New insights into the role of plasmids from probiotic Lactobacillus pentosus MP-10 in Aloreña table olive brine fermentation

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In silico analysis of Lactobacillus pentosus MP-10 plasmids (pLPE-1 to pLPE-5) suggests that plasmid-borne genes mediate the persistence of lactobacilli during olive fermentation and enhance their probiotic properties and their competitiveness in several ecological niches. The role of plasmids in the probiotic activities of L. pentosus MP-10 was investigated by plasmid-curing process which showed that plasmids contribute in increased metal tolerance and the biosequestration of several metals such as iron, aluminium, cobalt, copper, zinc, cadmium and mercury. Statistically significant differences in mucin adhesion were detected between the uncured and the cured L. pentosus MP-10, which possibly relied on a serine-rich adhesin (sraP) gene detected on the pLPE-2 plasmid. However, plasmid curing did not affect their tolerance to gastro-intestinal conditions, neither their growth ability under predetermined conditions, nor auto-aggregation and pathogen co-aggregation were changed among the cured and uncured L. pentosus MP-10. These findings suggest that L. pentosus MP-10 plasmids play an important role in gastro-intestinal protection due to their attachment to mucin and, thus, preventing several diseases. Furthermore, L. pentosus MP-10 could be used as a bioquencher of metals in the gut, reducing the amount of these potentially toxic elements in humans and animals, food matrices, and environmental bioremediation.

Table olive fermentation is the oldest practice by our ancestors to preserve vegetables and to also produce different flavours and textures. Additionally, fermented table olives remain an important economy for many production countries and a component of the Mediterranean diet (and recommended as part of the Healthy Eating Pyramid published in 2010, https://dietamediterranea.com/). The high nutritional value of fermented table olives (e.g., their content of carbohydrates, fiber, minerals, vitamins, fatty acids, and amino acids) and their role as potential source of probiotic lactobacilli of vegetable origin1–5 make them very attractive from an economic and social point of view. Lactobacillus genus is the most representative and heterogeneous member of lactic acid bacteria (LAB) group currently consisting of 237 species (as of December 2018 in www.bacterio.net) since they harbour in their genome a plethora of genes involved with a wide array of functional properties6,7. Lactobacillus spp. are principal bacteria in olive fermentation processes, possessing many biochemical and physiological traits to ferment several carbohydrates and tolerate stress8. These phenotypes are important as the brine environment represent harsh conditions for bacterial growth with low nutrient availability, saltiness, low pH and the presence of antimicrobials (e.g., phenolic compounds and oleuropein); thus, highly robust L. plantarum and L. pentosus are frequently isolated from the end of olive fermentation1,9. Furthermore, Perpetuini, et al.10 demonstrated by transposon mutagenesis that the high capacity of L. plantarum and L. pentosus to survive in the hostile, brine environments was due to critical genes encoding proteins involved in carbohydrate metabolism, membrane structure and function, and

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gene-expression regulation. They further suggested that the *obad* gene, which encodes a putative membrane protein strictly specific to *L. pentosus/L. plantarum* species, may be one of the key elements involved in their efficient adaptation to several conditions in many fermented food processes and natural ecosystems.

Aloreña green table olive fermentation is a spontaneous process relying on *L. pentosus* strains and yeasts. Resistance, persistence and predominance of *Lactobacillus* spp. in green table olive fermentation is due to their genetic variation and plasticity related to their chromosome and plasmids. In fact, *L. pentosus* species isolated from olive fermentation harbours the largest genome recognized to date and several plasmids (range: n = 5 to 7). However, *L. plantarum* contains the largest plasmids among the genus *Lactobacillus* such as *L. plantarum* 16, which harbors 10 plasmids ranging 6.46–74.08 kb. Most of the *Lactobacillus* plasmids are cryptic; however, they possess important properties such as antibiotic resistance, exopolysaccharide production, antimicrobial activity, bacteriocin synthesis, bacteriocin resistance, carbohydrate metabolism, host colonization and probiotic activity. On the other hand, megaplasmids were also detected in *Lactobacillus* sp., up to 490 kb. In this study, we analyzed in silico five plasmids harboured by *L. pentosus* MP-10 isolated from naturally fermented Aloreña green table olive. Moreover, we aimed to better understand the underlying functional and probiotic properties of these plasmids using curing plasmid experiments; in particular, we examined their physiological traits in metal tolerance and biosorption, antimicrobial activity and adaptation to gastro-intestinal conditions to determine possible probiotic applications of this bacterium.

**Results**

**General features of *L. pentosus* MP-10 plasmids.** We have already reported the sequencing of *L. pentosus* MP-10 genome, which consisted of a single circular chromosome of 3,698 kbp and five plasmids ranging 29–46 kbp (accession numbers FLYG01000001 to FLYG01000006). Sequence annotation was done using the Prokka annotation pipeline, version 1.11 as previously reported by Abriouel et al. The general features of the circular five plasmids are reported in Table 1. The average GC content of *L. pentosus* MP-10 plasmids ranged 39.52–42.50%, slightly lower than the host chromosome (with GC value of 46.32%). Furthermore, the GC contents of *L. pentosus* MP-10 plasmids were among the highest of known *L. pentosus* plasmids. All open reading frames in *L. pentosus* MP-10 plasmids are greater than 34 amino acids (Tables 2–6). Blast search for homology revealed lower identity with other plasmids in the database; however depending on coverage percentage, some regions harboring several genes in *L. pentosus* MP-10 plasmids were highly related with plasmids of *Lactobacillus* species isolated from foods like fermented olives, kimchi, koumiss, tofu or raw sausages and also from human saliva (Table 1).

**In silico analysis of plasmid properties in *L. pentosus* MP-10.** Analysis of the annotated CDs of each *L. pentosus* MP-10 plasmid revealed the presence of five genes involved in mobilization (*mobA*) gene) distributed in all plasmids except the pLPE-2 plasmid (Tables 2–6). These genes are likely required for plasmid relaxation and mobilization by conjugative plasmids. Also, conjugation-related genes were found, e.g., *traG_1* and *traG_2* and pLPE-5 (*traG_3*) plasmids (Tables 5 and 6). A gene encoding for a bacteriophage peptideglycan hydrolase that may have been involved in growth was found in pLPE-4 (*XX999_00013* and *XX999_00049*).

The presence of mobile genetic elements in *L. pentosus* MP-10 plasmids (pLPE-2, pLPE-3, pLPE-4 and pLPE-5) was already reported by Abriouel et al. such as four putative transposon Tn552 DNA-invertase bin3 (four different genes of the same family), transposase DDE domain proteins (4 genes in pLPE-2 and pLPE-5 plasmids), transposases of the mutator family (3 genes in pLPE2, pLPE3 and pLPE5 plasmids) and transposases (2 genes in pLPE-2 and pLPE-3 plasmids). Concerning integrases, one phage integrase family protein (pLPE-1 plasmid) and 9 integrase core domain proteins were detected in pLPE-2, pLPE-3 and pLPE-5 plasmids (Tables 3, 4 and 6). A gene *pinR* coding for DNA invertase from prophage was detected in pLPE-5 plasmid (Table 5).

