organoleptic properties of fermented food as well as exert beneficial effects on humans by increasing the content and activity of beneficial bacteria in the gut [7,8].

Despite the potential beneficial effects, none of the *Weissella* species has been granted the Qualified Presumption of Safety (QPS) status by the European Food Safety Agency (EFSA) [9]; therefore, the application of *Weissella* spp. as starting or adjunct cultures remains poorly explored. Moreover, rare but alarming reports have associated *Weissella* with a pathogenic lifestyle, such as the isolation of *W. cibaria* from the bloodstream and urinary tract infections (UTIs) [3]. High-throughput technologies can help to distinguish pathogenic from commensal bacteria via their thorough characterization [10]. The *Weissella* species remains understudied, with only 155 high-quality and taxonomically accurate genome sequences being publicly available in the NCBI assembly database, and the majority (12/19) of the *Weissella* species have less than four genome sequences (https://www.ncbi.nlm.nih.gov/assembly; accessed on 20 May 2022). In this regard, the aim of this study was to provide a high-resolution characterization of *W. paramesenteroides* strains isolated from different dairy products, such as raw sheep milk, artisanal Feta, and artisanal Kefalograviera cheese [11,12] using whole-genome sequencing (WGS), primarily with respect to their resistance and virulence repertoire but to other important genomic features as well. Given that our analysis significantly increased the number of genome sequences for *W. paramesenteroides*, we also aimed to conduct a comparative genomic analysis between dominant *Weissella* species and assess the genomic characteristics of this collection in the context of a broader and diverse set of sequenced isolates.

2. Materials and Methods

2.1. Microbial Strains and Culture Conditions

The *W. paramesenteroides* microbial collection (n = 9) of Dairy Research Department (DRD) of Hellenic Agricultural Organization "DIMITRA" (ELGO-DIMITRA) isolated from sheep milk and artisanal Feta and Kefalograviera cheeses [11] were used in this work. Storage and culture conditions of the strains are described in detail in the work of Tsigkrimani et al. (2022) [12]. In addition to this collection, 127 *Weissella* spp. genomes were parsed from the NCBI database to conduct a comparative genomic analysis. This dataset included *W. cibaria* (n = 72), *W. paramesenteroides* (n = 25), *W. viridescens* (n = 10), *W. soli* (n = 6), *W. koreensis* (n = 6), *W. hellenica* (n = 4), and *W. thailadensis* (n = 4). The total number of genomes described here (n = 136) makes up ~88% of the high-quality (excluding "anomalous" filter) and taxonomically accurate (taxonomy "OK" filter) *Weissella* spp. genomes available at the NCBI assembly database.

2.2. Whole Genome Sequencing, Assembly, and Quality Control

DNA extraction was based on the work of Syrokou et al. (2020) [13]. The GenElute Bacterial Genomic DNA Kit's manufacturer's recommended extraction procedure was followed (Sigma, Chemical Co., St. Louis, MO, USA). DNA sequencing was performed by Novogene Genomics Service (Novogene Co., Cambridge, UK). DNA quality was examined by agarose gel electrophoresis and quantified with the Qubit 2.0 (ThermoFisher Scientific,

Waltham, MA, USA). The steps followed for the library preparation were sonication for random DNA fragmentation, end polishing, A-tailing, ligation with Illumina's sequencing adapters, and PCR amplification with P5 and P7 oligos. PCR products were purified, and size selected using the AMPure XP system (Beckman Coulter, Brea, CA, USA). Size of the library was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified by qPCR. Sequencing of the qualified libraries was executed on the Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA) (2 × 150 bp). Quality of the adapter-free raw reads was checked with the software FastQC v.0.11 (Andrews, 2010; available online at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed on 27 May 2022) available in the KBase platform [14,15]. Polishing and de novo assembling of the raw reads into contigs were performed with the Unicycler assembler and Pilon, respectively, provided by the PATRIC v3.6.8 web platform [16–18]. The Multi-Draft based Scaffolder (MeDuSa) v1.6 [19] was used to organize the contigs into scaffolds. Scaffolds were ordered and oriented based on the reference genomes present in the NCBI database (https://www.ncbi.nlm.nih.gov/, accessed on 10 January 2022); *W. paramesenteroides* ATCC 33313 and *W. paramesenteroides* FDAARGOS 414. The CheckM tool v1.21 [20] of the PATRIC v3.6.8. system was employed for quality evaluation of the contigs and scaffolds to ensure that assembled genomes were of high quality, i.e., completeness (≥95%) and contamination (≤5%). Possible mis-assemblies after scaffolding were assessed by the mean of the Skew Index Test (SkweIT) v1.0 [21].

