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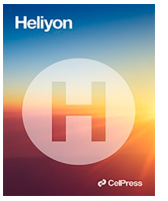

Research article

Dual effects of a bacteriocin-producing *Lactiplantibacillus pentosus* CF-6HA, isolated from fermented aloreña table olives, as potential probiotic and antimicrobial agent

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ABSTRACT

The probiotic potential of *Lactiplantibacillus pentosus* CF-6HA isolated from traditionally fermented Aloreña table olives was analyzed *in vitro* and *in silico*. Results obtained suggested that this strain can be catalogued as "talented" bacterium exhibiting bacteriocin production with antimicrobial activity against human/animal and plant pathogens, such as *Pseudomonas syringae* and *Verticillium dahliae*. The robustness, safety and probiotic potential of *L. pentosus* CF-6HA was confirmed by *in silico* analysis. In addition, a plethora of coding genes for defense and adaptability to different life styles besides functional properties were identified. In this sense, defense mechanisms of *L. pentosus* CF-6HA consist of 17 ISI elements, 98 transposases and 13 temperate phage regions as well as a CRISPR (clustered regularly interspaced short palindromic repeats)/cas system. Moreover, the functionality of this strain was confirmed by the presence of genes coding for secondary metabolites, exopolysaccharides and other bioactive molecules. Finally, we demonstrated the ability of *L. pentosus* CF-6HA to biotransform selenite to nanoparticles (SeNPs) highlighting its potential role in selenium bioremediation to be exploited in foods, agriculture and the environment; but also for the bio-enrichment of fermented foods with selenium.

1. Introduction

The World Health Organization defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1,2]. These include microorganisms of different origin (soil; plants; food; gastro-intestinal, urogenital and vaginal tracts; breast milk and feces of human subjects), taxonomy, bioavailability, metabolic activity and mode of action, although the key characteristic is the ability to promote human, animal or plant health [3]. Among these, the gram-positive Lactic acid bacteria (LAB) are a phylogenetically heterogenous and versatile group which comprise high probiotic potential [4]. Interestingly, LAB of vegetal origin represent a promising and attractive probiotic profile, including *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* (formerly known as *Lactobacillus plantarum* and *Lactobacillus pentosus*) isolated from raw and fermented vegetal foods such as table olives [5–8], cucumber [9], kimchi [10] and pickles [11] among others. The versatility of *L. plantarum* and *L. pentosus* is due to their genomic diversity, plasticity and functionality linked to their ecological niches which allow them to survive in harsh conditions such as gastrointestinal tracts and unstable environmental conditions [12–14]. Hence, functional characterization of probiotics may

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greatly depend on the specific strain and origin. In this regard, next generation genome sequencing ("NGS" within the toolbox offered by omics) provides valuable and interesting insights into the mechanisms and functionality of probiotics as well as their health-promoting functions.

Today, humankind is facing global health challenges related to a number of diseases caused by multidrug-resistant (MDR) pathogenic microorganisms in humans, animals and plants. The increased emergence of MDR pathogens is linked to several severe societal issues, such as the misuse/overuse of drugs/biocides in different ecosystems, the recent pandemic COVID-19 as well as climate change [15,16]. Thus, to tackle these challenges – notably by limiting the spread of pathogens and their antimicrobial/virulence genes throughout different ecosystems-it is pivotal to reduce the use of antimicrobial substances. In this sense, promoting and increasing the use of probiotics, especially those of vegetal origin, provides a good opportunity to optimize and balance the health of people, animal, plants and ecosystems in line with the integrated "One Health" approach.

A promising probiotic vehicle of *L. pentosus* are Aloreña table olives, which are naturally fermented green olives with a denomination of protection (DOP). Our previous studies have shown that these strains exhibit excellent growth characteristics and good survival when exposed to simulated gastro-intestinal conditions, besides other probiotic features (auto-aggregation and co-aggregation with pathogenic bacteria, adherence to intestinal and vaginal cell lines, etc.) [17]. It is noteworthy that *L. pentosus* strains isolated from Aloreña table olives such as *L. pentosus* MP-10 and *L. pentosus* CF2–10 N are masters of adaptation due to their genetic diversity and functionality [12,18,19]. Here, we analyzed *in silico* the genome of a related, highly promising potential probiotic strain isolated from Aloreña fermented table olives, *L. pentosus* CF-6HA. We expect that this strain could be used in isolation or in combination with *L. pentosus* MP-10 and *L. pentosus* CF2–10 N. Furthermore, we explored its role as probiotic and antimicrobial agent, targeting several plant pathogens including those affecting olive trees such as *Pseudomonas syringae* (agent of bacterial canker on olives) and *Verticillium dahlia* (causing Verticillium wilt disease). This is the first study broadening the role of *L. pentosus* isolated from fermented foods, to include its use as antimicrobial agent. The strain is proposed as potential probiotic for human, animal and plant capable to maintain or restore a host's natural microbiota, and reduce the spread of pathogens and their resistance genes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strain *Lactiplantibacillus pentosus* CF-6HA (deposited at Spanish Type Culture Collection as CECT 30896), is derived from naturally-fermented Aloreña green table olives and was selected for further studies due its robustness and probiotic profile such as good growth capacity and survival under simulated gastro-intestinal conditions (acidic pH of 1.5, up to 4% of bile salts), good ability to auto-aggregate and co-aggregate with pathogens, adhesion ability to host cells, strong capacity of biofilm formation, antagonistic activity against pathogenic bacteria and fermentation of lactose (data not shown). Bacteria were routinely grown aerobically at 37 °C in de Man, Rogosa and Sharpe (MRS) broth or agar (Fluka, Madrid, Spain) conditions for 24–48 h. Bacterial and fungi pathogens were obtained from the Spanish Culture Collection Type (CECT) except for *Pseudomonas syringae* strains which were kindly supplied by Dr. J. A. Gutiérrez (University of Malaga, Spain). Growth conditions of pathogens are shown in Table 1. All strains were stored at −20 °C or −80 °C in 20% glycerol.

2.2. In vitro analysis of antimicrobial activity of Lactiplantibacillus pentosus CF-6HA

The antimicrobial activity was screened *in vitro* against human and plant pathogens listed in Table 1. 10 μl of a *L. pentosus* CF-6HA

Table 1
Pathogen strains and growth conditions used in this study.

Strain	Culture media	Growth temperature (°C)	Growth time (h)
Pseudomonas syringae subsp. *syringae* UMAF0158 (PP)	TSB/TSA	25	24
Pseudomonas syringae subsp. *syringae* UMAF0294 (PP)	TSB/TSA	25	24
Pseudomonas fragi T81	TSB/TSA	25	24
Listeria innocua CECT 910	TSB/TSA	37	24
Staphylococcus aureus CECT 976	TSB/TSA	37	24
Staphylococcus aureus CECT 4465	TSB/TSA	37	24
Bacillus cereus LWL1	TSB/TSA	37	24
Bacillus cereus CECT 148	TSB/TSA	37	24
Enterococcus faecalis S-47	TSB/TSA	37	24
Enterococcus faecalis FI 9190	TSB/TSA	37	24
Escherichia coli CECT 432	TSB/TSA	37	24
Salmonella enterica S62	TSB/TSA	37	24
Saccharomyces cerevisiae CECT 1171	MHB/MHA	26	24
Candida albicans CECT 1001	MHB/MHA	26	24
Verticillium dahliae CECT 2694 (PP)	PDA	28	72–120
Verticillium dahliae CECT 2884 (PP)	PDA	28	72–120

MHB/MHA, Mueller Hinton Broth/Mueller Hinton; Agar TSB/TSA, Tryptic Soy Broth/Tryptic Soy Agar; PDA, Potato Dextrose Agar.
PP, Plant Pathogen.

overnight culture was spotted on MRS agar and then plates were overlayed with soft buffered Brain Heart Infusion agar (SF–BHI; at pH 7.0, 0.1 M) inoculated with indicator strains (Table 1) grown overnight. In a second step, the antimicrobial activity of the culture supernatant passed through a 0.22 μm nylon membrane filter was tested using the agar-well diffusion assay as described above. The antimicrobial activity was evaluated by measuring the diameter of inhibition zones from the edge of the well. In the case of *V. dahliae*, the antifungal activity of *L. pentosus* CF-6HA was assessed on potato dextrose agar (PDA) plates according to Mohamad et al. [20] evaluating growth inhibition by the following formula: 100 × [C−T]/C. Here, C is defined as the radial growth of fungus in control, while T is the same in dual culture [21].

2.3. In silico analysis of robustness, safety, antimicrobial activity and other functional properties of Lactiplantibacillus pentosus CF-6HA

2.3.1. DNA extraction, NGS, assembly, annotation and comparative analysis

L. pentosus CF-6HA was grown overnight in liquid medium, harvested by centrifugation and total genomic DNA was extracted using the PureGene core kit B (Qiagen, Spain). DNA was quantified as described by Abriouel et al. [19] and then stored at −20 °C until required.

Library preparation, genome sequencing, assembly and annotation was performed as reported by Abriouel et al. [19]. Briefly, 10- to 20-kb fragments of genomic DNA were obtained by shearing, the resulting libraries (22–24 kb) were purified and sequenced as described [19]. A total of 150292 reads was obtained with a median length of 12167 bp after filtering raw sequence data (Q20).

Genome sequencing, assembly, and annotation were performed at Biopolis-ADM (Valencia, Spain) and the complete *L. pentosus* CF-6HA sequence was deposited at the EMBL Nucleotide Sequence Database (accession number ERR12640876).