Chloride- (*clA* _2*) and sodium- (*nhaA3* _4*) transport genes harbourd by pLPE-2 plasmid (Table 3) indicated that this plasmid was involved in salt-tolerance in brine solutions (plasmid curing experiments). Furthermore, a
| Gene ID  | Gene     | Position | Strand | Gen length (bp) | Protein description | GO terms                                                                 | Similarity to proteins in Lactobacillus |
|---------|----------|----------|--------|-----------------|---------------------|--------------------------------------------------------------------------|----------------------------------------|
| XX999_03518 | XX999_03518 | 804–950  | −      | 147             | Hypothetical protein  | —                                                                        | 98% identity in *L. paracasei* subsp. *paracasei* Lpp70                    |
| XX999_03519 | XX999_03519 | 963–1271 | −      | 309             | Phage integrase family protein | —                                                                         | 87% identity in *Lactobacillus*                                               |
| XX999_03520 | XX999_03520 | 1238–1651 | −      | 414             | Hypothetical protein  | —                                                                        | 99% identity in *L. plantarum* IPLA88                                       |
| XX999_03521 | XX999_03521 | 1871–2215 | +      | 345             | Toxin MazF            | DNA binding (MF); RNA binding (MF); endoribonuclease activity (MF); 5′-phosphomonoesters (MF); negative regulation of cell growth (BP); regulation of mRNA stability (BP); RNA phosphodiester bond hydrolysis, endonucleolytic (BP) | 100% identity in *L. pentosus*                                              |
| XX999_03522 | XX999_03522 | 2675–3739 | −      | 1065            | Hypothetical protein  | —                                                                        | 99% identity in *L. xiangfangensis*                                         |
| XX999_03523 | XX999_03523 | 3901–4380 | −      | 480             | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                             |
| XX999_03524 | XX999_03524 | 4989–5576 | −      | 588             | Initiator Replication protein | —                                                                        | 98% identity in *L. plantarum*                                              |
| XX999_03525 | XX999_03525 | 6296–6490 | −      | 195             | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus* IG1                                           |
| XX999_03526 | mobA_4    | 7058–8221 | +      | 1164            | Mobilization protein A | Conjugation (BP); DNA binding (MF); DNA-directed RNA polymerase activity (MF); DNA topoisomerase type I activity (MF); cytoplasm (CC); metal ion binding (MF) | 100% identity in *L. pentosus*                                              |
| XX999_03527 | XX999_03527 | 8218–8910 | +      | 693             | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03528 | XX999_03528 | 9111–9866 | −      | 756             | Initiator Replication protein | —                                                                        | 100% identity in *L. plantarum* IPLA88                                      |
| XX999_03529 | XX999_03529 | 10508–10957 | +     | 450            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03530 | XX999_03530 | 10954–11157 | +    | 204            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03531 | XX999_03531 | 11306–11668 | −   | 363            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03532 | XX999_03532 | 11912–12271 | −  | 360            | Hypothetical protein  | —                                                                        | 99% identity in *L. brevis*                                                 |
| XX999_03533 | XX999_03533 | 12284–12871 | −  | 588            | Site-specific tyrosine recombinase XerC | —                                                                        | 99% identity in *L. plantarum* 2025                                         |
| XX999_03534 | XX999_03534 | 12949–13212 | +  | 264            | Putative regulator PrIF | Regulation of cell growth (BP); DNA binding (MF); sequence-specific DNA binding transcription factor activity (MF); cytoplasm (CC); transcription, DNA-templated (BP); enzyme binding (MF); negative regulation of transcription, DNA-templated (BP) | 100% identity in *L. plantarum*                                              |
| XX999_03535 | ndoA_2    | 13212–13559 | +  | 348            | mRNA interferase EndoA | DNA binding (MF); RNA binding (MF); endoribonuclease activity (MF); endoribonuclease activity, producing 5′-phosphomonoesters (MF); negative regulation of cell growth (BP); regulation of mRNA stability (BP); RNA phosphodiester bond hydrolysis, endonucleolytic (BP) | 98% identity in *Lactobacillus*                                             |
| XX999_03536 | XX999_03536 | 14021–15085 | −  | 1065            | Hypothetical protein  | —                                                                        | 99% identity in *L. xiangfangensis*                                         |
| XX999_03537 | XX999_03537 | 15164–15751 | −  | 588            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03538 | XX999_03538 | 15993–16928 | −  | 936            | Initiator Replication protein | —                                                                        | 99% identity in *L. plantarum* subsp. *plantarum*                           |
| XX999_03539 | XX999_03539 | 17648–17842 | −  | 195            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus* IG1                                           |
| XX999_03540 | mobA_5    | 18410–19573 | +  | 1164            | Mobilization protein A | Conjugation (BP); DNA binding (MF); DNA-directed RNA polymerase activity (MF); DNA topoisomerase type I activity (MF); cytoplasm (CC); metal ion binding (MF) | 95% identity in *L. plantarum*                                              |
| XX999_03541 | XX999_03541 | 19570–20262 | +  | 693            | Hypothetical protein  | —                                                                        | 98% identity in *L. plantarum* 2025                                         |
| XX999_03542 | XX999_03542 | 20463–21218 | −  | 756            | Initiator Replication protein | —                                                                        | 100% identity in *L. plantarum* IPLA88                                       |
| XX999_03543 | XX999_03543 | 21860–22309 | +  | 450            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03544 | XX999_03544 | 22306–22509 | +  | 204            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03545 | XX999_03545 | 22658–23020 | −  | 363            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03546 | XX999_03546 | 23264–23623 | −  | 360            | Hypothetical protein  | —                                                                        | 100% identity in *L. pentosus*                                              |
| XX999_03547 | XX999_03547 | 23636–24223 | −  | 588            | Site-specific tyrosine recombinase XerC | —                                                                        | 99% identity in *L. plantarum* 2025                                         |

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copy of the same genes clcA_1, nhaS3_1, nhaS3_2 and nhaS3_3 were also found in L. pentosus MP-10 chromosome with the aim to potentiate chloride and sodium tolerance in brines.

Genes related to carbohydrate metabolism were found on plasmids (besides on the chromosome) such as L-Lactate dehydrogenase in pLPE-5 plasmid (ldh_7 and ldl_8 genes) (Table 6), genes involved in glucose uptake and metabolism such as glcU_1 and gdhIV_1 genes in pLPE-3 plasmid (Table 4), and a gene involved in xylan catabolic process (axcAi_3) in pLPE-5 (Table 5). However, another gene involved in xylan catabolic process (XX999_00089) was only detected in pLPE-3 plasmid, but not on the chromosome (Table 4).

Toxins reported in L. pentosus MP-10 plasmids include mazF-toxin encoding gene (XX999_03521) detected in pLPE-1 plasmid, genes coding for Zeta toxins in pLPE-3 (XX999_00053) and pLPE-4 (XX999_00024) plasmids, and also for antitoxins such as RelB antitoxin (XX999_00026) in pLPE-4 plasmid and the bifunctional antitoxin/transcriptional repressor RelB in pLPE-5 plasmid (XX999_03554) (Tables 2, 4–6). MazF toxin is a desirable property in probiotic bacteria, and is only detected in plasmid DNA of L. pentosus MP-10, not in the chromosome. However, L. pentosus MP-10 has to protect itself from the MazF toxin without any MazE antitoxin. On the other hand, RelB antitoxins were found both on plasmids and on the chromosome; however, no RelB toxins were detected. Zeta toxins were detected both on the chromosome (one gene) and also on plasmid DNA (two genes); however, no antitoxin was detected.

Other coding genes for several functions, such as a serine-rich adhesin for platelets precursor (sraP gene), were detected in pLPE-2 plasmid but not on the chromosome (Table 3); genes coding for vitamin biosynthesis such as panE_1 and panE_2 genes coding for 2-dehydropantoate 2-reductase (biosynthesis of vitamin B5), a gene XX999_00068 coding for prephenate dehydratase (biosynthesis of phenylalanine, tyrosine and tryptophan), were such as genes coding for 2-dehydropantoate 2-reductase (biosynthesis of vitamin B5), a gene (Table 5). However, another gene involved in xylan catabolic process (XX999_00089) was only detected in pLPE-3 plasmid, but not on the chromosome (Table 4).

Regarding their responses to stress, in-silico analysis of plasmid sequences revealed the presence of yhdN_1 gene coding for a general stress protein 69 (in pLPE-3, Table 4) and several genes coding for metal tolerances, such as cadmium resistance transporter (XX999_03594) and a putative positive regulator of cadmium resistance (cadC) and two operons of arsenic resistance (in pLPE-5, Table 6). One of the operons consists of aarsR (arsenic resistance operon repressor ArsR) and aarsB (arsenic pump membrane protein (ArsB)), but lacks aarsC gene (arsenate reductase ArsC); the other aars operon contains aarsA (arsenic pump-driving ATPase (ArsA)) and aarsD gene (arsenic resistance operon trans-acting repressor (ArsD)). The synteny of arsenic-resistance genes was examined by comparing the annotated sequences of plasmid DNA and pWCFS103 plasmids (aligned by MAUVE algorithm) from L. pentosus MP-10 and L. plantarum WCFS1, respectively. Comparison revealed that the synteny of genes was similar (Fig. 2), being arsenic operons in pLPE-5 of L. pentosus MP-10 composed of two copies each gene: aarsB [coding for trivalent As(III) efflux permease ArsB], aarsA [coding for trivalent As(III)-stimulated ATPase ArsA], aarsD [coding for trivalent As(III) metallochaperone ArsD] and aarsR_3 gene [a trivalent As(III)-responsive repressor (ArsR)]. On the other hand, aarsC gene (aarsC2 coding for reductase ArsC), as a part of aars operon with aarsB and aarsR genes, was found in L. pentosus MP-10 chromosome, as well as two aarsR gene copies (aarsR_1 and aarsR_2).