2.3. In Silico Typing and Characterization

The quality of the assembled genomes was assessed with QUAST [22]. Species identification was performed with the Kraken2 taxonomic classifier [23] and the Type Strain Genome Server (TYGS) [24]. Genome relatedness was evaluated with OrthoANI [25]. The genomes were annotated using PROKKA [26], and further functional annotation and subsystem analysis of predicted open reading frames (ORFs) was done via the COG database [27]. Moreover, presence of clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) was evaluated with CRISPRCasFinder [28], whereas integrated prophages were identified with PHASTER [29]. Abricate [30] was used to determine the presence of resistance genes (RGs), virulence genes (VGs), mobile genetic elements (MGEs) and plasmids using the Resfinder [31], VFDB [32], MobileElementFinder [33] and PlasmidFinder [34] databases, respectively. Additionally, presence of bacteriocins was determined with BAGEL4 [32]. Lastly, we used the PathogenFinder [35] classifier to predict the pathogenicity of the isolates in our collection.

2.4. Phylogenetic Analysis and Comparative Genomics

The pangenome analysis and core-genome alignment of all *Weissella* spp. genomes (n = 136) was performed with Roary [35]. Proteins were assigned into the same family if their amino acid sequence identity was ≥90%. The threshold percentage of the isolates that needed to have a gene for it to be considered a core gene was set at 90%. Regions indicative of homologous recombination were removed with Gubbins [36], and a phylogenetic tree was built with FastTree [37]. The phylogenetic tree was annotated and visualized with the Interactive Tree Of Life (iTOL) program [38]. Furthermore, we conducted Carbohydrate-active enzyme (CAZyme) searches with the Run_dbcan V3 standalone tool of the dbCAN2 server [39], considering as positive hits only the genes found by both the Pfam Hidden Markov Models (HMMs) and DIAMOND. To further elucidate key genomic differences between *Weissella* species, a cluster heatmap was generated using a presence-absence matrix of the CAZymes, bacteriocins, MGEs and plasmids present in these isolates. Clustering observations on the heatmap were further explored with statistical analysis for gene-enrichment in the respective species. Moreover, *Weissella* spp. isolates were juxtaposed based on various predicted phenotypic traits (n = 67) using Traitar [40]. Lastly, we conducted a Gene Ontology (GO) over-representation analysis. Genes in the pangenome of *Weissella* spp. created with Roary were mapped to their respective GO terms using eggnog v5.0 [41], followed

by an enrichment analysis of identified GO terms in each species with ClusterProfiler [42] and visualization of results with Go-Figure! v1.0.1 [43]. Part of the bioinformatic analysis was done on the European public Galaxy [44] server (https://usegalaxy.eu/; accessed on 27 May 2022).The pangenome analysis and core-genome alignment of all *Weissella* spp. genomes (n = 136) was performed with Roary [36]. Proteins were assigned into the same family if their amino acid sequence identity was ≥90%. The threshold percentage of the isolates that needed to have a gene for it to be considered a core gene was set at 90%. Regions indicative of homologous recombination were removed with Gubbins [37], and a phylogenetic tree was built with FastTree [38]. The phylogenetic tree was annotated and visualized with the Interactive Tree Of Life (iTOL) program [39]. Furthermore, we conducted Carbohydrate-active enzyme (CAZyme) searches with the Run_dbcan V3 standalone tool of the dbCAN2 server [40], considering as positive hits only the genes found by both the Pfam Hidden Markov Models (HMMs) and DIAMOND. To further elucidate key genomic differences between *Weissella* species, a cluster heatmap was generated using a presence-absence matrix of the CAZymes, bacteriocins, MGEs, and plasmids present in these isolates. Clustering observations on the heatmap were further explored with statistical analysis for gene-enrichment in the respective species. Moreover, *Weissella* spp. isolates were juxtaposed based on various predicted phenotypic traits (n = 67) using Traitar [41]. Lastly, we conducted a Gene Ontology (GO) over-representation analysis. Genes in the pangenome of *Weissella* spp. created with Roary were mapped to their respective GO terms using eggnog v5.0 [42], followed by an enrichment analysis of identified GO terms in each species with ClusterProfiler [43] and visualization of results with GoFigure! [44]. Part of the bioinformatic analysis was done on the European public Galaxy [45] server (https://usegalaxy.eu/; accessed on 27 May 2022).

2.5. Statistical Analysis

Gene-enrichment analysis was conducted using presence-absence data matrices as input to Scoary [46] to determine which gene classes or GO terms were significantly enriched (over-represented) in each species. The significance level (alpha) was set at 0.01. The p-values were adjusted with the Benjamini–Hochberg's method for multiple comparisons correction.