Comparative genomic analysis was performed using *Lactiplantibacillus* spp. (NCBI database) as reference. Phylogenic relatedness was explored among *L. pentosus* CF2–10 N (ERR11550479), *L. pentosus* MP-10 (GCA_900092635.1), *L. pentosus* DSM 20314 (GCA_003641185.1), *L. pentosus* KCA1 (GCA_000271445.1) and *L. pentosus* CF-6HA (this study). Synteny analysis among species was performed using MAUVE alignment (DNASTAR LASERGENE v17.4.2). A phylogenetic tree was constructed by the maximum-likelihood: RAxML [22] using MAUVE alignment (DNASTAR LASERGENE v17.4.2). Bootstrap analysis of 1000 replicates was used to evaluate the statistical reliability of the tree. Furthermore, NDtree 1.2 was used to construct phylogenetic trees from Single-End or Pair-End FASTQ files of *Lactiplantibacillus* spp. (last access on march 2023) [23,24] and NCBI's Tree Viewer (Tree Viewer 1.19.4 available as NCBI application) for the graphical display for phylogenetic trees (https://www.ncbi.nlm.nih.gov/tools/treeviewer/).

2.3.2. In silico analysis of robustness and defense of Lactiplantibacillus pentosus CF-6HA

The mobilome (conjugative plasmids, transposase, transposon, IS elements and prophage coding genes) of *L. pentosus* CF-6HA was analyzed in the annotated genome sequence as detailed by Abriouel et al. [19]. For this, the search for Insertion Sequences (IS) was done using the ISfinder search tool [25] and for prophage analysis within *L. pentosus* CF-6HA genome we used PHASTER's version (PHAge Search Tool Enhanced Release, last updated March 2016) [26,27]. Furthermore, complementary information was obtained from the annotated genome sequence for prophage and transposon/transposase. Moreover, the CRISPRDetect program version 2.4 [28] was employed to screen the annotated *L. pentosus* CF-6HA genome sequence for CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) coding genes and CRISPR RNAs targets.

2.3.3. Safety aspects and antimicrobial resistance of Lactiplantibacillus pentosus CF-6HA

Besides the global annotation using the *BLAST2go* program, antibiotic resistance genes (ARGs) of *L. pentosus* CF-6HA were also screened using the Resistance Gene Identifier (RGI 6.0.1) software (as part of the CARD "The Comprehensive Antibiotic Resistance Database" tools) [29], with the CARD's curated AMR (antimicrobial resistance) detection models (last accessed in February 2023). ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) software version 4.1 [30–32] was used to screen for acquired ARGs/chromosomal mutations mediating antimicrobial resistance using the recommended selected %ID threshold and selected minimum length (last accessed in February 2023).

Annotation of the predicted CDS using reciprocal BLAST against the Virulence Factors of Bacterial Pathogens (VFDB) database was used to search for virulence factors (VFs). Hits were considering as positive when the results of reciprocal BLAST were similar and using an 80% sequence similarity cut-off [33].

2.3.4. In silico analysis of potential antimicrobial and probiotic activities of Lactiplantibacillus pentosus CF-6HA

The annotated genome sequence of *L. pentosus* CF-6HA was analyzed for the presence of genes coding for antimicrobial proteins/peptides. Genes coding for bacteriocin production were screened using the BAGEL 4 webserver (http://bagel4.molgenrug.nl/) [34] and confirmed by Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Additionally, antiSMASH version 7.0 [35] was used to search for secondary metabolite gene clusters. Both BAGEL and antiSMASH were employed for the identification of open reading frames (ORFs) involved in the production of antimicrobial compounds.

To determine probiotic features related to adhesins, exopolysaccharides, enzymes, antioxidants and selenium metabolism, the annotated genome sequence of *L. pentosus* CF-6HA was screened for the corresponding coding genes. Furthermore, Selenium (Se) reduction capacity of *L. pentosus* CF-6HA was tested *in vitro*. Bacteria were grown in 5 ml MRS broth or MRS-Se supplemented with 20 ppm Se (as Na_2SeO_3) at 37 °C for 24 h as described by Martínez et al. [36]. The remaining SeO_3^{2-} concentration in the supernatants (after centrifugation of overnight cultures in both conditions at 5600×*g* for 5 min) was quantified by the modified method of Brown and Watkinson [37] using a microplate assay measured spectrophotometrically [36]. Briefly, 500 μl of each culture supernatant were added to 200 μl of 0.1% 2,3-diaminonaphthalene in 0.1 M HCl and then mixed in microtubes with 160 μl of the following mixture: 0.5

M HCl, 80 μl of 0.1 M EDTA, 40 μl of 0.1 M NaF, and 40 μl of 0.1 M dipotassium oxalate. The microtubes were incubated at 40 °C for 40 min, cooled in ice bath and then mixed with 500 μl of cyclohexane under shaking horizontally for 10 min at 850×*g*. The centrifuged samples at 3000×*g* for 10 min (150 μl) were measured spectrophotometrically in a 96-wellmicroplate at 377 nm. On the other hand, the biosorption capacity of selenium on the *L. pentosus* CF-6HA surface as nanoparticles (SeNPs) was analyzed by scanning electron microscopy (SEM) in combination with energy dispersive X-ray spectroscopy in the presence and absence of Se in culture broth. For this, *L. pentosus* CF-6HA was grown in MRS broth or MRS-Se supplemented with 5 ppm Se (as Na_2SeO_3) at 37 °C for 24 h [36]. A drop of the bacterial pellet was allowed to dry on SEM Specimen Stubs (ANAME, Spain), followed by a series of dehydration steps in 20, 40, 60, 80, and 100% ethanol solutions (15 min each) before suspension in acetone for 1 h. After this, the stubs were subjected to critical-point drying before imaging by SEM (FESEM, MERLIN de Carl Zeiss, Oxford) equipped with Energy Dispersive X-Ray Analyzer (EDX).

2.4. Statistical analysis

Averages and standard deviations were determined using Excel 2016 (Microsoft Corporation, Redmond, WA, United States). All analyses were performed in triplicate.

3. Results

3.1. Screening of antimicrobial activity of Lactiplantibacillus pentosus CF-6HA

We evaluated bacteriocin production assaying the antimicrobial activity of *L. pentosus* CF-6HA cell free supernatant against several human and phytopathogens, obtaining promising results (Table 2). *P. syringae* subsp. *syringae* UMAF0291 and UMAF0158 strains and *Verticillium dahliae* CECT 2694 and CECT 2884 strains were sensitive to the antimicrobial substances produced *ex-situ* by *L. pentosus* CF-6HA, since the supernatant of this strain was active against bacterial and some fungal phytopathogens. Furthermore, other pathogens were also sensitive to antimicrobial substances produced by *L. pentosus* CF-6HA such as the spoilage bacteria *P. fragi* T81 and other human/animal pathogens, i.e. *Bacillus cereus* strains, *Staphylococcus aureus* strains, *Enterococcus faecalis* strains, *Listeria innocua*, *Escherichia coli*, *Salmonella enterica* and *Candida albicans*. On the other hand, no activity was detected against *Saccharomyces cerevisiae* CECT 1171 (Table 2).

3.2. Exploring in silico the probiotic and antimicrobial potential of Lactiplantibacillus pentosus CF-6HA

3.2.1. Genome analysis of Lactiplantibacillus pentosus CF-6HA and comparison with other Lactiplantibacillus spp.

L. pentosus CF-6HA has a genome size of 4,148,739 bp and an estimated mol% G + C content of 45.7%. As shown in Fig. 1 (A, B), the genome sequence contained a single circular chromosome and 6 plasmids ranging 38.5–74.5 kb, namely pLPE6-1 (38,502 bp), pLPE6-2 (73,263 bp), pLPE6-3 (72,520 bp), pLPE6-4 (62,787 bp), pLPE6-5 (74,498 bp) and pLPE6-6 (61,672 bp). The RNAmmer (version 1.2)

Table 2
Antimicrobial activity of *Lactiplantibacillus pentosus* CF-6HA against phytopathogens and other pathogens.

Indicator strains	Antimicrobial activity of *L. pentosus* CF-6HA against indicator strains (inhibition zone)	
	Bacteria	Cell-free supernatant (mm±SD)
Pseudomonas syringae subsp. *syringae* UMAF0158	+	12 ± 0,85
Pseudomonas syringae subsp. *syringae* UMAF0291	+	12 ± 1,35
Pseudomonas fragi T81	+++	12 ± 1,03
Listeria innocua CECT 910	+++	W
Staphylococcus aureus CECT 976	+++	10 ± 0,43
Staphylococcus aureus CECT 4465	+++	10 ± 1,10
Bacillus cereus LWL1	+++	14 ± 0,95
Bacillus cereus CECT 148	+++	11 ± 2,01
Enterococcus faecalis S-47	+++	W
Enterococcus faecalis FI 9190	++	W
Escherichia coli CECT 432	+++	10 ± 1,45
Salmonella enterica S62	+++	W
Saccharomyces cerevisiae CECT 1171	–	–
Candida albicans CECT 1001	+	9 ± 0,65
Indicator strains	**Bacteria (%*±SD)**	**Cell-free supernatant (%*±SD)**
Verticillium dahliae CECT 2694	36 ± 0,67	8 ± 0,89
Verticillium dahliae CECT 2884	9 ± 1,02	0 ± 0

(+), diameter of inhibition zone from the edge of growth is < 10 mm.
(++), diameter of inhibition zone from the edge of growth is in the rage of 10–13 mm.
(+++), diameter of inhibition zone from the edge of growth is ≥ 14 mm.
(−), absence of inhibition.
(W: Weak), diameter of inhibition zone from the edge of growth is ≤ 8 mm.
*% of growth inhibition according to Aeron et al. (2011).
±SD, standard deviations of three independent experiments.

prediction produced 16 rRNA genes and 84 tRNA encoding sequences in the *L. pentosus* CF-6HA genome.