### Table 2. Genes determined in pLPE-1 plasmid of Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives. BP, biological process; CC, cellular component; MF, molecular function.

| Gene ID | Gene | Position | Strand | Gen length (bp) | Protein description | GO terms | Similarity to proteins in Lactobacillus |
|---------|------|----------|--------|----------------|---------------------|----------|--------------------------------------|
| XX999_03548 | XX999_03548 | 24300–24563 | + | 264 | Putative regulator PrIP | Regulation of cell growth (BP); DNA binding (MF); sequence-specific DNA binding transcription factor activity (MF); cytoplasm (CC); transcription, DNA-templated (BP); enzyme binding (MF); negative regulation of transcription, DNA-templated (BP) | 100% identity in L. plantarum MP-10 |
| ndoA_3 | XX999_03549 | 24563–24910 | + | 348 | mRNA interferase EndoA | DNA binding (MF); RNA binding (MF); endoribonuclease activity (MF); endonucleolytic (BP); RNA phosphodiester bond hydrolysis, endonucleolytic (BP) | 98% identity in Lactobacillus |
| XX999_03550 | XX999_03550 | 25372–26436 | − | 1065 | Hypothetical protein | — | 99% identity in L. xiangiangensis |
| XX999_03551 | XX999_03551 | 26515–27102 | − | 588 | Hypothetical protein | — | 100% identity in L. pentosus |
| XX999_03552 | XX999_03552 | 27344–28279 | − | 936 | Initiator Replication protein | — | 99% identity in L. plantarum subsp. plantarum |

**In vitro detection of functional properties in L. pentosus MP-10 plasmids.** Effect of plasmid curing on growth of L. pentosus MP-10. The MIC of acridine orange (AO) was of 0.15 mg/ml; as such, we used 0.1 mg/ml as the sub-MIC for plasmid curing in this strain. After confirming L. pentosus MP-10 being cured of plasmids (data not shown), we compared the growth kinetics of uncured and cured L. pentosus MP-10C. The presence of plasmids did not affect the growth in MRS broth at 37 °C in any experimental conditions: presence/absence of plasmids did not significantly affect the growth in MRS broth at 37 °C in any experimental conditions.
| Gene ID   | Gene    | Position | Strand | Gen length (bp) | Protein description                                                                 | GO terms                                                                 | Similarity to proteins in Lactobacillus |
|----------|---------|----------|--------|-----------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------------|
| XX999_03611 | clcA_2   | 2632–2853 | +      | 951             | Ribonucleoside-diphosphate reductase subunit beta ndF2                                  | ATP binding (MF); oxidoreductase activity (MF); hydrolyase activity (MF); sporulation resulting in formation of a cellular spore (BP); negative regulation of sporulation resulting in formation of a cellular spore (BP) | 99% identity in L. pentosus DSM 20314 |
| XX999_03612 | soj_3    | 20479–20826 | +    | 486             | Hypothetical protein                                                                  | —                                                                        | 100% identity in L. plantarum           |
| XX999_03613 | yusO     | 1539     | —      | 523             | Hypothetical protein                                                                  | —                                                                        | 100% identity in L. plantarum DSM 20314 |
| XX999_03614 | nrdF2_2  | 24087–25037 | −    | 951             | Ribonucleoside-diphosphate reductase subunit beta ndF2                                  | Ribonucleoside-diphosphate reductase activity, threonin diphosphate as acceptor (MF); ribonucleoside-diphosphate reductase complex (CC); DNA replication (BP); deoxyribonucleoside diphosphate metabolic process (BP); deoxyriboadenosine synthesis (BP) | 100% identity in L. pentosus IG1 |
| XX999_03615 | sraP     | 25052–25978 | −    | 927             | Ribonucleoside-diphosphate reductase 2 subunit beta                                   | Ribonucleoside-diphosphate reductase activity, threonin diphosphate as acceptor (MF); ribonucleoside-diphosphate reductase complex (CC); DNA replication (BP); deoxyribonucleoside diphosphate metabolic process (BP); deoxyriboadenosine synthesis (BP) | 100% identity in L. pentosus IG1 |
| XX999_03616 | nrdF     | 26085–28253 | −    | 2169            | Ribonucleoside-diphosphate reductase 2 subunit alpha                                   | Ribonucleoside-diphosphate reductase activity, threonin diphosphate as acceptor (MF); ATP binding (MF); DNA replication (BP) | 100% identity in L. pentosus DSM 20314 |

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absence of 6.5% NaCl, different pH ranges (1.5 to 7.0), nor the presence of bile salts (1.8 or 3.6%) - no differences in 600 nm absorbances were detected over 24 h of incubation - (Figs S1–A,B, S2). In a similar manner, pH monitoring during their incubation also did not exhibit any significant differences between cured and uncured strains in regards to their acidification capacity (Fig. S1–C). Furthermore, no differences in the growth were detected between the cured and uncured L. pentosus MP-10 strains in the presence of xylan as the only carbohydrate source (Fig. S1–D). However, at high salt concentration of 8% usually found in brine, significant differences were detected between the cured and uncured L. pentosus MP-10 strains, with the uncured strain being the most tolerant (Fig. S1–E).

Table 7 shows that curing had no significant effect on the growth of uncured and cured L. pentosus MP-10 in the presence of phenolic compounds naturally present in the brines; both the cured and uncured strains tolerated more than 200 mg/ml of olive-leaf extract.

**Effect of plasmid curing on antimicrobial resistance and probiotic features.** We determined the MIC of different antibiotics and biocides between uncured and cured strains, and the results did not show any significant differences in response between both strains except for clindamycin, which exhibited 20 fold increase in the MIC in the uncured L. pentosus MP-10. Thus, plasmids have no role in the susceptibility to the antibiotics and biocides tested, except clindamycin (Table 7).

Regarding the probiotic features, the uncured and the cured L. pentosus MP-10 had performed similarly in auto-aggregation and co-aggregation with all pathogens tested (Table 7), which suggest that plasmids had neither

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### Table 3. Genes determined in pLPE-2 plasmid of Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives.

| Gene ID  | Gene     | Position (bp) | Strand | Gen length (bp) | Protein description | GO terms | Similarity to proteins in Lactobacillus L. plantarum |
|----------|----------|---------------|--------|----------------|---------------------|----------|-----------------------------------------------|
| XX999_03638 | XX999_03638 | 28260–28697  | –      | 438           | Putative Ndi1-like protein | —        | 100% identity in L. plantarum AV01 |
| XX999_03639 | XX999_03639 | 29395–29496   | –      | 102           | Hypothetical protein | —        | 100% identity in L. plantarum 2165 |
| XX999_03640 | XX999_03640 | 29486–29845   | –      | 360           | Putative hydrolase   | —        | 99% identity in L. plantarum 2165 |
| XX999_03641 | XX999_03641 | 30683–30943   | –      | 261           | Hypothetical protein | Recombinase activity (MF); DNA binding (MF); DNA integration (BP) | 100% identity in L. plantarum AV01 |
| XX999_03642 | XX999_03642 | 30999–31250   | +      | 252           | Transposase          | —        | 100% identity in L. pentosus |
| XX999_03643 | XX999_03643 | 31304–32146   | +      | 843           | Integrase core domain protein | —        | 99% identity in L. plantarum |
| XX999_03644 | XX999_03644 | 32416–32805   | –      | 390           | Integrase core domain protein | —        | 99% identity in L. plantarum |
| XX999_03645 | XX999_03645 | 32896–33381   | –      | 486           | Hypothetical protein | —        | 100% identity in L. plantarum |
| XX999_03646 | nhaS3_4   | 33487–34641   | +      | 1155          | High-affinity Na(+)/H(+) antiporter NhaS3 | —        | 100% identity in L. pentosus IG1 |