3. Results and Discussion

3.1. Species Identification, Assembly Statistics, and Subsystem Analysis

Taxonomic classification with Kraken2 and TYGS corroborated the strain identification ofTsigkrimani et al. (2022) [11], as all nine strains were identified as *W. paramesenteroides*. Details and assembly statistics as well as orthoANI values are presented in Tables 1 and 2, respectively. The average genome size, GC content, and number of coding sequences (CDSs) along with the corresponding standard deviation were 1.92 ± 0.06, 38.03% ± 0.12% and 1926 ± 86, respectively. Of note, compared with the other genomes strain, weis_C142 had the shortest genome length, the smallest number of CDS, and the highest GC content ratio (Table 1). A subsystem is a set of CDSs that together implement a specific biological process or structural complex [47]. Subsystem analysis with the COG database revealed the presence of 11 enriched subsystem categories (Figure 1). The process category of metabolism was the most enriched one with 284 (± 2) genes, on average. Together with metabolism, protein processing (176 ± 21), energy (91 ± 2), DNA processing (69 ± 0), and stress-response-virulence (56 ± 4) processes, made the majority of CDSs with known functions (Figure 1).

Table 1. Species identification and assembly statistics for the nine *W. paramesenteroides* isolates.

Strain ID	Genus & Species	Genome Size (Mb)	GC Content (%)	No of Scaffolds	N50 (Mb)	No of CDSs
weis_C39	*W. paramesenteroides*	1.95	37.98	25	1.37	1965
weis_C194	*W. paramesenteroides*	1.95	37.99	26	1.92	1969
weis_C187	*W. paramesenteroides*	1.95	37.98	24	1.92	1968
weis_C172	*W. paramesenteroides*	1.91	38	21	1.87	1915
weis_C169	*W. paramesenteroides*	1.91	38	26	1.50	1920
weis_C149	*W. paramesenteroides*	1.95	37.98	25	1.13	1964
weis_C142	*W. paramesenteroides*	1.75	38.37	7	1.75	1690
weis_C137	*W. paramesenteroides*	1.95	37.98	25	1.34	1969
weis_C105	*W. paramesenteroides*	1.95	37.98	27	1.92	1975

Table 2. OrthoANI values for the nine *W. paramesenteroides* isolates.

Strain ID	weis_C39	weis_C105	weis_C137	weis_C142	weis_C149	weis_C169	weis_C172	weis_C187	weis_C194
weis_C39		99.99	99.99	99.91	99.99	99.99	99.99	99.99	99.99
weis_C105			99.99	99.91	99.99	99.98	99.99	99.99	99.97
weis_C137				99.89	99.99	99.99	99.98	99.98	99.99
weis_C142					99.89	99.90	99.89	99.90	99.89
weis_C149						99.98	99.99	99.99	99.98
weis_C169							99.99	99.98	99.99
weis_C172								99.99	99.99
weis_C187									99.98