Regarding the functional classification, the annotated genome sequence [Fig. 1 (A, B)] yielded 3960 open reading frames (ORFs). Here, 73.3% (2902) were attributed to a COG (Cluster of Orthologous Groups) family and/or were given a functional description (Supplementary Table S1). Moreover, the genome of *L. pentosus* CF-6HA contains 1245 KEGG orthology (KO) assignments, mainly enzymes (699 genes) and transporters (212 genes). BlastKOALA (KEGG tool; last updated May 15, 2019; Kanehisa et al. [38]) assigned approximately half (47.4%) of the genes to KEGG annotations corresponding to genetic information processing (248 genes), carbohydrate metabolism (244), signaling and cellular processing (214 genes), environmental information processing (193 genes), genetic information processing (165 genes), unclassified: metabolism (113 genes), unclassified: genetic information processing (106 genes), amino acid metabolism (100 genes), nucleotide metabolism (69 genes), metabolism of cofactors and vitamins (68 genes) and metabolism (49 genes) among others (Fig. 2).

Furthermore, the genome of *L. pentosus* CF-6HA was aligned and compared to other *L. pentosus* strains using the MAUVE algorithm

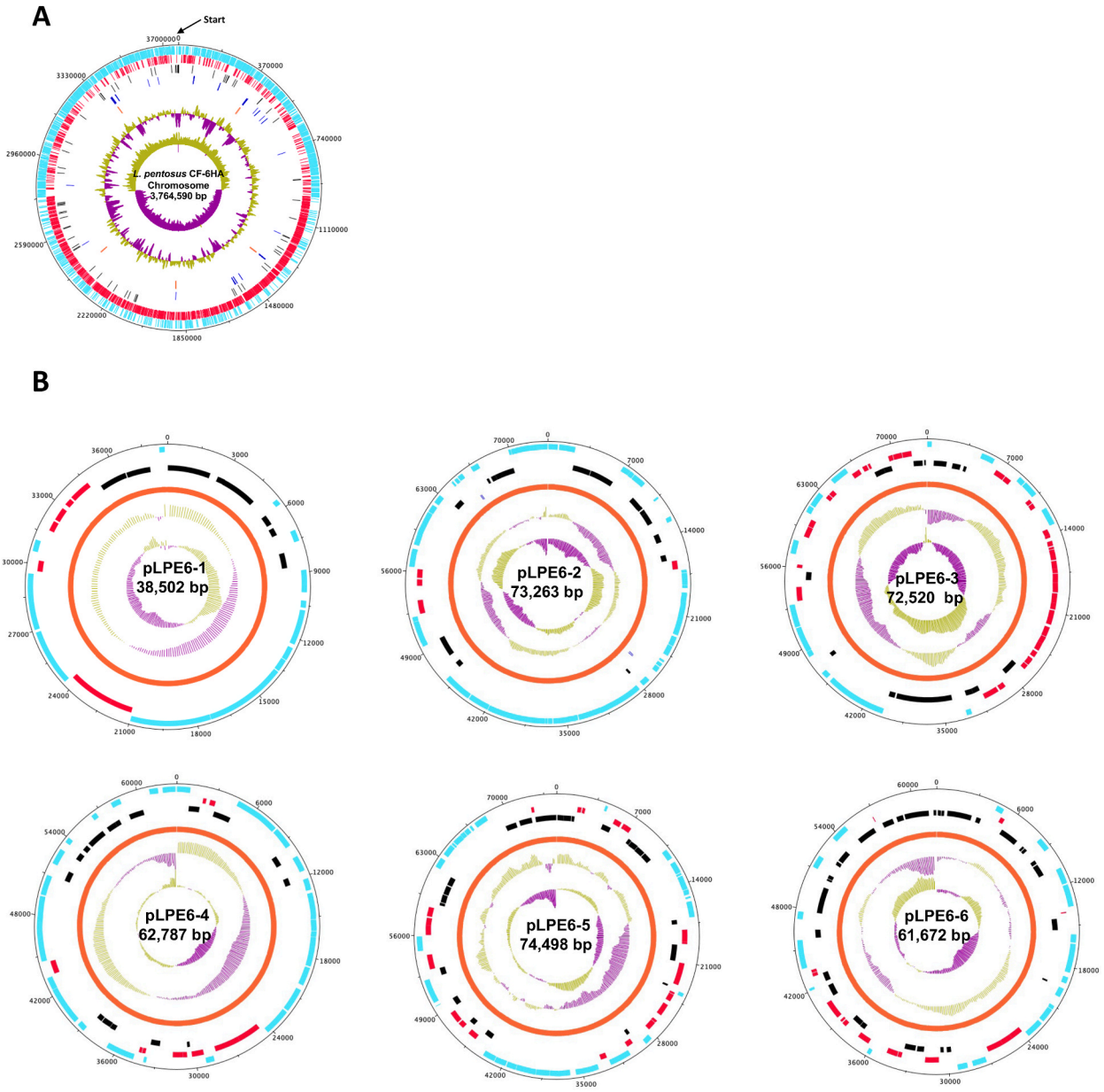

Fig. 1. *Lactiplantibacillus pentosus* CF-6HA presented as circular map for the chromosome **(A)** and six plasmids **(B)**. The circles represented (outside to inside) are the annotated Coding DNA Sequences (CDS) elements in forward orientation (blue); the annotated CDS elements in the reverse orientation (red); the Pseudogenes (black); the tRNA (dark blue); the rRNA (orange); the %GC plot and the GC skew. The genome plot was generated using DNAPlotter version 18.2.0 from Artemis 18.2.0, Sanger Institute.

(Fig. 3A): *L. pentosus* MP-10 and CF2–10 N (isolated from the same ecological niche, i.e. Aloreña table olives), *L. pentosus* DSM 20314 (Type Strain) isolated from corn silage and *L. pentosus* KCA1 isolated form the vaginal tract. Although inversion and rearrangements occurred among all *L. pentosus* strains the synteny linkage of *L. pentosus* CF-6HA against other *L. pentosus* strains indicated the presence of highly conserved syntenic blocks (Fig. 3A). The construction of a maximum-likelihood core genome tree (RaxML) revealed that *L. pentosus* CF-6HA overall shared 97% identity (evolutionary distance "ED", ED = 0.02) with *L. pentosus* strains MP-10 (97.62%), CF2–10 N (97.62%) and *L. pentosus* DSM 20314 (97.53%), while *L. pentosus* KCA1 only exhibited 92.16% identity (ED = 0.08) (Fig. 3B).

Based on the number of nucleotide differences between strains, the NDtree tool and NCBI's Tree Viewer showed a clear distinction between 48 *Lactiplantibacillus* spp. strains with clustered strains belonging to the same species (Fig. 3C).

3.2.2. Safety aspects and antimicrobial resistance of Lactiplantibacillus pentosus CF-6HA

To carry out an *in silico* analysis of the *L. pentosus* CF-6HA genome with respect to antibiotic resistance genes (ARGs), we employed first the RGI tool v3.2.1 available in the CARD database (curated antimicrobial resistance "AMR" detection models). Only one strict hit was detected, which was defined within the similarity cut-offs of the individual AMR detection models and represented likely homologs of AMR genes according to Alcock et al. [29]. In this sense, the RGI revealed that the *L. pentosus* CF-6HA genome contained a *vanY* gene in a vanB cluster conferring glycopeptide resistance.

BLAST2go annotation yielded hits potentially involved in non-specific antimicrobial resistance mechanisms, such as efflux transporters or transmembrane proteins involved in response to drugs including antibiotics (Supplementary Table S1).

Regarding antimicrobial resistance genes (ARGs) acquired by horizontal gene transfer, no relevant genes were identified (data not shown), while equally no known virulence factors including toxins were identified.

3.2.3. Lifestyle adaptation to different stresses

3.2.3.1. Mobilome analysis. The L. *pentosus* CF-6HA mobilome was represented by ISI elements, transposases and prophages. With regards to ISI elements, 17 CDS belonging to nine families were identified and distributed in 12 different bacteria, with IS3 as the most family originating from *Acinetobacter baumannii/A. lwoffii* (Table 3).

Furthermore, the *L. pentosus* CF-6HA genome harbored 98 transposases (31 putative) of 10 IS families (ISL3 and IS30 family transposases most prevalent) (Supplementary Table S2). These transposases were equally distributed both on the chromosome (50 CDS) and plasmids (48 CDS) - mainly on pLPE6-6, appearing as multiple copies (Table 4).

13 temperate phage regions were identified within the *L. pentosus* CF-6HA genome (7 intact, 1 questionable, 5 incomplete) (Supplementary Table S3). Intact regions were mainly related to Lactob_Sha1_NC_019489 (Regions 1, 2, 7, 8 and 9; GC content, 30.30–42.12%; region length, 27.1–46 kb), while the incomplete regions were associated with different phages such as Lactob_Sha1_NC_019489 (Region 6, 17.2 Kb), Lactob_phig1e_NC_004305 (Region 4, 15.7 Kb), Erwini_pEp_SNUABM_01_NC_048807 (Region 10, 12 Kb), Photob_PDCC_1_NC_048821 (Region 5, 7.6 Kb) and Paenib_Tripp_NC_028930 (Region 11, 5.5 Kb). The highest protein match among the identified prophages was determined for Lactob_Sha_1. Only five prophage regions have attL/attR sequences and integrase such as regions 1, 2, 4, 6 and 7 located on bacterial chromosome and identified in intact and incomplete regions.

3.2.3.2. CRISPR. CRISPR I and II systems (both signature genes for the Type I "*cas3*" and Type II "*cas9*" systems) detected in the *L. pentosus* CF-6HA genome (12 genes, five exclusives to *L. pentosus* CF-6HA), are organized in two operons (CRISPR-I and CRISPR-II) (fig. 6 and Fig. S1, Supplementary Material Table S4). With regards to CRISPR arrays (CR), we identified three CRISPR unquestionable arrays, located dispersed on the chromosome (678102–2943655) (Table 4).