**Table 3.** Genes determined in pLPE-2 plasmid of Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives. BP, biological process; CC, cellular component; MF, molecular function.
| Gene ID   | Gene    | Position | Strand | Gen length (bp) | Protein description                                                                 | GO terms                                                                 |
|----------|---------|----------|--------|-----------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| XX999_00053 | XX999_00053 | 146–412  | −      | 267             | Zeta toxin                                                                           | —                                                                        |
| XX999_00054 | XX999_00054 | 586–783  | −      | 198             | Hypothetical protein                                                                 | —                                                                        |
| XX999_00055 | XX999_00055 | 1002–1931 | +      | 930             | Integrase core domain protein                                                         | —                                                                        |
| XX999_00056 | XX999_00056 | 1934–2152 | +      | 219             | Hypothetical protein                                                                 | —                                                                        |
| XX999_00057 | soj_1  | 3395–4204 | +      | 810             | Chromosome-partitioning ATPase Soj                                                    | DNA binding (MF); ATP binding (MF); chromosome segregation (BP); hydrolase activity (MF) |
| XX999_00058 | XX999_00058 | 4197–4532 | −      | 336             | Hypothetical protein                                                                 | —                                                                        |
| XX999_00059 | XX999_00059 | 4598–4771 | +      | 174             | Hypothetical protein                                                                 | —                                                                        |
| XX999_00060 | XX999_00060 | 5611–6453 | −      | 843             | Integrase core domain protein                                                         | —                                                                        |
| XX999_00061 | XX999_00061 | 6507–6758 | −      | 252             | Transposase                                                                           | —                                                                        |
| XX999_00062 | XX999_00062 | 6826–7092 | −      | 267             | Divergent AAA domain protein                                                         | —                                                                        |
| XX999_00063 | ivE_1 | 7372–8394 | −      | 1023            | Putative branched-chain-amino acid aminotransferase                                   | Isoleucine biosynthetic process (BP); leucine biosynthetic process (BP); valine biosynthetic process (BP); L-leucine transaminase activity (MF); L-valine transaminase activity (MF); L-isoleucine transaminase activity (MF) |
| XX999_00064 | panE_1 | 8444–9463 | −      | 1020            | 2-dehydropantoate 2-reductase                                                        | Cytoplasm (CC); 2-dehydropantoate 2-reductase activity (MF); pantothenate biosynthetic process from valine (BP); NADP binding (MF) |
| XX999_00065 | yvdD_1 | 9990–10559 | −      | 570             | LOG family protein YvdD                                                               | —                                                                        |
| XX999_00066 | XX999_00066 | 10970–11968 | +      | 999             | Integrase core domain protein                                                         | —                                                                        |
| XX999_00067 | panE_2 | 12688–13698 | +      | 1011            | 2-dehydropantoate 2-reductase                                                        | Cytoplasm (CC); 2-dehydropantoate 2-reductase activity (MF); pantothenate biosynthetic process from valine (BP); NADP binding (MF) |
| XX999_00068 | XX999_00068 | 13686–14087 | +      | 402             | Prephenate dehydratase                                                                | —                                                                        |
| XX999_00069 | XX999_00069 | 14032–14613 | −      | 582             | Transposase, Mutator family                                                          | —                                                                        |
| XX999_00070 | aonB_1 | 14954–16543 | −      | 1590            | Asparagine synthetase B [glutamine-hydrolyzing]                                       | Asparagine synthase (glutamine-hydrolyzing) activity (MF); ATP: aspartate-ammonia ligase activity (MF); ATP binding (MF); cytoplasm (CC); asparagine biosynthetic process (BP); glutamine metabolic process (BP); cellular amino acid biosynthetic process (BP); cellular amino acid catabolic process (BP); amino acid binding (MF); identical protein binding (MF); L-asparagine biosynthetic process (BP) |
| XX999_00071 | bin3_2 | 17298–17972 | −      | 675             | Putative transposon Tn552 DNA-invertase bin3                                           | Recombinase activity (MF); DNA binding (MF); DNA integration (BP); transposition (BP) |
| XX999_00072 | ltrA_1 | 18520–19686 | +      | 1167            | Group II intron-encoded protein LtrA                                                   | RNA-directed DNA polymerase activity (MF); endonuclease activity (MF); intron homing (BP); mRNA processing (BP) |
| XX999_00073 | hsaA_1 | 20060–20479 | +      | 420             | Transcriptional regulator HsaA                                                        | DNA binding (MF); sequence-specific DNA binding (MF); transcription, DNA-templated (BP); pathogenesis (BP) |
| XX999_00074 | XX999_00074 | 20536–20991 | +      | 456             | hypothetical protein                                                                 | —                                                                        |
| XX999_00075 | XX999_00075 | 20988–21206 | +      | 219             | hypothetical protein                                                                 | —                                                                        |
| XX999_00076 | XX999_00076 | 21421–21912 | +      | 492             | hypothetical protein                                                                 | —                                                                        |
| XX999_00077 | XX999_00077 | 22017–22805 | +      | 789             | flavodoxin                                                                           | —                                                                        |
| XX999_00078 | XX999_00078 | 22823–23476 | +      | 654             | NmrA-like family protein                                                              | —                                                                        |
| XX999_00079 | XX999_00079 | 23512–24384 | +      | 873             | Alpha/beta hydrolase family                                                           | —                                                                        |
| XX999_00080 | hsrA_2 | 24631–24924 | +      | 294             | putative transport protein HsrA                                                       | Plasma membrane (CC); integral component of membrane (CC); transmembrane transport (BP) |
| XX999_00081 | efpA | 24921–25958 | +      | 1038            | putative MFS-type transporter EfpA                                                    | Plasma membrane (CC); integral component of membrane (CC); transmembrane transport (BP) |
| XX999_00082 | XX999_00082 | 26043–26618 | +      | 576             | flavodoxin                                                                           | —                                                                        |
| XX999_00083 | glcU_1 | 26631–27491 | +      | 861             | Glucose uptake protein GlcU                                                           | Plasma membrane (CC); rhamnose transmembrane transporter activity (MF); integral component of membrane (CC); sporulation resulting in formation of a cellular spore (BP) |
| XX999_00084 | yygN_1 | 27580–28431 | +      | 852             | Glyoxal reductase                                                                   | Methylglyoxal reductase (NADPH-dependent) activity (MF) |
| XX999_00085 | gdhIV_1 | 28460–29245 | +      | 786             | Glucose 1-dehydrogenase 4                                                            | Identical protein binding (MF); glucose 1-dehydrogenase (NADP)(S); activity (MF) |
| XX999_00086 | adhR_1 | 29308–29703 | +      | 396             | HTH-type transcriptional regulator AdhR                                              | DNA binding (MF); transcription, DNA-templated (BP); regulation of transcription, DNA-templated (BP) |
| XX999_00087 | XX999_00087 | 29700–30434 | +      | 735             | putative oxidoreductase                                                              | Oxidoreductase activity (MF) |
| XX999_00088 | yhdN_1 | 30459–31436 | +      | 978             | General stress protein 69                                                             | Oxidoreductase activity (MF) |
| XX999_00089 | XX999_00089 | 31514–32101 | +      | 588             | Polysaccharide decactylase                                                           | Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds (MF); polysaccharide binding (MF); endo-1,4-beta-xylanase activity (MF); xylan catabolic process (BP) |
| XX999_00090 | XX999_00090 | 32681–33805 | −      | 1125            | hypothetical protein                                                                 | —                                                                        |
| XX999_00091 | XX999_00091 | 33809–34024 | −      | 216             | hypothetical protein                                                                 | —                                                                        |

Continued
any role in auto-aggregation nor co-aggregation processes. Regarding acid and bile tolerance, no differences were detected between the uncured and the cured L. pentosus MP-10 (Table 7).

Adhesion to mucin was measured in both the uncured and the cured L. pentosus MP-10, and the results showed a statistically significant increase in adhesion capacity to mucin in the uncured L. pentosus MP-10 (Table 7).

### Discussion

Olive brine represents a stressful environment for the growth and survival of many bacteria due to the harsh conditions (i.e., high salt concentration, presence of phenolic compounds and low-nutrient availability), which provide selective pressures for the maintenance of LAB. As such, L. plantarum and L. pentosus have the genetic tools to survive and grow in the hostile olive-brine conditions\(^{26}\), and these genetic traits are widely distributed on both the chromosome and the plasmids, with several genes having multiple copies to enhance their adaptability and fitness in different ecological niches.

In this study, L. pentosus MP-10, isolated from Aloreña green table olives, harboured five plasmids with an average GC content (39.52–42.50%) slightly lower than the host chromosome (46.32%), this difference was less than 10% as reported by Nishida\(^ {25}\) for the majority of plasmids. pLPE-5 had remarkably the lowest average GC content (39.52%) than the other four plasmids (pLPE-1, pLPE-2, pLPE-3 and pLPE-4), suggesting it is possibly a recent acquisition from another bacterium. *In-silico* analysis of plasmid sequences revealed the presence of genes involved in mobilization (mobA) and conjugation (traG) distributed in several plasmids, which suggest their role in gene mobilization and secretion using a type-IV secretion mechanism\(^ {26}\). Furthermore, mobile genetic elements (e.g., transposon, transposase, integrase and invertase) were also found in several plasmids\(^ {24}\) suggesting a frequent genetic diversification among the L. pentosus MP-10. Furthermore, bacteriophage peptidoglycan hydrolases were found in pLPE-4 and pLPE-5 plasmids; these lysozyme-like proteins may play a key role in L. pentosus MP-10 growth, its cell-wall structure, and immunomodulatory properties as reported by Rolain, et al.\(^ {27}\).