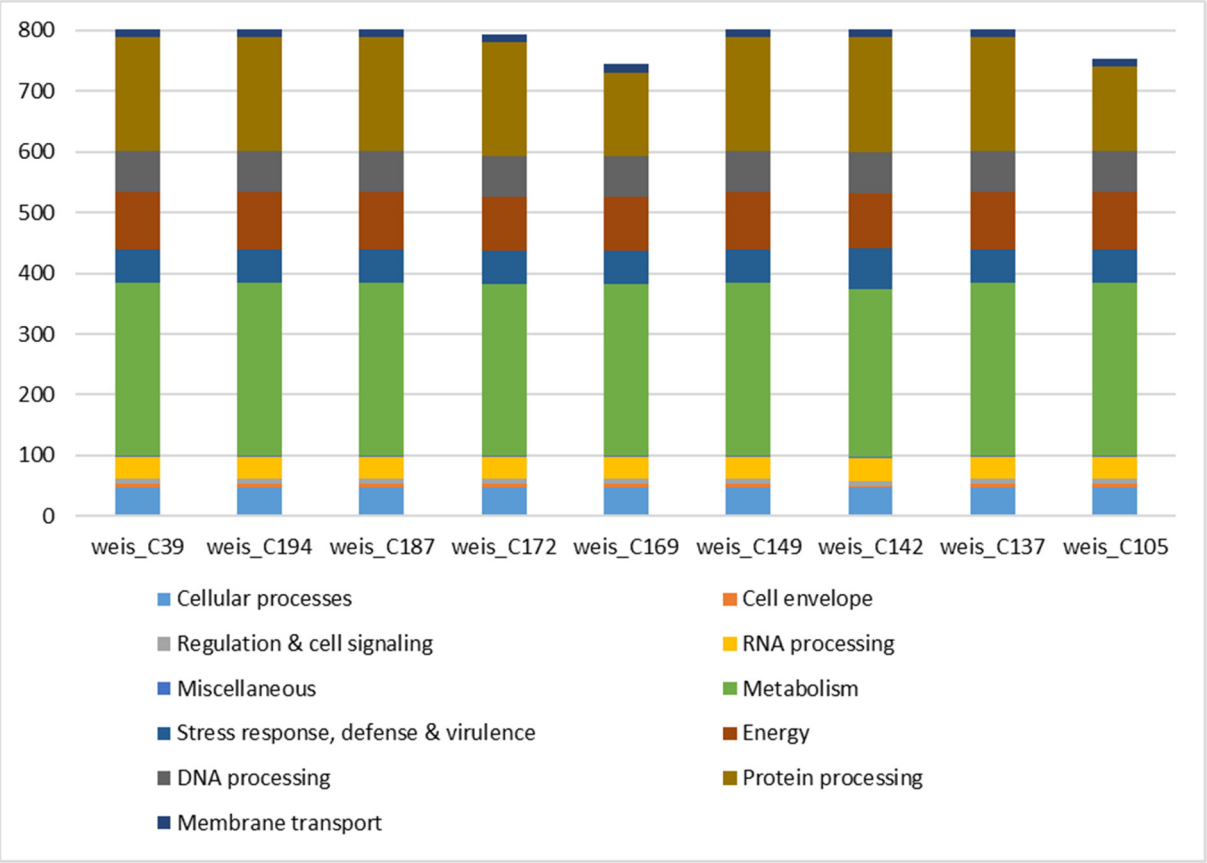

Figure 1. Overview of the subsystems in *Weissella paramesenteroides* genomes.

3.2. Presence of Resistance and Virulence Genes

Analysis with the ResFinder and VFDB databases for the presence of resistance and virulence genes (RGs, VGs) using an identity and coverage threshold of 80% showed absence of relevant genes.

3.3. Other Genomic Features

3.3.1. Bacteriocins, Prophages, and CRISPR-Cas

Bacteriocins are ribosomally synthesized peptides that are produced by bacteria and are active against other bacteria. They are classified into two groups, class I and class II, based on their structure and mode of action. Class I bacteriocins are small, heat-stable, cationic peptides that are active against a wide range of bacteria. Class II bacteriocins are larger, heat-labile, and have a narrower spectrum of activity [48]. Bacteriocins can be applied to foods as natural preservatives to inhibit the growth of pathogenic and spoilage bacteria [49]. None of the nine *W. paramesenteroides* harbored genes encoding for the production of bacteriocins.

With regard to the prophage content, only two isolates (weis_C172 and weis_C179) had intact prophage regions in their genomes (27.4 Kb and 34.2 Kb, respectively). Both regions corresponded to *Staphylococcus* phage *SPbeta*-like (NCBI accession: NC_029119.1). Prophages that integrate in bacterial genomes often harbor resistance or virulence genes that can be transferred to the host bacterium [49]; the existence of CRISPR/Cas systems can help to protect bacterial genomes from prophage integration [50]. In this regard, none of the *W. paramesenteroides* isolates had robust evidence (evidence level = 4) of CRISPR sequences and *cas* genes in their genomes. With regard to the prophage content, only two isolates (weis_C172 and weis_C179) had intact prophage regions in their genomes (27.4 Kb and 34.2 Kb, respectively). Both regions corresponded to *Staphylococcus* phage *SPbeta*-like (NCBI accession: NC_029119.1). Prophages that integrate in bacterial genomes often harbor resistance or virulence genes that can be transferred to the host bacterium [50], therefore, the existence of CRISPR/Cas systems can help to protect bacterial genomes from prophage integration [51]. In this regard, none of the *W. paramesenteroides* isolates had robust evidence (evidence level = 4) of CRISPR sequences and *cas* genes in their genomes.

3.3.2. Plasmids and Other MGEs

All isolates but one (n = 8) contained one 1755 bp *Inc11* plasmid with a GC-content of 40.4%, as well as an 808 bp *ISS1N* insertion sequence (IS) of the *IS26* family [52]. This plasmid and IS element were initially described in *Lactococcus lactis* and are considered to play an important role to the conjugal transfer of genes (e.g., phospho-p-galactosidase) involved in lactose metabolism between various lactic acid bacteria species [53].

Moreover, we used the PathogenFinder machine-learning algorithm to predict the pathogenicity of the isolates in our collection and, thus, classify them as human pathogens or commensals. All isolates in our collection were predicted as non-pathogenic, which further corroborates the absence of pathogenic determinants such as RGs, VGs, and MGEs known to harbor pathogenic determinants.