3.2.4. Antibiotic and probiotic properties of Lactiplantibacillus pentosus CF-6HA

3.2.4.1. Antimicrobial activity. A gene cluster coding for bacteriocin biosynthesis was determined to contain 26 genes with a total

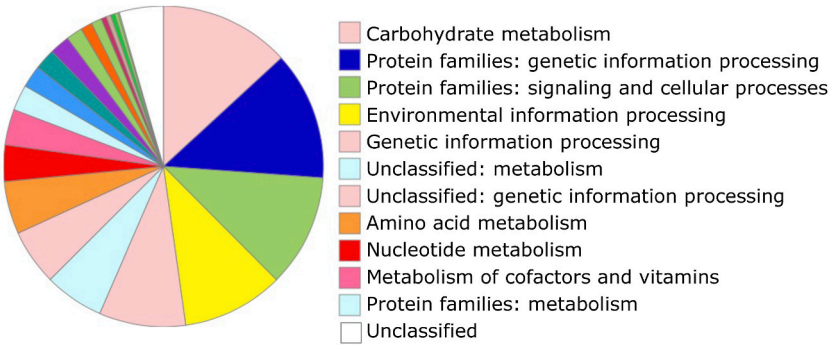

Fig. 2. BlastKOALA results of functional categories predicted in *Lactiplantibacillus pentosus* CF-6HA genome and their frequencies.

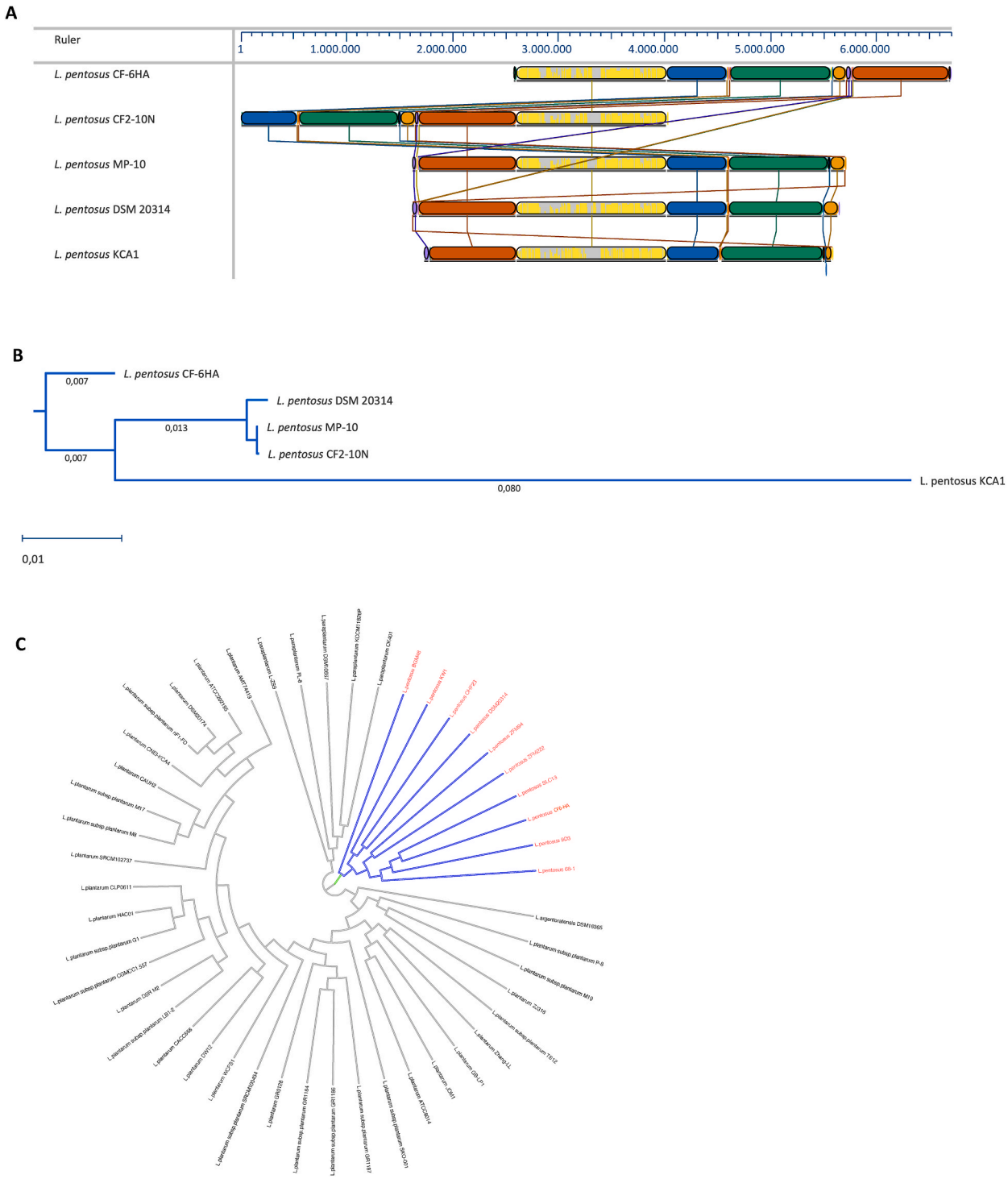

Fig. 3. MAUVE alignment and phylogenetic relationships among *Lactiplantibacillus pentosus* CF-6HA and other *L. pentosus* strains. **(A)** MAUVE visualization of whole genome alignment of *L. pentosus* CF-6HA and other *L. pentosus* strains. The color blocks represent the synteny blocks (locally collinear blocks) of the genomes of *L. pentosus* CF-6HA and four *L. pentosus* strains (CF2–10 N, MP-10, DSM 20314 and KCA1). **(B)** Phylogenetic tree based on the MAUVE-aligned five genome sequences with branch lengths as estimated by RaxML. The scale bar indicates the branch length (Evolution Distance). **(C)** Phylogenetic relationships among *Lactiplantibacillus* spp. genomes. An NDtree was constructed by mapping *L. pentosus* CF-6HA genome sequence against the genomic sequences of other *Lactiplantibacillus* spp. (*L. pentosus*, *L. plantarum*, *L. paraplantarum*) which was visualized by NCBI's Tree Viewer.

Table 3
Characterization of IS elements found within the genome of *Lactiplantibacillus pentosus* CF-6HA using the ISfinder search tool.

Sequences producing significant alignments	IS Family	Group	Origin	Score (bits)	*E.* value
ISHvo2	IS5	ISH1	Haloferax volcanii	40.1	0.53
ISPa108	IS21		Pseudomonas aeruginosa	38.2	2.1
ISAbe14	ISL3		Acinetobacter bereziniae	38.2	2.1
ISMba13	IS1634		Methanosarcina barkeri	38.2	2.1
ISSme3	IS66		Sinorhizobium medicae	38.2	2.1
ISCtet1	IS1202	IS1202	Clostridium tetanomorphum	36.2	8.2
ISAba66	IS3	IS51	Acinetobacter baumannii	36.2	8.2
ISPmo2	IS5	IS5	Pseudomonas monteilii	36.2	8.2
ISAlw23	IS3	IS51	Acinetobacter lwoffii	36.2	8.2
ISAlw5	IS3	IS51	Acinetobacter lwoffii	36.2	8.2
ISAlw4	IS3	IS51	Acinetobacter lwoffii	36.2	8.2
ISMba1	IS4	ISH8	Methanosarcina barkeri	36.2	8.2
ISVbsp4	IS1202	ISTde1	Vibrio sp.	36.2	8.2
ISIse1	IS1595	IS1016	Ichthyobacterium seriolicida	36.2	8.2
ISMod1	IS1595		Myroides odoratimimus	36.2	8.2
ISAba19	IS3	IS51	Acinetobacter baumannii	36.2	8.2
ISAba18	IS3	IS51	Acinetobacter baumannii	36.2	8.2

length of approximately 20 kb and was located on the chromosome (Fig. 4A). In this gene cluster, only one biosynthetic bacteriocin gene coding for pediocin (core peptide, pediocin; location 2,511,611–2,511,967 bp) was detected (Fig. 4A), however no pediocin immunity/transport genes were identified although several genes coding for other transporters or resistance genes were detected such as MFS transporter, Cadmium transporting P family ATPase or MerR family transcriptional regulator (Table S5). Furthermore, *in silico* analysis of the *L. pentosus* CF-6HA genome using the *BLAST2go* program showed the presence of a putative gene (*LPENT_01393*) coding for the biosynthesis of Plantaricin Y (PlnY; location 3,248,967–3,249,260 bp) (Table S1).

On the other hand, the presence of genes responsible for the synthesis of secondary metabolites with antimicrobial potential related to T3PKS (Type III polyketide synthase) (Fig. 4B) was determined by genome analysis using the antiSMASH software tool. In this sense, one region 2.1 (location:1,169,377–1,210,546 nt): T3PKS was identified within chromosome. This cluster was represented by one core biosynthetic gene (ctg*2_1084*) coding for hydroxymethylglutaryl-CoA synthase; six biosynthetic additional genes (ctg*2_1067,* ctg*2_1074,* ctg*2_1076,* ctg*2_1079,* ctg*2_1087* and ctg*2_1105*) coding for Peptidase_M50, SMCOG1115:HAD-superfamily hydrolase, SMCOG1072:dehydrogenase, SMCOG1141:acyltransferase, SMCOG1123:polyprenol-monophosphomannose synthase ppm1 and SMCOG1001:short-chain dehydrogenase/reductase SDR, respectively; one transport gene (ctg*2_1100*) coding for an MFS transporter and other genes (Table S6, Fig. 4B). Detailed gene information is available in Table S6. AntiSMASH blastp output results indicate the closest compound from the MiBIG database and showed the best hits corresponding to terpene, Type III polyketide synthase (T3PKS), polyketides (PKS), non-ribosomal peptide (NRP), the hybrid peptide-polyketide products (PK + NRP), post translationally modified Peptide (RiPP) and others being NRP and PKS the most abundant classes of biosynthetic gene clusters (Fig. 4B).