Metabolic profile within L. pentosus MP-10 plasmids include carbohydrate enzymes such as L-lactate dehydrogenase, glucose uptake and metabolism and xylan catalytic enzymes. L-lactate dehydrogenase was codified by two genes (ldh\(_7\) and ldh\(_8\)) located on pLPE-5 plasmid; however, six L-lactate dehydrogenase (ldh\(_1\), ldh\(_2\), ldh\(_3\), ldh\(_4\), ldh\(_5\) and ldh\(_6\)) and four D-lactate dehydrogenase (XX999\_00315, XX999\_00955, XX999\_02047 and XX999\_02719) coding genes were also present on the chromosome. Both enantiomers (L-lactate and D-lactate) are produced by L. pentosus MP-10 being D-and L-lactate dehydrogenases involved in the reversible metabolism of D- and L-lactate, respectively. This finding is of great interest suggesting that the use of L. pentosus MP-10 as a probiotic may help human to metabolise D-lactate obtained from exogenous sources (e.g., diet and the carbohydrate-fermenting bacteria normally present in the gastrointestinal tract) since mammalian cells lack sufficient D-lactate dehydrogenase required to utilise D-lactic acid—leading to chronic fatigue syndrome and D-lactic acidosis or D-lactate encephalopathy associated with short bowel syndrome\(^ {28–30}\). Further, L-lactate dehydrogenase genes present on the plasmids may enhance their metabolic activity during the fermentation process to produce more L-lactate and energy. However, the presence of L-lactate dehydrogenase (ldh\(_7\) and ldh\(_8\)) coding genes on pLPE-5 plasmid did not enhance the acidification capacity, as results were similar after 8 and 24 h incubation in both cured and uncured L. pentosus MP-10, suggesting that these genes either have a minor role in lactate production or they are regulated. Further experiments, based on differential relative expression of ldh\(_7\) gene in both the cured and uncured L. pentosus MP-10 strains, revealed lower expression level in the cured strain (Fig. S3), thus the low activity of lactate dehydrogenase gene in the cured strain is enough to give rise to a substantial lactate accumulation in the fermentation broth in a manner similar as the uncured strain. Regarding glucose uptake and metabolism, glucose uptake and metabolism and xylan catalytic enzymes. L-lactate dehydrogenase was codified by two genes (ldh\(_7\) and ldh\(_8\)) located on pLPE-5 plasmid; however, six L-lactate dehydrogenase (ldh\(_1\), ldh\(_2\), ldh\(_3\), ldh\(_4\), ldh\(_5\) and ldh\(_6\)) and four D-lactate dehydrogenase (XX999\_00315, XX999\_00955, XX999\_02047 and XX999\_02719) coding genes were also present on the chromosome. Both enantiomers (L-lactate and D-lactate) are produced by L. pentosus MP-10 being D-and L-lactate dehydrogenases involved in the reversible metabolism of D- and L-lactate, respectively. This finding is of great interest suggesting that the use of L. pentosus MP-10 as a probiotic may help human to metabolise D-lactate obtained from exogenous sources (e.g., diet and the carbohydrate-fermenting bacteria normally present in the gastrointestinal tract) since mammalian cells lack sufficient D-lactate dehydrogenase required to utilise D-lactic acid—leading to chronic fatigue syndrome and D-lactic acidosis or D-lactate encephalopathy associated with short bowel syndrome\(^ {28–30}\).

Among defense mechanisms found on plasmids, genes encoding the mazF toxin (pLPE-1), Zeta toxins (pLPE-3 and pLPE-4), and also antitoxins such as RelB antitoxin (pLPE-4) and the bifunctional antitoxin/tran
scriptional repressor RelB (pLPE-5) were detected in L. pentosus MP-10 plasmids. RelBE and MazEF are known as sequence-specific endo-ribonucleases that inhibit the global translations of cellular mRNAs\(^ {31}\). MazF toxin is a desirable trait for probiotic bacteria, as its antimicrobial property inhibits several pathogens in foods and the gastrointestinal tract\(^ {32}\). However, L. pentosus MP-10 must protect itself from the mazF toxin, as no MazE antitoxin was detected. Either their protection relies on other mechanisms because mazEF is functional being only expressed in the uncured strain (Fig. S3). On the other hand, genes for RelB antitoxins were found both on plasmids and on the chromosome; however, no RelB-toxin genes were detected. So this antitoxin may contribute a greater defense against other bacteria possessing RelB toxins, possibly increasing its competitiveness and survival in

### Table 4. Genes determined in pLPE-3 plasmid of Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives. BP, biological process; CC, cellular component; MF, molecular function.

| Gene ID   | Gene     | Position | Strand | Gen length (bp) | Protein description | GO terms                                                                 |
|----------|----------|----------|--------|-----------------|---------------------|-------------------------------------------------------------------------|
| XX999\_00092 | topB\_4  | 34147–35697 | –      | 1551           | DNA topoisomerase 3 | **Magnesium ion binding (MF); DNA binding (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomerase type I activity (MF); DNA topoisomera... |
several ecological niches including gastrointestinal tract. This feature was mainly linked to plasmid being relB antitoxin gene over-expressed in the uncured strain (Fig. S3). Zeta toxins, which are kinases that kill bacteria through global inhibition of peptidoglycan synthesis, are detected both on the chromosome and also on plasmid DNA of \emph{L. pentosus} MP-10, however no antitoxin was detected. Overall, \emph{L. pentosus} MP-10 harbored in their plasmids incomplete toxin-antitoxin systems unlike what occur naturally in bacterial genomes, since several toxins or antitoxins were detected without self protection.

Data obtained by \emph{in-silico} analysis suggests that plasmid-borne genes mediate the persistence of lactobacilli under olive fermentation conditions and enhance their probiotic properties; however, this hypothesis requires further studies for confirmation. As such, plasmid curing experiments carried out with \emph{L. pentosus} MP-10 showed several differences between the uncured and the cured strains regarding metal tolerances, removal and mucin adhesion. However, plasmid curing did not affect their tolerance to gastro-intestinal conditions (e.g., acids and bile salts); neither their ability to grow under determined conditions (i.e., different pH intervals, bile salts or sodium chloride of 6.5%) nor their colony morphology were changed after plasmid curing (data not shown). However, at high concentration of chloride of 8% (commonly added to brines), \emph{L. pentosus} pLPE-2 plasmid plays a key role in salt tolerance. In this sense, the results suggest that the plasmids did not govern the fermentation of carbohydrates under these conditions, however different results were obtained by Adeyemo and Onilude which showed that plasmid curing had a significant negative effect on growth, physiological characteristics and colony morphology of \emph{L. plantarum} isolated from fermented cereals. In this study, plasmids in \emph{L. pentosus} MP-10 may confer a selective advantage, providing other physiological properties in certain environments such as gut and brines and thus allowing metal tolerance and removal, salt tolerance and adherence to mucin and thus their persistence in competitive ecological niches. Mucin adhesion declined in the cured \emph{L. pentosus} MP-10 since a serine-rich adhesin for platelets precursor gene (sraP, detected in pLPE-2 plasmid) may be involved in mucin adhesion mechanisms similarly as reported by Hevia, \textit{et al.} for an extracellular serine/threonine-rich protein as a novel aggregation-promoting factor with affinity to mucin in \emph{Lactobacillus plantarum} NCIMB 8826. The role of \emph{L. pentosus} MP-10 plasmids in mucin adhesion was confirmed by relative expression gene analysis as reported by Pérez Montoro \textit{et al.}, since \textit{recA} and \textit{pgm} genes considered as potential biomarkers of mucin adhesion were over-expressed in the uncured strain (Fig. S3). However, auto-aggregation and co-aggregation with some pathogens were not changed after plasmid curing of \emph{L. pentosus} MP-10.