3.4. Phylogenetic Analysis and Comparative Genomics

Sequencing of the nine *W. paramesenteroides* genomes of our collection increased the number of published, high-quality genomes of this species by 25%, leading to a total of 36 genome assemblies available in the NCBI database (ncbi.nlm.nih.gov/assembly; accessed on 20 May 2022). We conducted a phylogenetic and comparative genomic analysis in order to gain deeper insights into the genetic relationships of the most dominant *Weissella* species (*W. cibaria*, *W. paramesenteroides*, *W. viridescens*, *W. soli*, *W. koreensis*, *W. hellenica*, and *W. thailadensis*) in terms of the number of high-quality and taxonomically accurate sequenced genomes. The pangenome of the seven *Weissella* species consisted of 15,949 clusters of orthologous genes (COGs), whereas the core-genome comprised 86 COGs. The phylogenetic tree based on the alignment of core genes revealed the genomic relat-

edness of the analyzed *Weissella* spp. isolates (Figure 2). Isolates clustered according to their species assignment with no overlaps between clusters. This observation is in contrast with the results of Surachat et al. (2022) [8], who found extensive misplacement of *Weissella* strains in the phylogenetic tree. The underlying reason is that, contrary to our analysis, this study included genomes with "inconclusive" taxonomic status, i.e., strains that have conflict between the user-declared species and the average nucleotide identity (ANI) with the respective representative (reference) genome of the species in the NCBI database [53]. As the authors of this study concluded, the use of 16S rRNA gene sequencing [54] may not always be sufficient for taxonomic classification, and the species with inconclusive status need to be further explored for assignment to different or novel *Weissella* species [8]. Furthermore, sub-clusters were formed within each species cluster especially for *W. cibaria* and *W.* paramesenteroides, indicating the phylogenetic diversity of *Weissella* species. Although information regarding the origin of isolation was not available for the majority of the isolates, core-genome variance may explain the adaptation of *Weissella* in different niches and environmental conditions [7].