3.2.4.2. Probiotic functions. Several genes potentially coding for probiotic activity were detected in the annotated genome sequence (Table 5). These were notably related to adhesion (20 genes) exopolysaccharide biosynthesis (4 genes) and antioxidants (11 genes). Regarding key probiotic properties such as transferases, oxidoreductases, lyases, hydrolases, ligases, isomerases and translocases, we confirmed the presence of 1 bile salt hydrolase coding gene, 2 coding genes for tannase, 1 coding gene for alpha-amylase, 1 coding gene for amylopullulanase, 1 coding gene for catalase and 23 different coding genes for lactase (beta-galactosidase or beta-glucosidase).

Finally, we screened the *L. pentosus* CF-6HA genome for genes involved in selenium metabolism and identified 4 coding genes, i.e. Serine–tRNA ligase, Homocysteine methyltransferase, Cysteine sulfinate desulfinase and MULTISPECIES: serine–tRNA ligase (Table 5). The ability of *L. pentosus* CF-6HA to grow and biotransform selenite (Na_2SeO_3) was confirmed *in vitro,* measuring spectrophotometrically the residual Se in the supernatant after 24 h growth in MRS broth supplemented with Se. Results obtained showed its growth capacity producing 100% Se removal since no detectable Se was registered (data not shown). Furthermore, we visualized and confirmed the presence of Se on the cell surface of *L. pentosus* CF-6HA as white nanoparticles (SeNPs) by SEM and EDX microanalysis [Fig. 5 (A-G)]. Moreover, no transformation of the cell shape was observed after growth in the presence of Se and subsequent formation of SeNPs [Fig. 5 (D, F)].

4. Discussion

The emergence of new pathogens with increased antimicrobial resistance due to the abuse and misuse of antimicrobials (antimicrobials, antifungal, antivirals, antiparasitics, biocides) in different ecosystems was exacerbated by the last pandemic COVID-19 and thus will have a great impact on human life quality as well as globally on animal, plant and environment. Several strategies within the "One Health" context are ongoing to fight these so-called "superbugs" [39]. However, it is becoming clear that these strategies are not sufficient, given the high adaptation capacity of microorganisms. We thus need to adopt more collaborative and smart solutions to compensate the emergence of multidrug bacteria. In this sense, probiotics are one of the most promising strategies [40]. That said, the same probiotic strain seldom exerts beneficial effects on different hosts. Thus, a promising future area of research is the search for

Table 4
Summary of CRISPR arrays predicted in the *Lactiplantibacillus pentosus* CF-6HA genome.

CRISPR array (CR)	Start position	End position	Array orientation	CRISPR lenght (bp)	Number of repeats	DR consensus[a]	Array family
CR 1	678377	678102	Reverse	276	29	CTATTCCCCGTGTATACGGGGGTGATCCT	I-E
CR 2	689546	689697	Forward	152	29	TGTACTCCCCGTGTATACGGGGGTGATCC	NA
CR 3	2942770	2943655	Forward	886	36	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC	NA

[a] The same DR consensus sequences are indicated.

probiotics which can interact with different hosts and accomplish different functions simultaneously. In this study, we explored for the first time a potential dual effect of a probiotic strain as beneficial bacteria for humans and also for plants.

Lactiplantibacillus pentosus CF-6HA -isolated throughout the natural fermentation process of Aloreña table olives-exhibited probiotic potential *in vitro* (data not shown) as well as antimicrobial activity against a range of pathogens of human, animals and plants. Due to the dual antimicrobial activity of *L. pentosus* CF-6HA this strain can be classified as "talented" strain. Furthermore, although the bacterium shared many phenotypical and genotypical characteristics with close relatives of the same ecological niche (*L. pentosus* MP-10 and CF2–10 N) [5,17] its uniqueness is evident. Using whole genome sequencing as an unequivocal diagnostic tool [41], the strain was shown to harbor a single circular chromosome of 3,764,590 bp and an estimated mol% G + C content of 46.19% similar to its counterpart strains (*L. pentosus* MP-10 [3,698,214 bp; GC content of 46.32%] and *L. pentosus* CF2–10 N [3,645,747 bp; GC content of 46.42%]) [18,19]. All of these strains possess plasmids [18,19] which may play a key role in fermentation process and hint towards the adaptation to the ecosystem (soil, plant and brine), including heavy metal detoxification [42] via these genetic elements. In this sense, the evolutionary distance (ED) between *L. pentosus* CF-6HA, *L. pentosus* CF2–10 N and *L. pentosus* MP-10 was lower (ED = 0.02) than with other *L. pentosus* strains isolated from other ecological niches. However, besides shared genetic diversity and many functional

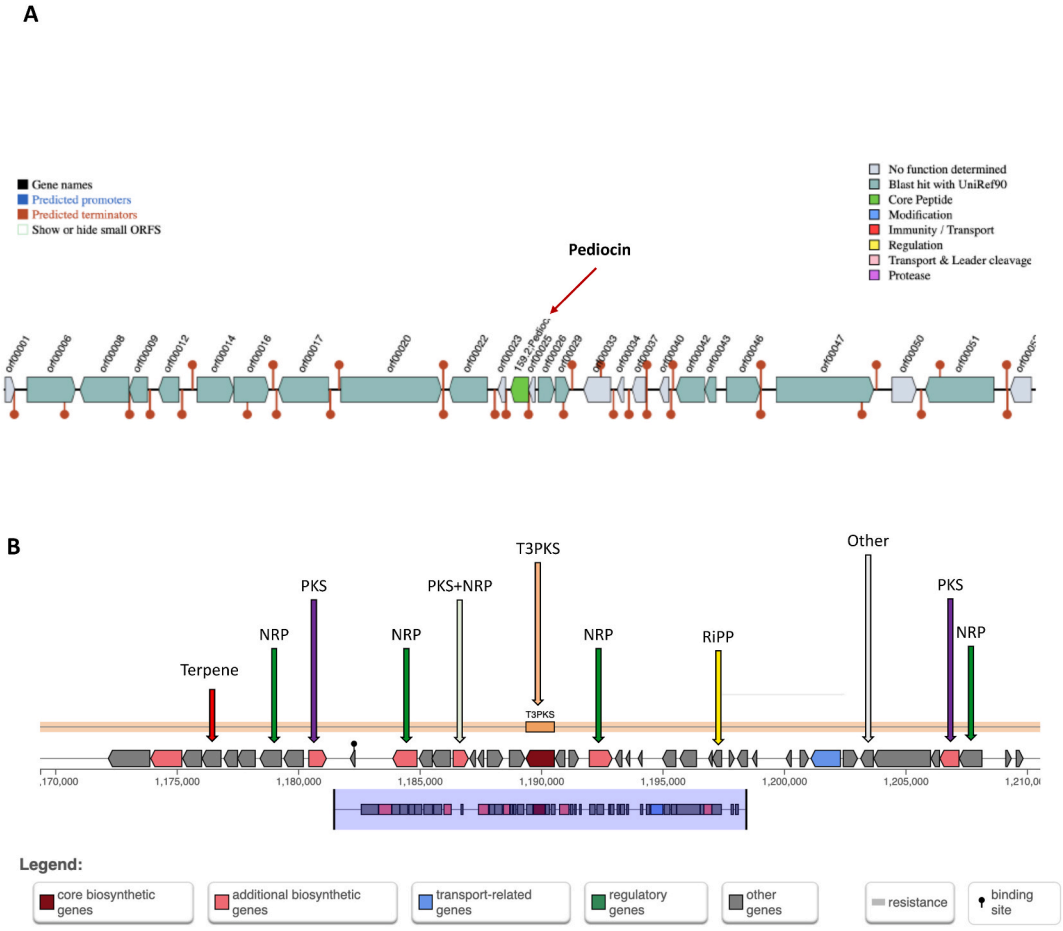

Fig. 4. Organization of biosynthetic gene clusters of antimicrobials predicted in *Lactiplantibacillus pentosus* CF-6HA genome. **(A)** bacteriocin gene cluster predicted using the BAGEL4 webserver. **(B)** T3PKS (Type III PKS, region 2.1) gene cluster predicted using antiSMASH software tool. Genes are represented by arrows with different colors corresponding to different functions.

Table 5
Characterization of genes associated with probiotic properties predicted in the *Lactiplantibacillus pentosus* CF-6HA genome.

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
Adhesion	gene_267	*FD24_GL000107*	151284–152156	+	873	Metal ABC transporter permease	Plasma membrane, integral component of membrane, ATP binding, ATPase activity, coupled to transmembrane movement of substances, transmembrane transport (GO:0005886, GO:0016021, GO:0005524, GO:0042626, GO:0055085)	ABC-type Mn2+/Zn2+ transport systems, permease components (COG1108)
	gene_268	*FD24_GL000106*	152153–153094	+	942	Manganese ABC transporter substrate-binding protein	metal ion binding, cell adhesion, metal ion transport (GO:0046872, GO:0007155, GO:0030001)	ABC-type metal ion transport system, periplasmic component/ surface adhesin (COG0803)
	gene_320	*gene_320*	219154–220599	+	1446	Hypothetical protein	–	–
	gene_566	*gene_566*	472433–485428	+	12996	Beta-fructosidase	–	Type V secretory pathway, adhesin AidA (COG3468)
	gene_643	*LPENT_00563*	550575–552623	+	2049	ABC transporter, ATP-binding and permease protein	Plasma membrane, integral component of membrane, ATP binding, ATPase activity, transport, response to antibiotic (GO:0005886, GO:0016021, GO:0005524, GO:0016887, GO:0006810, GO:0046677)	ABC-type antimicrobial peptide transport system, permease component (COG0577)
	gene_686	*FD24_GL002081*	605850–606218	–	369	Cell surface protein	Integral component of membrane (GO:0016021)	–
	gene_687	*FD24_GL002082*	606264–607310	–	1047	Cell surface protein	Integral component of membrane (GO:0016021)	–
	gene_875	*gene_875*	800768–807652	+	6885	Mucus-binding protein	–	RNA-binding protein of the Puf family, translational repressor (COG5099)
	gene_1016	*gene_1016*	947073–948779	–	1707	Adherence protein	–	Fibronectin-binding protein A N-terminus (FbpA) (COG1293)
	gene_1332	*gene_1332*	1291493–1292944	–	1452	Cell surface protein	–	Bacterial surface proteins containing Ig-like domains (COG5492)
	gene_1333	*LPENT_03051*	1292995–1293984	–	990	Cell surface protein	integral component of membrane (GO:0016021)	–
	gene_1334	*gene_1334*	1294042–1294770	–	729	Cell surface protein	–	–
	gene_1602	*gene_1602*	1571153–1574347	–	3195	Mucus-binding protein	–	Predicted solute binding protein (COG3889)
	gene_2014	*gene_2014*	2014376–2017015	–	2640	Cell surface protein	–	Autotransporter adhesin (COG5295)
	gene_2040	*FD24_GL000462*	2042688–2044532	–	1845	Cell surface protein	Extracellular region, cell wall, collagen binding, cell adhesion (GO:0005576, GO:0005618, GO:0005518, GO:0007155)	Predicted outer membrane protein (COG4932)
	gene_2098	*LPE_02200*	2102216–2103109	–	894	ABC superfamily ATP binding cassette transporter, binding protein	Metal ion binding, cell adhesion, metal ion transport (GO:0046872, GO:0007155, GO:0030001)	ABC-type metal ion transport system, periplasmic component/ surface adhesin (COG0803)
	gene_2180	*N692_03575*	2194347–2200730	–	6384	Mucus-binding protein	Integral component of membrane (GO:0016021)	Periplasmic protein TonB, links inner and outer membranes (COG0810)
	gene_2196	*N692_03630*	2217952–2219733	+	1782	Mucus-binding protein, LPXTG-motif cell wall anchor	Cell wall, integral component of membrane (GO:0005618, GO:0016021)	Type IV secretory pathway, TrbL components (COG3846)