With respect to metals, which are considered non-biodegradable and non-thermodegradable and are of high concern in both developing and developed countries because of their impact on the environment and health (water and food), the wild strain \emph{L. pentosus} MP-10 showed greater tolerance to their increased concentrations (MICs higher than 1 mg/ml, except for cadmium and mercury) of iron, cobalt, copper, aluminium and zinc. This suggests that high contamination of metals in the environment from natural and anthropogenic sources may be tolerable by the bacteria. The self-protective mechanisms displayed by \emph{L. pentosus} MP-10 as a response to metals is promoted by their architecture (cell wall and membrane) and also by their resistance determinants located on the chromosome and the plasmids. Moreover, several chromosomally encoded cation transporters (e.g., encoded by \textit{czcD} gene) have a predicted substrate range, including cadmium, cobalt and zinc; although the increased
| Gene ID     | Gene Description | GO terms                                                                 | Similarity to proteins in Lactobacillus |
|------------|------------------|--------------------------------------------------------------------------|----------------------------------------|
| XX999_0001 | XX999_00000      | hypothetical protein                                                     | 100% identity in L. plantarum 90ak    |
| XX999_0002 | XX999_00002      | DNA topoisomerase III                                                    | 100% identity in L. paraplantarum DSM 10667 |
| XX999_0003 | topB_1           | DNA topoisomerase 3                                                      | 100% identity in L. paraplantarum DSM 10667 |
| XX999_0004 | topB_2           | DNA topoisomerase 3                                                      | 98% identity in L. pentosus IG1       |
| XX999_0005 | XX999_00005      | hypothetical protein                                                     | 100% identity in L. plantarum 1p1610  |
| XX999_0006 | XX999_00006      | hypothetical protein                                                     | 100% identity in L. sakei WkKim0063   |
| XX999_0007 | XX999_00007      | hypothetical protein                                                     | 100% identity in L. pentosus          |
| XX999_0008 | traG_1           | Conjugation transfer protein TraG                                         | 99% identity in L. kefirtranslaciens subsp. kefirtranslaciens DSM 5016 |
| XX999_0009 | XX999_00009      | hypothetical protein                                                     | 96% identity in L. fermentum MTC2 8711 |
| XX999_0010 | XX999_0010       | hypothetical protein                                                     | 97% identity in L. paraplantarum      |
| XX999_0011 | XX999_0011       | hypothetical protein                                                     | 91% identity in L. plantarum          |
| XX999_0012 | XX999_0012       | hypothetical protein                                                     | 99% identity in L. brevis DmCS_003    |
| XX999_0013 | XX999_0013       | hypothetical protein                                                     | 99% identity in L. brevis KB290       |
| XX999_0014 | XX999_0014       | hypothetical protein                                                     | 98% identity in L. plantarum Nizo2239 |
| XX999_0015 | XX999_0015       | hypothetical protein                                                     | 99% identity in L. parabuchneri DSM 15352 |
| XX999_0016 | XX999_0016       | hypothetical protein                                                     | 100% identity in L. plantarum 2023    |
| XX999_0017 | XX999_0017       | hypothetical protein                                                     | 100% identity in L. plantarum CMPG5300 |
| XX999_0018 | XX999_0018       | hypothetical protein                                                     | 100% identity in L. plantarum Nizo2239 |
| XX999_0019 | XX999_0019       | hypothetical protein                                                     | 98% identity in L. paracollinoides DSM 15502 |
| XX999_0020 | XX999_0020       | hypothetical protein                                                     | 100% identity in L. parakefiri JCM 8573 |
| XX999_0021 | mobA_1           | Mobilization protein A                                                   | 100% identity in L. pentosus         |
| XX999_0022 | XX999_0022       | hypothetical protein                                                     | 100% identity in L. pentosus         |
| XX999_0023 | XX999_0023       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0024 | XX999_0024       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0025 | XX999_0025       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0026 | XX999_0026       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0027 | XX999_0027       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0028 | XX999_0028       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0029 | XX999_0029       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0030 | XX999_0030       | hypothetical protein                                                     | 100% identity in L.                  |
| XX999_0031 | dpmM             | Modification methylase DpnIA                                             | 100% identity in L.                  |
| XX999_0032 | bin3_1           | Putative transposon Tn552 DNA-invertase bin3                             | 100% identity in L.                  |
| XX999_0033 | XX999_0033       | FRG domain protein                                                       | 100% identity in L.                  |
| XX999_0034 | hrsA_1           | putative transport protein HrsA                                           | 100% identity in L.                  |
| XX999_0035 | XX999_0035       | putative hydrolase                                                       | 100% identity in L.                  |
| XX999_0036 | XX999_0036       | hypothetical protein                                                     | 100% identity in L.                  |

**Note:** The table above lists the gene IDs, their descriptions, and their similarity to proteins in Lactobacillus species. The GO terms column indicates the biological processes, molecular functions, and cellular components associated with the gene products. The similarity to proteins in Lactobacillus is indicated with the percentage identity and the specific strain or species the identity was observed in. The table is an excerpt from a scientific report, and the full content would include additional details and context not shown here.
| Gene ID  | Gene    | Position | Strand | Gen length (bp) | Protein description                                                                 | GO terms                                                                                   | Similarity to proteins in Lactobacillus |
|----------|---------|----------|--------|----------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|------------------------------------------|
| XX999_00037 | XX999_00037 | 29820–30473 | +      | 654            | S-adenosyl-L-homocysteine hydrolase                                                      | Adenosylhomocysteine activity (MF); cytoplasm (CC); one-carbon metabolic process (BP)         |                                          |
| XX999_00038 | XX999_00038 | 31017–32141 | –      | 1125           | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00039 | XX999_00039 | 32145–32360 | –      | 216            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00040 | topB_3   | 32482–34617 | –      | 2136           | DNA topoisomerase 3                                                                     | Magnesium ion binding (MF); DNA binding (MF); DNA topoisomerase type I activity (MF); DNA topological change (BP); DNA recombination (BP); chromosome separation (BP) |                                          |
| XX999_00041 | XX999_00041 | 34624–35034 | –      | 411            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00042 | XX999_00042 | 35050–35907 | –      | 858            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00043 | XX999_00043 | 35913–36287 | –      | 375            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00044 | traG_2   | 36302–37846 | –      | 1545           | Conjugation transfer protein TraG                                                         | Conjugation (BP); DNA binding (MF); plasma membrane (CC); integral component of membrane (CC) |                                          |
| XX999_00045 | XX999_00045 | 37890–38060 | –      | 171            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00046 | XX999_00046 | 38076–38546 | –      | 471            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00047 | XX999_00047 | 38549–38917 | –      | 369            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00048 | XX999_00048 | 38904–39521 | –      | 618            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00049 | XX999_00049 | 39536–40690 | –      | 1155           | Bacteriophage peptidoglycan hydrolase                                                    | —                                                                                           |                                          |
| XX999_00050 | XX999_00050 | 40691–42004 | –      | 1314           | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00051 | XX999_00051 | 42001–42108 | –      | 108            | hypothetical protein                                                                    | —                                                                                           |                                          |
| XX999_00052 | XX999_00052 | 42101–43795 | –      | 1695           | AAA-like domain protein                                                                  | —                                                                                           |                                          |

Table 5. Genes determined in pLPE-4 plasmid of Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives. BP, biological process; CC, cellular component; MF, molecular function.

resistance towards different metals are displayed by plasmids (especially the pLPE-5 plasmid). Similar results were obtained by van Kranenburg et al., which reported that the plasmid-borne (pWCFS103) cadC gene coding for a transcription regulator of the cadmium operon was responsible of the increased resistance to cadmium in L. plantarum WCFS1. Furthermore, the synteny of arcs genes in both L. pentosus MP-10 and L. plantarum WCFS1 was similar suggesting their evolutionary relatedness. Arsenic and cadmium are among the most toxic elements widely occurring in the environment, often a threat to food and water supply. Arsenic is known as a group A “known” carcinogen according to the United States Environmental Protection Agency (USEPA) and contributes to a range of other illnesses such as cardiovascular and peripheral vascular diseases, neurological disorders, diabetes mellitus and chronic kidney disease. Detoxification of this metal was earlier established by bacteria. Thus, tolerance of L. pentosus MP-10 is necessary to prevent damage to their cells.

The ability of L. pentosus MP-10 to bind different metals was demonstrated by SEM and EDX analysis. This is of great importance with regards to their application as an adjunct to improve food safety and quality by bio-remediating metals and probiotically reduce metal toxicity among human intestinal microbiota and thus protecting the host. Also, we demonstrated that L. pentosus MP-10 contributed to metal removal, especially mercury and cadmium (81 and 67%, respectively).

Metal- and antibiotic-resistance genes often co-exist on the same plasmid, however in this case, we did not find any genes coding for clindamycin resistance on plasmids, which was the only antibiotic with different susceptibility after plasmid curing. Thus, clindamycin resistance in L. pentosus MP-10 may rely on other plasmid-associated genes that we could not deciphered yet.

Conclusions

In-silico analysis of L. pentosus MP-10 plasmids suggests that plasmid-borne genes mediate the persistence of lactobacilli under olive-fermentation conditions and enhance their probiotic properties with genes encoding for carbohydrate metabolism, defense mechanisms, metal tolerance and mobilization increasing subsequently its competitiveness and survival in several ecological niches. Plasmid curing demonstrated the role of plasmids in the increased metal tolerance, and bioremoval of several metals (e.g., iron, aluminium, cobalt, copper, zinc, cadmium and mercury). This probiotic property by L. pentosus MP-10 should be exploited to detoxify metals in intestines; basically they could bioquench the metals in the gut thus reducing their toxic exposure to humans and animals, in the food matix and in environmental bioremediation.