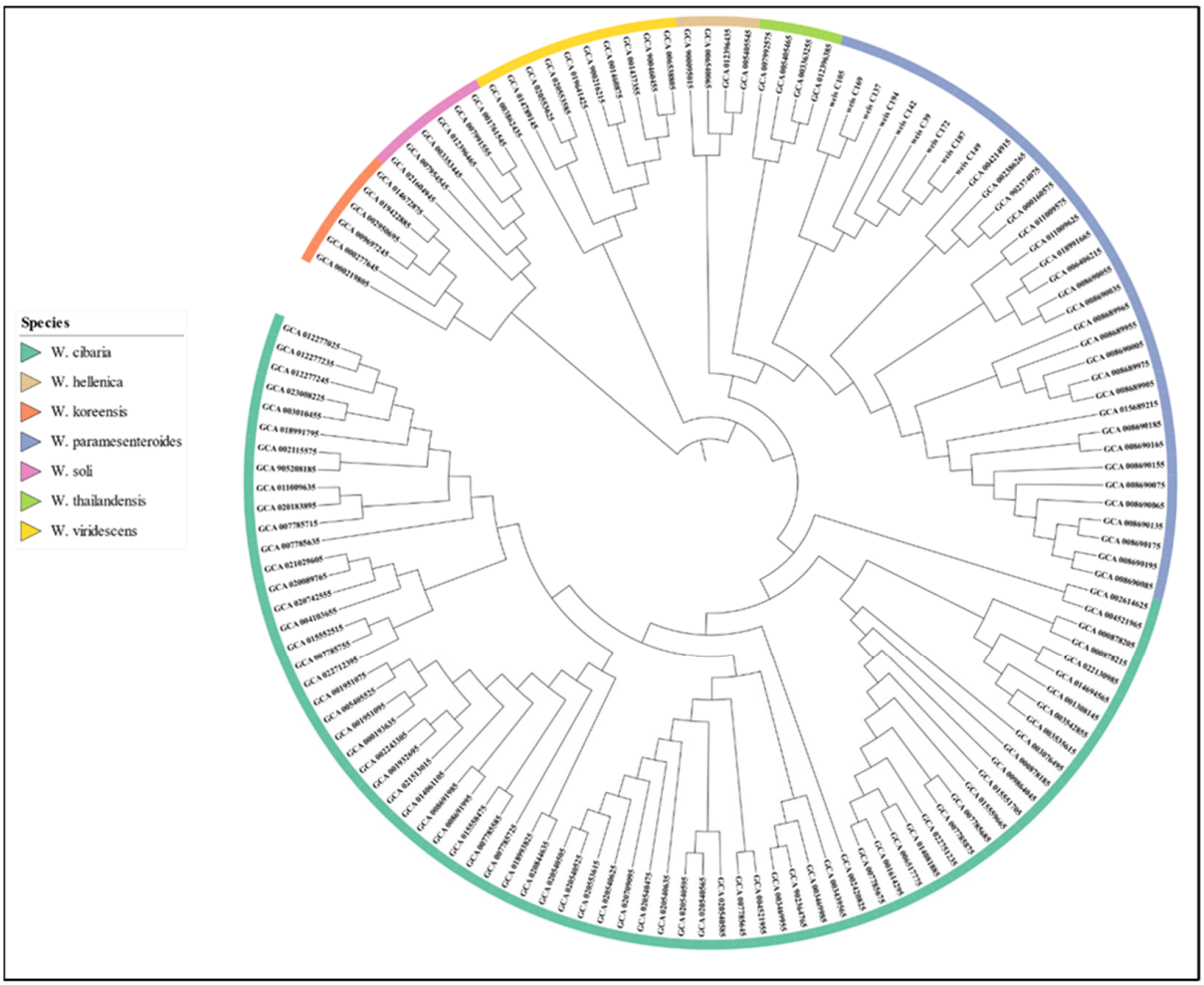

Figure 2. Phylogenetic tree including the 163 *Weissella* spp. isolates. The colored ring indicates the isolates' species according to the legend.

Analysis for RGs and VGs showed an overall absence of relevant genes except for one *W. cibaria* isolate (assembly accession No: GCA_012277245) that harbored $bla_{\text{TEM-181}}$, a beta-lactamase gene that confers resistance to ampicillin. The presence of RGs can be a significant issue for microorganisms that are used as food additives (e.g., starter or adjunct cultures), especially if they are located in MGEs (plasmids and IS elements), which can

be exchanged between pathogenic and commensal microorganisms [3,55]. The tendency of a bacterial species to harbor mobile genetic elements is a key factor in the evolution of bacterial pathogens. The presence of these elements in the genome is a reflection of the ability of the species to acquire and maintain pathogenicity and microbial resistance determinants [56].

Enzymes responsible for the metabolism of carbohydrates are known as carbohydrate-active enzymes (CAZymes). These enzymes are involved in the degradation and synthesis of polysaccharides, oligosaccharides, and glycoconjugates [57]. Apart from being interesting in biotechnological applications, the biotransformation of food carbohydrates by bacteria can produce valuable metabolites. Additionally, the combination of pre- and probiotics can lead to significant beneficial effects such as the inhibition of inflammatory processes and the reduction of cholesterol levels [5]. In the next analysis, we aimed to juxtapose all *Weissella* isolates with respect to their functional (CAZymes and bacteriocin content) and pathogenic-potential (presence of MGEs) and elucidate whether the presence-absence patterns of these genes can distinguish isolates of different species.

The heatmap and hierarchical clustering showed distinct clusters for *W. cibaria*, *W. koreensis* and *W. viridescens*, whereas *W. paramesenteroides*, *W. hellenica*, *W. soli*, and *W. thailadensis* overlapped (Figure 3). The majority of the CAZymes identified in the analyzed *Weissella* isolates belonged to the glycoside hydrolase (GH) families with the GH13 family being predominant, whereas 38 different families were identified. *Weissella cibaria* showed the strongest association with CAZyme content, as 19 families were significantly enriched [Odds Ratio (OR) < 1, p-value < 0.05] in this species, predominantly of the GH family but also of the Glycosyltransferase (GT) and Carbohydrate-binding module (CBM) families. Interestingly, CBMs act as catalytic modules of long CAZymes, such as glycoside hydrolases, with the latter being essential in the degradation of complex carbohydrates such as lactose and starch [57]. This CAZyme family was also significantly associated with *W. soli*. Compared to *W. cibaria*, the rest of *Weissella* species had weaker association with CAZymes, with seven and five GH families being significantly enriched in *W. paramesenteroides* and *W. koreensis*, respectively, and less than three in the other species (Figure 3).

Only 32 out of 136 (23.5%) *Weissella* strains were found to harbor bacteriocin-encoding genes. Zoocin_A, the predominant identified bacteriocin was significantly enriched (OR > 1, p-value < 0.05) in *W. thailadensis* (Figure 3). This bacteriocin was initially purified from *Streptococcus equi* and is involved in the growth inhibition of pathogenic bacteria, such as pathogenic Streptococci and *Listeria monocytogenes* [58]. The Enterocin_L50b and MR10B were each found in seven isolates and were significantly associated with *W. viridescens*. Enterocin_L50b and MR10B are respectively active against *L. monocytogenes* and *S. aureus*, and they were first extracted from *Lactobacillus lactis* [48]. Lastly, closticin_574 was identified in six isolates of *W. soli*; this 82-amino-acid bacteriocin initially retrieved from *Clostridium tyrobutyricum* shows a broad range of antimicrobial activity and is especially active against *Clostridium* spp. [59].

With regard to MGEs, only *ISS1N* was found to be significantly associated with *W. paramesenteroides*, present only in 8 out of 34 isolates of this species (all of them belonging to our collection). As described previously, this IS element mediates the transport of genes involved in lactose metabolism between various lactic acid bacteria species [52] and, to our knowledge, has not been described to mediate the transfer of resistance or virulence genes.