(*continued on next page*)

Table 5 (*continued*)

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
	gene_2297	*FD24_GL003217*	2322251–2323189	+	939	Hypothetical protein FD24_GL003217	Integral component of membrane (GO:0016021)	–
	gene_2378	*gene_2378*	2403766–2404674	+	909	Metal ABC transporter substrate-binding protein	–	ABC-type metal ion transport system, periplasmic component/ surface adhesin (COG0803)
Exopolysaccharides	gene_435	*LPENT_00748*	310856–311584	+	729	Exopolysaccharide biosynthesis protei	Extracellular polysaccharide biosynthetic process (GO:0045226)	ATPases involved in chromosome partitioning (COG0489)
	gene_444	*LPENT_00741*	318075–319058	+	984	Exopolysaccharide phosphotransferase cps2G	Transferase activity, transferring phosphorus-containing groups (GO:0016772)	–
	gene_1276	*LPENT_02997*	1226608–1227324	–	717	Capsular exopolysaccharide family protein	extracellular polysaccharide biosynthetic process (GO:0045226)	ATPases involved in chromosome partitioning (COG0489)
	gene_1277	*LPENT_02998*	1227339–1228094	–	756	Exopolysaccharide biosynthesis protein	Integral component of membrane, lipopolysaccharide biosynthetic process (GO:0016021, GO:0009103)	Capsular polysaccharide biosynthesis protein (COG3944)
Enzymes	gene_2429	*LPENT_02042*	2461421–2462407	–	987	Bile salt hydrolase	Choloylglycine hydrolase activity (GO:0045302)	Penicillin V acylase and related amidases (COG3049)
	gene_1132	*gene_1132*	1081444–1083327	–	1884	Tannase	–	–
	gene_2039	*gene_2039*	2040950–2042362	–	1413	Tannase	–	–
	gene_2912	*FD24_GL003074*	2998722–3000044	+	1323	Alpha-amylase	Alpha-amylase activity, carbohydrate metabolic process (GO:0004556, GO:0005975)	Glycosidases (COG0366)
	gene_2733	*LPE_01041*	2811399–2813216	+	1818	Amylopullulanase	Alpha-amylase activity, carbohydrate metabolic process (GO:0004556, GO:0005975)	Glycosidases (COG0366)
	gene_2616	*LPENT_01867*	2668277–2669743	+	1467	Catalase	Catalase activity, heme binding, metal ion binding, response to oxidative stress, hydrogen peroxide catabolic process, oxidation-reduction process, cellular oxidant detoxification (GO:0004096, GO:0020037, GO:0046872, GO:0006979, GO:0042744, GO:0055114, GO:0098869)	Catalase (COG0753)
	gene_1886	*FD48_GL001133*	1867622–1867969	–	348	MULTISPECIES: PTS lactose/cellobiose transporter subunit IIA	Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity, D-glucosamine PTS permease activity, phosphoenolpyruvate-dependent sugar phosphotransferase system, carbohydrate transmembrane transport (GO:0008982, GO:0103111, GO:0009401, GO:0034219)	Phosphotransferase system cellobiose-specific component IIA (COG1447)
	gene_2207	*pts25B*	2230122–2230424	–	303	PTS lactose transporter subunit IIB	Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity, phosphoenolpyruvate-dependent sugar phosphotransferase system, carbohydrate transmembrane transport (GO:0008982, GO:0009401, GO:0034219)	Phosphotransferase system, galactitol-specific IIB component (COG3414)
	gene_2507	*LPENT_01969*	2536551–2538515	–	1938	Lactose transport protein	Integral component of membrane, transporter activity, kinase activity, sodium ion transport, phosphoenolpyruvate-dependent sugar phosphotransferase	Phosphotransferase system IIA components (COG2190)

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Table 5 (*continued*)

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
							system, phosphorylation (GO:0016021, GO:0005215, GO:0016301, GO:0006814, GO:0009401, GO:0016310)	
	gene_2680	*gene_2680*	2742526–2742873	–	348	PTS lactose/cellobiose transporter subunit IIA	–	Phosphotransferase system cellobiose-specific component IIA (COG1447)
	gene_2682	*gene_2682*	2744176–2744484	–	309	PTS lactose transporter subunit IIB	–	Phosphotransferase system cellobiose-specific component IIB (COG1440)
	gene_1884	*pbg4*	1864533–1865975	–	1443	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_1885	*LPE_01553*	1865993–1867432	–	1440	Aryl-phospho-beta-D-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_2091	*LPE_02206*	2095494–2096876	–	1383	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_2130	*gene_2130*	2138219–2138689	+	471	YhcH/YjgK/YiaL family protein	–	Beta-galactosidase, beta subunit (COG2731)
	gene_2212	*bglH*	2236718–2238169	–	1452	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_2260	*FD24_GL003184*	2283325–2284758	–	1434	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_2508	*N692_07380*	2538534–2540585	–	2052	MULTISPECIES: beta-galactosidase	Beta-galactosidase complex, beta-galactosidase activity, metal ion binding, galactose metabolic process (GO:0009341, GO:0004565, GO:0046872, GO:0006012)	Beta-galactosidase (COG1874)
	gene_2524	*FD24_GL001068*	2556975–2558855	+	1881	Beta-galactosidase	Hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process (GO:0004553, GO:0005975)	Beta-galactosidase/beta-glucuronidase (COG3250)
	gene_2525	*LPENT_01954*	2558839–2559798	+	960	Beta-galactosidase small subunit	Beta-galactosidase complex, beta-galactosidase activity, carbohydrate binding, carbohydrate metabolic process (GO:0009341, GO:0004565, GO:0030246, GO:0005975)	Beta-galactosidase/beta-glucuronidase (COG3250)
	gene_2534	*FD24_GL001059*	2570230–2572635	–	2406	Glycoside hydrolase family 2	hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process (GO:0004553, GO:0005975)	Beta-galactosidase/beta-glucuronidase (COG3250)
	gene_2566	*pbg9*	2608589–2610037	–	1449	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_2567	*LPE_02502*	2610125–2611588	–	1464	6-phospho-beta-glucosidase	hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process (GO:0004553, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)

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Table 5 (*continued*)

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
	gene_2654	*FD24_GL001144*	2708929–2710314	–	1386	6-phospho-beta-glucosidase	hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process (GO:0004553, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_3149	*FD24_GL002865*	3267530–3268966	+	1437	Aryl-phospho-beta-D-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_3557	*LPENT_00991*	3713910–3715418	+	1509	6-phospho-beta-glucosidase	beta-glucosidase activity, scopolin beta-glucosidase activity, carbohydrate metabolic process (GO:0008422, GO:0102483, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_3563	*KCA1_0727*	3723017–3724489	+	1473	6-phospho-beta-glucosidase	6-phospho-beta-glucosidase activity, carbohydrate metabolic process (GO:0008706, GO:0005975)	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_3614[£]	*gene_3614*	8012–9502	+	1491	6-phospho-beta-glucosidase	–	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
	gene_3655[£]	*gene_3655*	49454–50944	+	1491	6-phospho-beta-glucosidase	–	Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase (COG2723)
Antioxidant	gene_480	*LPENT_00708*	355621–356943	–	1323	Glutathione reductase	Cell, glutathione-disulfide reductase activity, flavin adenine dinucleotide binding, cell redox homeostasis, oxidation-reduction process, cellular oxidant detoxification (GO:0005623, GO:0004362, GO:0050660, GO:0045454, GO:0055114, GO:0098869)	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes (COG1249)
	gene_1040	*LPENT_00184*	971815–973167	+	1353	Pyridine nucleotide-disulfide oxidoreductase	Cell, glutathione-disulfide reductase activity, flavin adenine dinucleotide binding, cell redox homeostasis, oxidation-reduction process, cellular oxidant detoxification (GO:0005623, GO:0004362, GO:0050660, GO:0045454, GO:0055114, GO:0098869)	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes (COG1249)
	gene_1471	*FD24_GL000371*	1448226–1448720	–	495	2-Cys peroxiredoxin	Peroxidase activity, oxidation-reduction process, cellular oxidant detoxification (GO:0004601, GO:0055114, GO:0098869)	Peroxiredoxin (COG2077)
	gene_1472	*FD24_GL000372*	1448881–1450110	–	1230	Glutamate–cysteine ligase	Glutamate-cysteine ligase activity, glutathione biosynthetic process (GO:0004357, GO:0006750)	Gamma-glutamylcysteine synthetase (COG2918)
	gene_1486	*LPENT_03203*	1462134–1464383	–	2250	Glutathione biosynthesis bifunctional protein GshAB	Glutamate-cysteine ligase activity, ATP binding, glutathione biosynthetic process (GO:0004357, GO:0005524, GO:0006750)	D-alanine-D-alanine ligase and related ATP-grasp enzymes (COG1181)
	gene_1641	*gene_1641*	1608318–1608698	+	381	VOC family virulence protein	–	Lactoylglutathione lyase and related lyases (COG0346)
	gene_1652	*LPE_00514*	1622222–1623577	+	1356	NAD(FAD)-dependent dehydrogenase	Cell, NADH peroxidase activity, flavin adenine dinucleotide binding, cell redox homeostasis, oxidation-reduction process, cellular oxidant detoxification	Uncharacterized NAD(FAD)-dependent dehydrogenases (COG0446)