Materials and Methods

Bacteria and growth conditions. Lactobacillus pentosus MP-10 isolated from naturally-fermented Aloreña green table olives‘ were cultured in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) at 37 °C for 24 h. Pathogenic bacteria used in this study included Listeria innocua CECT 910, Staphylococcus aureus CECT 4468, Escherichia coli CCUG 47553, and Salmonella Enteritidis UJ3449, which were cultured in Tryptone...
| Gene ID | Gene | Position | Strand | Gen length (bp) | Protein description | GO terms | Similarity to proteins in *Lactobacillus* |
|---------|------|----------|--------|----------------|---------------------|----------|---------------------------------------|
| XX999_03555 | XX999_03555 | 763–1230 | + | 468 | Hypothetical protein | — | 95% identity in *L. plantarum Nizo2814* |
| XX999_03554 | XX999_03554 | 1634–1915 | + | 282 | Bifunctional antitoxin/transcriptional repressor RelB | DNA binding (MF); transcription, DNA-templated (BP); regulation of transcription, DNA-templated (BP) | 99% identity in *L. plantarum* |
| XX999_03555 | XX999_03555 | 1956–2147 | + | 192 | Hypothetical protein | — | 98% identity in *L. plantarum Nizo2814* |
| XX999_03556 | XX999_03556 | 2224–2502 | − | 279 | Hypothetical protein | — | 100% identity in *L. farraginis DSM 18382* |
| XX999_03557 | XX999_03557 | 2525–2734 | − | 210 | Hypothetical protein | — | 100% identity in *L. delivorans DSM 14421* |
| XX999_03558 | mobA_6 | 3004–5061 | + | 2058 | Mobilization protein A | Conjugation (BP); DNA binding (MF); DNA-directed RNA polymerase activity (MF); DNA topoisomerase type I activity (MF); cytoplasm (CC); metal ion binding (MF) | 99% identity in *L. plantarum 2025* |
| XX999_03559 | XX999_03559 | 5168–5461 | + | 294 | Hypothetical protein | — | 100% identity in *L. plantarum 2025* |
| XX999_03560 | XX999_03560 | 5501–6115 | + | 615 | Hypothetical protein | — | 100% identity in *L. plantarum* |
| XX999_03561 | XX999_03561 | 6117–6455 | + | 339 | Hypothetical protein | — | 100% identity in *L. plantarum 2025* |
| XX999_03562 | XX999_03562 | 6476–6838 | + | 363 | Hypothetical protein | — | 100% identity in *L. plantarum IPLA88* |
| XX999_03563 | XX999_03563 | 6807–7466 | + | 660 | Hypothetical protein | — | 100% identity in *L. plantarum CMPG5300* |
| XX999_03564 | XX999_03564 | 7478–9496 | + | 2019 | AAA-like domain protein | Conjugation (BP); plasma membrane (CC) | 99% identity in *L. plantarum* |
| XX999_03565 | XX999_03565 | 9489–10904 | + | 1416 | Hypothetical protein | — | 99% identity in *L. plantarum TMW 1.25 pl125–4* |
| XX999_03566 | XX999_03566 | 10906–12075 | + | 1170 | Bacteriophage peptidoglycan hydrolase | — | 99% identity in *L. paraplantarum* |
| XX999_03567 | XX999_03567 | 12090–12767 | + | 618 | Hypothetical protein | — | 100% identity in *L. plantarum 2025* |
| XX999_03568 | XX999_03568 | 12685–13059 | + | 375 | Hypothetical protein | — | 99% identity in *L. plantarum Nizo2814* |
| XX999_03569 | XX999_03569 | 13060–13518 | + | 459 | Hypothetical protein | — | 100% identity in *L. plantarum 2025* |
| XX999_03570 | trcG_3 | 13515–15044 | + | 1530 | Conjugal transfer protein TraG | Conjugation (BP); DNA binding (MF); plasma membrane (CC); integral component of membrane (CC) | 99% identity in *L. plantarum* |
| XX999_03571 | XX999_03571 | 15057–15470 | + | 414 | Hypothetical protein | — | 99% identity in *L. plantarum Nizo2814* |
| XX999_03572 | XX999_03572 | 15483–16352 | + | 870 | Hypothetical protein | — | 99% identity in *L. L. plantarum Nizo2814* |
| XX999_03573 | topR_5 | 16369–18507 | + | 2139 | DNA topoisomerase 3 | Magnesium ion binding (MF); DNA binding (MF); DNA topoisomerase type I activity (MF); DNA topological change (BP); DNA recombination (BP); chromosome separation (BP) | 98% identity in *L. plantarum SRCM1101060* |
| XX999_03574 | XX999_03574 | 18629–18844 | + | 216 | Hypothetical protein | — | 100% identity in *L. plantarum Nizo1838* |
| XX999_03575 | XX999_03575 | 18848–19039 | + | 192 | Hypothetical protein | — | 83% identity in *L. collinoides* |
| XX999_03576 | XX999_03576 | 18993–19250 | + | 258 | Hypothetical protein | — | 99% identity in *L. plantarum Nizo2029* |
| XX999_03577 | aexA1_3 | 19530–20243 | + | 714 | Acetylxylin esterase precursor | Xylan catabolic process (BP); acetylxylin esterase activity (MF) | 100% identity in *L. plantarum Nizo2029* |
| XX999_03578 | XX999_03578 | 20326–20994 | − | 669 | Integrase core domain protein | — | 100% identity in *L. tuccti DSM 20183* |
| XX999_03579 | XX999_03579 | 20957–21163 | − | 207 | Hypothetical protein | — | 100% identity in *L. brevis 47 f* |
| XX999_03580 | ldh_7 | 21343–22305 | + | 963 | L-lactate dehydrogenase | L-lactate dehydrogenase activity (MF); cytoplasm (CC); glycolytic process (BP); cellular carbohydrate metabolic process (BP) | 100% identity in *L. plantarum* |
| XX999_03581 | XX999_03581 | 22735–22869 | + | 135 | Hypothetical protein | — | 95% identity in *L. backii TMW 1.1991* |
| XX999_03582 | XX999_03582 | 23089–23295 | − | 207 | Hypothetical protein | — | 100% identity in *L. brevis 47 f* |
| XX999_03583 | ldh_8 | 23475–24437 | + | 963 | L-lactate dehydrogenase | L-lactate dehydrogenase activity (MF); cytoplasm (CC); glycolytic process (BP); cellular carbohydrate metabolic process (BP) | 100% identity in *L. plantarum* |
| XX999_03584 | XX999_03584 | 24867–25001 | + | 135 | Hypothetical protein | — | 95% identity in *L. backii TMW 1.1991* |
| XX999_03585 | XX999_03585 | 24998–25501 | − | 504 | Transposase DDE domain protein | — | 100% identity in *L. plantarum subsp. plantarum P-8* |