Moreover, analysis with Traitar for predicted phenotypic characteristics showed that, irrespective of their species, all isolates can utilize sugars such as glucose, maltose, and sucrose (Figure 4). The clustered heatmap of predicted traits indicated an overlap of species clusters, suggestive of shared phenotypic profiles between *Weissella* spp. A statistical analysis for phenotype association provided more insights; *W. cibaria* isolates were found to be significantly related (OR > 1, p-value < 0.05) with the catabolism of salicin, trehalose, L-rhamnose, and raffinose. The fermentation process of the latter two sugars has been linked with nosocomial, pathogenic strains of *E. faecium* [60,61], and this may explain the fact that several *W. cibaria* strains have been described as opportunistic pathogens involved in

bloodstream infections as well as cases of dog ear otitis [4]. In contrast, *W. paramesenteroides* and *W. hellenica* showed significant association with the utilization of starch and malonate, suggesting that these species could be used in both dairy and vegetable fermentation [62]. Lastly, we found that *W. soli* was significantly associated with the production of hydrogen sulfide. Recent studies propose that bacterial-derived H_2S plays a pivotal role as a defense system against antibiotics and oxidative stress [63], but we found no reports of *W. soli* being related with disease in humans or animals. It is important to note, however, that *W. soli*, first isolated from soil and then from fermented vegetables [3], remains an understudied species with only six genome sequences available in the NCBI database.

Figure 3. Cluster heatmap generated using an MGE (plasmids and insertion sequences), CAZyme, and bacteriocin gene presence-absence data matrix of all *Weissella* spp. isolates (n = 136).

Figure 4. Predicted phenotypic characteristics of *Weissella* spp. with Traitar.

Furthermore, the analysis for significantly enriched GO terms provided further insights into the biological processes of *Weissella* spp. that are accomplished by multiple molecular activities. For *W. cibaria*, the most populated clusters of unique GO terms were related to dGTP catabolic processes and L-alpha-amino acid transmembrane transport (Figure 5). Interestingly, *W. paramesenteroides* was significantly associated with the biosynthesis of extracellular polysaccharides, a process that can play a significant role in the adaptation and symbiotic relationship of probiotic bacteria [64]. Moreover, processes for the metabolism of mannitol were significantly enriched in *W. hellenica*, and, in contrast, were reported to be absent from *W. confusa*. Lack of mannitol pathways in LAB may promote non-alcoholic fatty liver disease in host mammals that receive a high-fructose, high-fat diet [65]. Lastly, a notable insight was discovered for *W. thailadensis*, which was only associated with the spheroidene biosynthetic process. Carotenoids like spheroidene have multiple applications, such as in the production of pharmaceuticals and food/feed additives, due to their robust antioxidant capabilities. In this context, bacterial species that can accumulate carotenoids in their microbial cells, e.g., through sequential nutrition starvation, have been proposed as viable competitors of existing carotenoid sources [66].