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Table 5 (*continued*)

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
							(GO:0005623, GO:0016692, GO:0050660, GO:0045454, GO:0055114, GO:0098869)	
	gene_1660	*FD24_GL003416*	1630176–1630496	–	321	MULTISPECIES: 4-carboxy-muconolactone decarboxylase	Peroxiredoxin activity, oxidation-reduction process, cellular oxidant detoxification (GO:0051920, GO:0055114, GO:0098869)	Uncharacterized homolog of gamma-carboxymuconolactone decarboxylase subunit (COG0599)
	gene_1945	*LPE_03260*	1943153–1943473	–	321	MULTISPECIES: 4-carboxy-muconolactone decarboxylase	4-carboxymuconolactone decarboxylase activity, peroxiredoxin activity, oxidation-reduction process, cellular oxidant detoxification (GO:0047575, GO:0051920, GO:0055114, GO:0098869)	Uncharacterized homolog of gamma-carboxymuconolactone decarboxylase subunit (COG0599)
	gene_2203	*FD24_GL003129*	2225610–2225993	–	384	4-carboxymuconolactone decarboxylase	Peroxiredoxin activity, oxidation-reduction process, cellular oxidant detoxification (GO:0051920, GO:0055114, GO:0098869)	Uncharacterized homolog of gamma-carboxymuconolactone decarboxylase subunit (COG0599)
	gene_2225	*LPENT_02218*	2248328–2249086	–	759	Putative carboxymuconolactone decarboxylase	4-carboxymuconolactone decarboxylase activity, peroxiredoxin activity, oxidation-reduction process, cellular oxidant detoxification (GO:0047575, GO:0051920, GO:0055114, GO:0098869)	Uncharacterized homolog of gamma-carboxymuconolactone decarboxylase subunit (COG0599)
	gene_2333	*LPENT_02125*	2357373–2358707	+	1335	Glutathione reductase	Cell, glutathione-disulfide reductase activity, flavin adenine dinucleotide binding, cell redox homeostasis, oxidation-reduction process, cellular oxidant detoxification (GO:0005623, GO:0004362, GO:0050660, GO:0045454, GO:0055114, GO:0098869)	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes (COG1249)
	gene_2470	*LPENT_02002*	2502088–2503041	+	954	Putative iron dependent peroxidase	Peroxidase activity, heme binding, oxidation-reduction process, cellular oxidant detoxification (GO:0004601, GO:0020037, GO:0055114, GO:0098869)	Predicted iron-dependent peroxidase (COG2837)
	gene_2554	*N692_07595*	2596555–2597346	–	792	Peptidase M13	S-formylglutathione hydrolase activity, formaldehyde catabolic process (GO:0018738, GO:0046294)	Predicted esterase (COG0627)
	gene_2856	*LPENT_01661*	2935576–2936358	+	783	Halo peroxidase	Peroxidase activity, cellular oxidant detoxification (GO:0004601, GO:0098869)	Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily) (COG0596)
	gene_2951	*N692_11435*	3051203–3051688	+	486	MULTISPECIES: glutathione peroxidase	Glutathione peroxidase activity, response to oxidative stress, oxidation-reduction process, cellular oxidant detoxification (GO:0004602, GO:0006979, GO:0055114, GO:0098869)	Glutathione peroxidase (COG0386)
	gene_2856	*LPENT_01661*	2935576–2936358	+	783	Glutathione reductase	Peroxidase activity, cellular oxidant detoxification (GO:0004601, GO:0098869)	Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily) (COG0596)
	gene_2951	*N692_11435*	3051203–3051688	+	486	MULTISPECIES: glutathione peroxidase	Glutathione peroxidase activity, response to oxidative stress, oxidation-reduction process, cellular oxidant detoxification (GO:0004602, GO:0006979, GO:0055114, GO:0098869)	Glutathione peroxidase (COG0386)

(*continued on next page*)

Table 5 (*continued*)

Probiotic function	Gene ID	Gene	Position	Strand	Gene length	Protein description	Ontology term (Ontology ID)	COG class description (COG class)
	gene_3102	*LPENT_01420*	3212808–3214139	+	1332	Glutathione reductase	Cell, glutathione-disulfide reductase activity, flavin adenine dinucleotide binding, cell redox homeostasis, oxidation-reduction process, cellular oxidant detoxification (GO:0005623, GO:0004362, GO:0050660, GO:0045454, GO:0055114, GO:0098869)	Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes (COG1249)
Selenium metabolism	gene_198	*serS*	82854–84131	+	1278	Serine–tRNA ligase	Cytoplasm, serine-tRNA ligase activity, ATP binding, seryl-tRNA aminoacylation, selenocysteine biosynthetic process, selenocysteinyl-tRNA(Sec) biosynthetic process (GO:0005737, GO:0004828, GO:0005524, GO:0006434, GO:0016260, GO:0097056)	Seryl-tRNA synthetase (COG0172)
	gene_523	*LPENT_00672*	413379–414293	+	915	Homocysteine methyltransferase	Methyltransferase activity, methylation (GO:0008168, GO:0032259)	Homocysteine/selenocysteine methylase (S-methylmethionine-dependent) (COG2040)
	gene_712	*FD24_GL002107*	632920–634158	+	1239	Cysteine sulfinate desulfinase	Pyridoxal phosphate binding, cysteine desulfurase activity, cysteine metabolic process (GO:0030170, GO:0031071, GO:0006534)	Selenocysteine lyase (COG0520)
	gene_3190	*serS1*	3311036–3312307	+	1272	MULTISPECIES: serine–tRNA ligase	Cytoplasm, serine-tRNA ligase activity, ATP binding, seryl-tRNA aminoacylation, selenocysteine biosynthetic process, selenocysteinyl-tRNA(Sec) biosynthetic process (GO:0005737, GO:0004828, GO:0005524, GO:0006434, GO:0016260, GO:0097056)	Seryl-tRNA synthetase (COG0172)

*: The best hit was indicated.
£: sequences of pLPE6-4 plasmid.
§: sequences of pLPE6-plasmid.
&: sequences of pLPE6-plasmid.

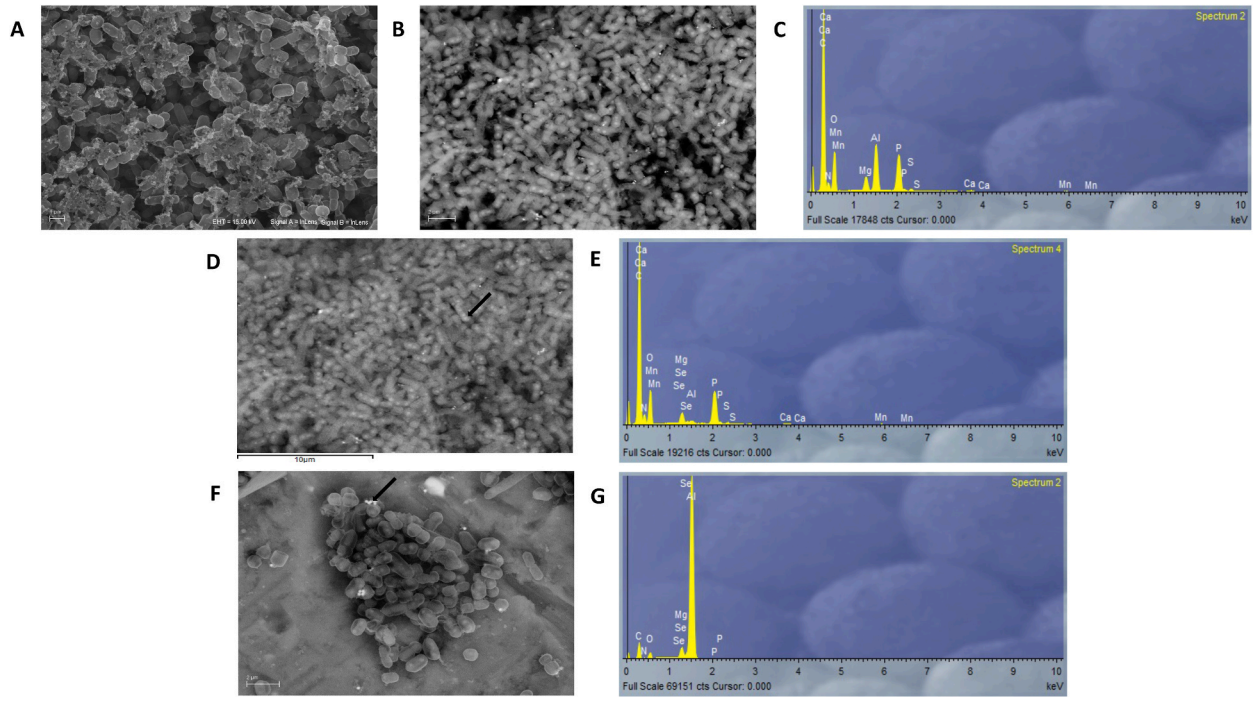

Fig. 5. Scanning electron micrographs **(A, B, D and F)** and EDX microanalysis **(C, E and G)** of *L. pentosus* CF-6HA cells grown in absence **(A–C)** and presence of 5 ppm Se **(D–G)**. SEM images were taken at different magnification. Arrows indicate the sites of the EDX spectra.

properties, each *L. pentosus* strain may have a characteristic hallmark which distinguishes it from the other. In fact, each strain should be investigated for its safety aspects, as an inherent trait dependent on the species and strain which must be addressed following the guidelines of FAO/WHO [43]. Thus, *in silico* analysis of the *L. pentosus* CF-6HA genome with respect to antibiotic resistance genes (ARGs by RGI database) showed the presence of a *vanY* gene in vanB cluster conferring glycopeptide resistance (100% of identity with *vanZ* gene in other *L. pentosus* strains which was located on the chromosome) and several non-specific antimicrobial resistance mechanisms. These mechanisms could rely on transmembrane proteins or efflux transporters involved in response to drugs and antibiotics. However, no acquired antimicrobial resistance gene(s) and no virulence factors (VFs) including toxins were identified in the genome sequence. These data confirmed the safety status of *L. pentosus* CF-6HA. It also confirms that an intrinsic antimicrobial resistance is not transferable horizontally, as requested in probiotic strains, although it contributes to defense and fitness against drugs [44].