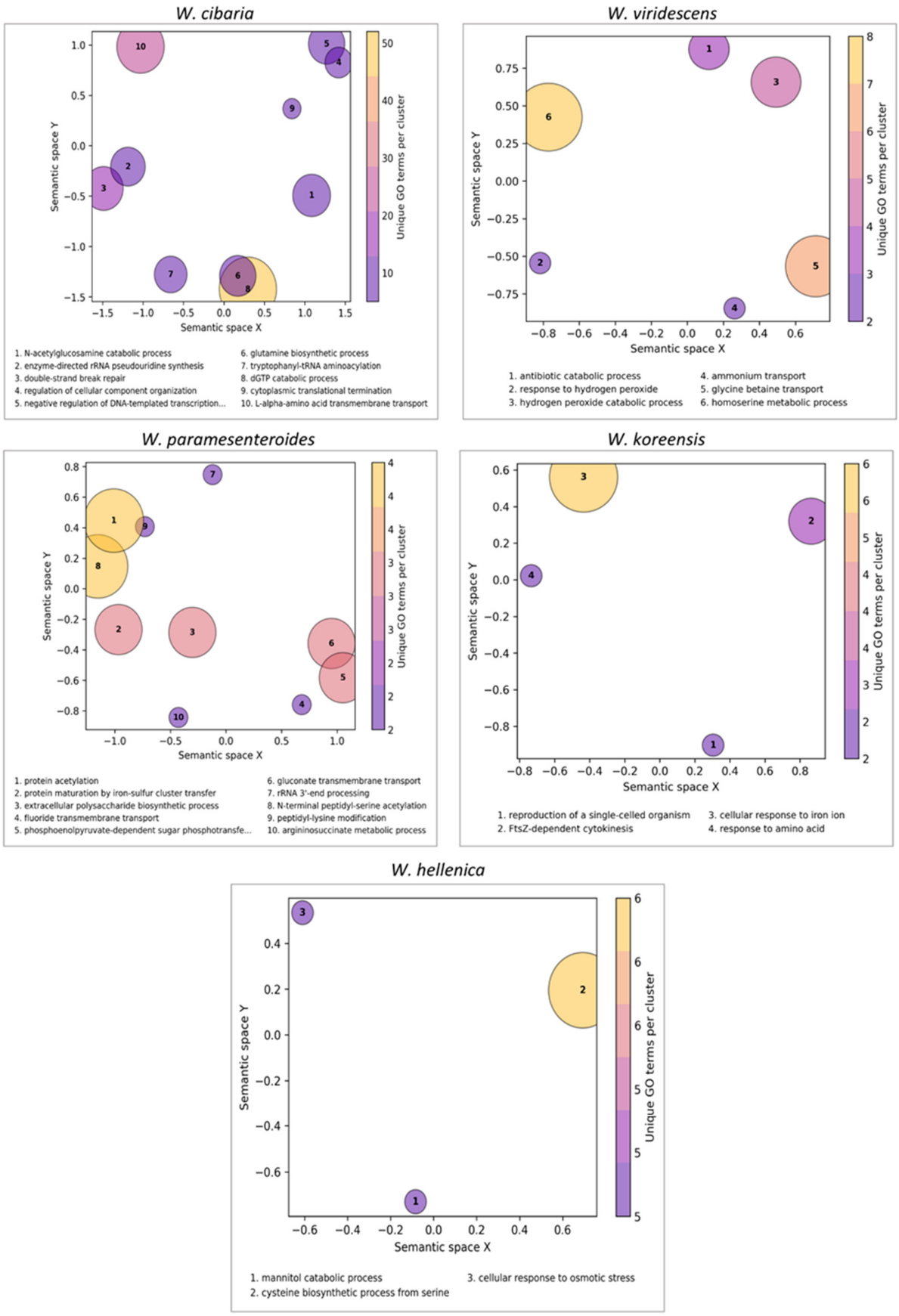

Figure 5. Clusters of unique Gene Ontology (GO) terms that were enriched in *Weissella* spp.

4. Conclusions

The genus of *Weissella* comprises versatile strains able to adapt in different niches and environmental conditions. Their functional, microbial-modulating, and probiotic traits enhance not only the sensorial properties but also the nutritional value, beneficial effects, and safety of spontaneously-fermented foods, in which they are frequently found [7,8]. However, sporadic cases of opportunistic pathogenicity have deprived the QPS status for all *Weissella* species, meaning that strains may not be used freely as food additives (e.g., as starter cultures). For this reason and in contrast to other LAB, *Weissella* spp. Remain understudied.

Our study increased the number of available, high-quality *W. paramesenteroides* genomes by 25%. We conducted a phylogenetic and comparative genomic analysis of the most dominant *Weissella* species (*W. cibaria*, *W. paramesenteroides*, *W. viridescens*, *W. soli*, *W. koreensis*, *W. hellenica*, and *W. thailadensis*), focusing on high-quality and taxonomically accurate sequenced genomes. The phylogenetic tree based on the alignment of 86 conserved core-genes corroborated species assignment but also revealed phylogenetic diversity within *Weissella* species, which is likely related to the adaptation of *Weissella* in different niches and environmental conditions [7]. Notably, using robust alignment criteria ($\geq$80% gene coverage and identity), we showed the overall absence of resistance and virulence genes in *Weissella* spp., with the exception of one *W. cibaria* isolate carrying $bla_{\text{TEM-181}}$. Enrichment analysis for important genomic traits provided more insights; all studied *Weissella* species showed association with several CAZyme families, which are essential for biotechnological applications and, in combination with probiotics, can promote health [5]. Bacteriocins were less abundant; however, *W. thailadensis* and *W. viridescens* showed significant association with specific bacteriocin-encoding genes. Thus, to fully exploit the beneficial functional properties of *Weissella*, a combination of strains as food additives may be necessary [2]. Furthermore, MGEs were rare among *Weissella* spp., although *ISS1N*, an IS so far related with the transfer of functional and not pathogenic genes, was found to be significantly associated with *W. paramesenteroides* [53]. Lastly, analysis of phenotypic traits underlined the need to carefully evaluate *W. cibaria* strains before use as food additives and suggested the possibility of employing *W. paramesenteroides* and *W. hellenica* in the fermentation process of vegetable products.

Several LAB species are used as food additives despite their implication in infections and association with antibiotic resistance [3]. Given that the majority of *Weissella* population does not harbor virulence or resistance genes and has only sporadically been linked with disease, their GRAS status needs to be reconsidered. To this end, more studies providing high-resolution characterization of *Weissella* strains are necessary.

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