The L. *pentosus* CF-6HA genome sequence was further explored for determinants involved in robustness and functionality allowing this strain to withstand several stresses in its habitat, during fermentation and also in the gastrointestinal tract. *In silico* analysis of the genome sequence showed the frequent genetic diversification inferred by the mobilome (17 ISI elements, 98 transposases, 13 temperate phage regions) and CRISPR elements (CRISPR I and II systems). The high number of mobile genetic elements in the *L. pentosus* CF-6HA genome was comparable to other *L. pentosus* strains derived from the same source – i.e. *L. pentosus* MP-10 (29 transposases, 5 temperate phage regions) and *L. pentosus* CF2–10 N (66 transposases, 45 IS elements and 8 temperate phage regions) [5,12,19]. However, the number greatly exceeded other *L. pentosus* strains isolated from other ecological niches such as *L. pentosus* IG1 (Spanish-style green olive fermentations; 5 genes coding for transposases), *L. pentosus* KCA1 (vagina; 25 genes coding for transposases) and *L. pentosus* DSM 20314 (corn silage; 14 genes coding for transposases) [12]. These features indicated that *L. pentosus* strains isolated from Aloreña table olives possess a large number of elements with adaptive function playing a significant role in their habitat by means of chromosomal rearrangements, with especial focus on *L. pentosus* CF-6HA. Moreover, other selective advantages for survivability of *L. pentosus* CF-6HA are conferred by 13 temperate phage regions detected in its genome, seven of them were intact and are mainly lactobacilli prophages, although the other questionable and incomplete prophages were related to other bacteria (*Erwinia*, *Photobacterium*, *Paenibacillus* or *Staphylococcus*). As reported by Zhou et al. [45], prophage behaviors can increase or decrease host bacteria virulence, thus this issue may depend on the harboring genome of temperate phage and also the target being in this case more effective than antimicrobials (antibiotics and biocides) in pathogen inhibition and at the same time preventing antimicrobial resistance. For this, it is necessary to carry out specific *in vivo* studies of each probiotic bacterium to explore the impact of temperate phages in the target environment. In fact, although *L. pentosus* CF-6HA possess the machinery able to face the risk of a phage infection in different ecosystems [46], *in vivo* studies are needed to elucidate the role of temperate phage in different environments where this bacterium is going to be applied. CRISPR system (CRISPR-I and CRISPR-II) as another defense mechanism was also detected in the *L. pentosus* CF-6HA genome. Notably, 12 genes were detected providing protection against mobile genetic elements in a similar way as *L. pentosus* CF2–10 N and *L. pentosus* MP-10. However, both the genes and their organization were strain specific, suggesting that

adaptation and fitness may rely on the specific strain [12,19].

In silico analysis of the genome sequence of *L. pentosus* CF-6HA and also *in vitro* tests determined a plethora of desired functional properties, such as antimicrobial activity allowing this "talented" strain to successfully outcompete undesired and pathogenic microbial species in its habitat and also in the gut. First, bacteriocin genes analysis revealed the presence of a gene cluster responsible for pediocin (class IIa bacteriocin) production in addition to a plantaricin gene (*LPENT_01393*) coding for the biosynthesis of Plantaricin Y (PlnY; class IId bacteriocin) [47]. Besides bacteriocins, the presence of other gene clusters with putative secondary metabolite biosynthetic capabilities were also predicted in the *L. pentosus* CF-6HA genome related to T3PKS. In this context, we identified a list of putative gene clusters coding for terpene, Type III polyketide synthase (T3PKS), polyketides (PKS), non-ribosomal peptide (NRP), the hybrid peptide-polyketide products (PK + NRP) and post translationally modified Peptide (RiPP). These secondary metabolites, including bacteriocins and exopolysaccharides, are of particular interest, due to their diverse structure and specific bioactivities highlighting promising probiotic potential of *L. pentosus* CF-6HA as a source of antimicrobial and bioactive compounds. Similarly, Tenea and Ortega [48] predicted in the *L. plantarum* UTNGt2 (isolated from White Cacao of Ecuadorian Amazon) genome two bacteriocin clusters (class IIc; the sactipeptide class and plantaricin E class), and also several polyketides (PKs), RiPP-like peptides and terpenes. It is noteworthy to highlight that some LAB are able to produce a wide arsenal of beneficial secondary metabolites such as bacteriocins, diacetyl, acetoin, exopolysaccharides, vitamins (B-group), cyclic dipeptides, Mevalonic acid, Mevalonolactone and butanediol among others. These accomplish several functions such as antimicrobial activity, antioxidant, interaction with host, immunomodulation, etc. [49,50]. Nevertheless, only few LAB strains are able to produce secondary metabolites, while only some of them are secreted externally or they are produced in low concentration. Hence, further analysis is required to identify and characterize secondary metabolites both *in silico* and *in vitro*, notably to enhance the functionality of the studied strain.

Other detected probiotic features are related to the presence of genes coding for adhesion proteins involved in the interaction and adherence to the host (e.g. epithelial cells) such as cell surface proteins, mucus-binding proteins, adherence protein, Metal ABC transporter permease, ABC transporters, Beta-fructosidase and hypothetical proteins. Some of these proteins are also involved in other functions such as survivability, competitive exclusion of pathogen to epithelial cells, stress response, drug efflux and others [51–53]. In a similar way, Abriouel et al. ([12,18,19]) reported that *L. pentosus* MP-10 and CF2–10 N strains possess several moonlighting proteins responsible of their functionality. Once the probiotic bacteria interact and adhere to the host cells, a large variety of functional activities may occur due to the enzymatic machinery. In this regard, the *L. pentosus* CF-6HA genome revealed the presence of oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases and translocases remarking the presence of coding genes for bile salt hydrolase, tannase, alpha-amylase, amylopullulanase, catalase and multiple coding genes for lactase (beta-galactosidase or beta-glucosidase). Furthermore, genes coding for antioxidants required for reducing oxidative stress were detected (i.e. glutathione biosynthesis, 4-carboxymuconolactone decarboxylase, 2-Cys peroxiredoxin, NAD(FAD)-dependent dehydrogenase, Putative iron dependent peroxidase and Halo peroxidase).

Both *in silico* and *in vitro* analysis showed the important selenite bioremediation role of *L. pentosus* CF-6HA. This strain tolerated selenium in culture broth and metabolized selenium in accordance with the presence of relevant coding genes (Serine–tRNA ligase, Homocysteine methyltransferase, Cysteine sulfinate desulfinase and MULTISPECIES: serine–tRNA ligase) for proteins able to biotransform and reduce toxic selenite (SeO_3^{2-}) into elemental selenium (Se^0). The strain could thus completely remove Se from the culture supernatant. This fact was confirmed by scanning electron microscopy and EDX microanalysis as white nanoparticles (SeNPs) on the cell surface of *L. pentosus* CF-6HA. Similarly, other lactobacilli were also able to reduce selenite such as *L. plantarum* CRL 2030 [33] and *L. casei* ATCC 393 [54]. This important and effective approach for bioremediation of selenite contamination by *L. pentosus* CF-6HA presents an additional attractive feature which could be exploited in food, agriculture and environment applications where accumulation of this highly soluble and bioavailable micronutrient may cause a great challenge. On the other hand, as starter culture, *L. pentosus* CF-6HA could also be used for the bio-enrichment of fermented foods with selenium.

5. Conclusion

This study demonstrated beneficial properties and antimicrobial activity of *L. pentosus* CF-6HA against human/animal and plant pathogens. The safety, robustness and functional properties of *L. pentosus* CF-6HA was confirmed by *in silico* analysis, also yielding a wide range of coding genes for defense and adaptability to different life styles besides functional properties. The functionality of this strain was evidenced by the presence of genes coding for secondary metabolites as well as proteins involved in adhesion, antioxidant activity and other functions. We also highlight the potential use of *L. pentosus* CF-6HA in selenite bioremediation.

In summary, results from this study suggest that *L. pentosus* CF-6HA can be catalogued as a "talented" bacterium, however *in vivo* studies are required to confirm its application in different environments.

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CRediT authorship contribution statement

Hikmate Abriouel: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Natacha Caballero Gómez:** Validation, Methodology, Investigation. **Julia Manetsberger:** Writing – review & editing, Validation, Methodology, Investigation. **Nabil Benomar:** Writing – review & editing,

Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interestsHikmate Abriouel reports was provided by University of Jaen. Hikmate Abriouel has patent pending to In course.

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Appendix A. Supplementary data

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