Continued
| Gene ID      | Gene        | Position | Strand | GO terms                                                                 | Similarity to proteins in Lactobacillus |
|-------------|-------------|----------|--------|---------------------------------------------------------------------------|----------------------------------------|
| XX999_03586 | XX999_03586 | 25459–25797 | –      | 339 Hypothetical protein                                                  | 98% identity in L. plantarum IPLA8     |
| XX999_03587 | XX999_03587 | 26046–26384 | –      | 339 Hypothetical protein                                                  | 100% identity in L. plantarum IPLA8     |
| XX999_03588 | XX999_03588 | 26499–27041 | +      | 543 Hypothetical protein                                                  | 100% identity in L. pentosus           |
| XX999_03589 | XX999_03589 | 27059–27859 | +      | 801 Adenylate and Guanylate cyclase catalytic domain protein                | 100% identity in L. pentosus           |
| XX999_03590 | adeE_3      | 27925–28629 | +      | 705 Demethylmenaquinone methyltransferase                                 | 84% identity in L. parakeferi DSM 10518|
| XX999_03591 | XX999_03591 | 28680–28781 | +      | 102 Hypothetical protein                                                  | 100% identity in L. parakeferi DSM 10518|
| XX999_03592 | XX999_03592 | 28794–29021 | +      | 228 ASCH domain protein                                                   | 100% identity in L. plantarum Nizo2029  |
| XX999_03593 | XX999_03593 | 29337–30368 | +      | 1032 Integrate core domain protein                                         | 99% identity in L. plantarum WCFS1     |
| XX999_03594 | XX999_03594 | 30453–31067 | –      | 615 Cadmium resistance transporter                                        | 100% identity in L. plantarum SF2A35B  |
| XX999_03595 | cadC        | 31069–31437 | –      | 369 putative positive regulator of cadmium resistance                     | 100% identity in L. plantarum WCFS1     |
| XX999_03596 | npr_2       | 31787–33187 | +      | 1401 NADH peroxidase                                                      | 100% identity in L. plantarum Nizo1839  |
| XX999_03597 | XX999_03597 | 33361–33615 | +      | 255 Hypothetical protein                                                  | —                                       |
| XX999_03598 | XX999_03598 | 33533–33862 | –      | 330 Hypothetical protein                                                  | 100% identity in L. plantarum WCFS1     |
| XX999_03599 | arsB        | 33879–35174 | –      | 1296 Arsical pump membrane protein                                        | 99% identity in L. plantarum SF2A35B    |
| XX999_03600 | arsA        | 35233–36963 | –      | 1731 Arsenical pump-driving ATPase                                        | 100% identity in L. plantarum WCFS1     |
| XX999_03601 | arsD        | 37047–37409 | –      | 363 Arsenical resistance operon trans-acting repressor ArsD               | 100% identity in L. plantarum WCFS1     |
| XX999_03602 | arsR_3      | 37396–37755 | –      | 360 Arsenical resistance operon repressor                                | 100% identity in L. plantarum WCFS1     |
| XX999_03603 | pinR        | 39098–39679 | +      | 582 Putative DNA-invertase from lambdoid prophage Rac                    | 100% identity in L. backii TMW 1.1992   |
| XX999_03604 | bin3_3      | 40077–40709 | +      | 633 Putative transposon Tn552 DNA-invertase bin3                          | 100% identity in L. backii TMW 1.1992   |
| XX999_03605 | XX999_03605 | 40806–41168 | +      | 363 Hypothetical protein                                                  | 98% identity in L. backii TMW 1.1992    |
| XX999_03606 | XX999_03606 | 41577–41990 | –      | 414 Hypothetical protein                                                  | 100% identity in L. backii TMW 1.1992   |
| XX999_03607 | parA        | 41987–42871 | –      | 885 Chromosome partitioning protein ParA                                  | 100% identity in L. hokkaidonensis ICM 18461|
| XX999_03608 | XX999_03608 | 43459–44988 | +      | 1530 Hypothetical protein                                                 | 100% identity in L. backii TMW 1.1992   |
| XX999_03609 | XX999_03609 | 45128–45235 | –      | 108 Hypothetical protein                                                  | —                                       |
| XX999_03610 | XX999_03610 | 45885–46475 | –      | 591 Transposase, Mutator family                                          | 99% identity in L. brevis TMW 1.2113    |

**Table 6.** Genes determined in pLPE-5 plasmid of *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives. BP, biological process; CC, cellular component; MF, molecular function.

Soya Broth (TSB; Fluka, Madrid, Spain) at 37°C for 24 h. Cultures were maintained in 20% glycerol at −20°C and −80°C for short- and long-term storage, respectively.

**In silico analysis of *L. pentosus* MP-10 plasmid sequences.** The genome sequence of *L. pentosus* MP-10 consisted of a single circular chromosome of 3,698,214 bp, with an estimated mol% G + C content of...
Figure 2. MAUVE visualization of the alignment of the pLPE-5 plasmid from *L. pentosus* MP-10 with the pWCF1303 plasmid from *L. plantarum* WCFS1. Arsenic- and cadmium-resistance genes are indicated.

| Antibiotic          | MIC (µg/ml) |
|---------------------|-------------|
|                      | *L. pentosus* MP-10 (uncured) | *L. pentosus* MP-10C (cured) |
| Amoxicillin         | 0.2         | 0.2         |
| Ampicillin          | 2           | 2           |
| Chloramphenicol     | 8           | 8           |
| Ciprofloxacin       | 16          | 16          |
| Clindamycin         | 2           | 0.1         |
| Gentamicin          | 0.1         | 0.1         |
| Kanamycin           | 4           | 4           |
| Streptomycin        | 4           | 4           |
| Teicoplanin         | 256         | 256         |
| Tetracycline        | 16          | 16          |
| Trimethoprim        | 0.125       | 0.125       |
| Trimethoprim/sulfometoxazole | 0.125/2.38 | 0.125/2.38 |
| Vancomycin          | 2048        | 2048        |

| Biocide             | MIC (µg/ml) |
|---------------------|-------------|
| Benzalconium Chloride | 2           | 2           |
| Triclosan           | 32          | 32          |

| Phenolic compounds | MIC (µg/ml) |
|--------------------|-------------|
| Auto-aggregation   | 20.58 ± 2.54a | 13.49 ± 0.54a |
| Co-aggregation + *L. innocua* CECT 910 (%) | 32.87 ± 2.14a | 36.13 ± 2.33a |
| Co-aggregation + *S. aureus* CECT 4468 (%) | 28.61 ± 0.99a | 28.69 ± 0.72a |
| Co-aggregation + *E. coli* CCUG 47553 (%) | 16.14 ± 2.09a | 14.15 ± 3.24a |
| Co-aggregation + *S. Enteritidis* UJ 3449 (%) | 12.27 ± 1.50a | 13.17 ± 2.87a |
| Acid tolerance pH 2.0 (%) | 100 ± 0.04a | 100 ± 0.01a |
| Acid tolerance pH 2.5 (%) | 100 ± 0.03a | 100 ± 0.02a |
| Acid tolerance pH 3.0 (%) | 100 ± 0.01a | 100 ± 0.02a |
| Bile tolerance at 1% | + | + |
| Bile tolerance at 2% | + | + |
| Bile tolerance at 3% | + | + |
| Bile tolerance at 4% | + | + |
| Mucin adhesion (%) | 55.93 ± 0.34a | 51.92 ± 1.06a |

Table 7. Antibiotic and biodice susceptibility, and probiotic properties of cured and uncured *L. pentosus* MP-10 isolated from Aloreña Green table olives. ±SD, standard deviations of three independent experiments. *Different lowercase letters represent significant differences according to 2-sided Tukey’s HSD between strains (p < 0.05). +, Presence of growth in MRS-agar with different concentrations of bile salts.
In vitro analysis of *L. pentosus* MP-10 plasmid properties. 

Plasmid curing. First, we determined the minimum inhibitory concentrations (MIC) of acridine orange (AO) to *L. pentosus* MP-10 using the broth micro-dilution method. Overnight cultures, grown in MRS broth at 37 °C for 24 h, were diluted 1/10 (v/v) in fresh MRS broth and 20 μl were added to each well of 96-well microtiter plates. 180 μl of MRS broth supplemented with AO at different concentrations (12.5–400 μg/ml) were then added to the wells and incubated at 37 °C under aerobic conditions for 24 h. Bacterial growth was evaluated by the presence of turbidity. MIC was defined as the lowest concentration of AO that inhibited visible growth. Each experiment was done in triplicate.

Plasmid curing (eliminating the plasmid from cells) of *L. pentosus* MP-10 was done as described by Adeyemo and Onilude with some modifications. Briefly, MRS broth (4 ml) supplemented with the sub-MIC of AO, as determined in this study, was inoculated with a selected colony of *L. pentosus* MP-10 grown onto MRS agar; then the cultures were incubated at 37 °C for 72 h. Serial dilutions of bacterial cultures in NaCl (0.85%) were plated onto MRS agar, and the resulting colonies, obtained after incubation for 48 h at 37 °C, were inoculated into MRS broth to obtain a pure culture. Cultures were maintained in 20% glycerol at −20 °C and −80 °C for short- and long-term storage, respectively.

To confirm that the resulting colonies were cured of plasmids, bacterial cultures (uncured and cured) were subjected to plasmid isolation as described by Abriouel, *et al.* and visualized on 0.8% agarose gel electrophoresis (iNtRON Biotechnology) in 1×TBE (Tris-Boric acid-EDTA).

In addition to plasmid isolation, plasmid-borne genes were amplified by PCR using the Prokka annotation pipeline, version 1.11 (Seemann, 2014) as previously reported by Abriouel, *et al.* The predicted CDSs of plasmids were annotated by using BLAST (Basic Local Alignment Search Tool) and the associated GO (Gene Ontology) terms were obtained using Swiss-Prot database.

The general metabolic pathways of *L. pentosus* MP-10 plasmids were reconstructed using BlastKOALA (last updated March 4, 2016) as part of the KEGG (Kyoto Encyclopedia of Genes and Genome) tool in the pathway database (http://www.genome.jp/kegg/pathway.html) for annotating genomes; here, we used the annotated genes predicted in each *L. pentosus* MP-10 plasmid as the input query.

To evaluate the alignment and the synteny of genes between the *L. pentosus* MP-10 and *L. plantarum* WCFS1 plasmid data sets, comparison was done by using Mauve algorithm in Lasergene’s MegAlign Pro software (Lasergene 14).

| Metal          | MIC (μg/ml) | L. pentosus MP-10 (uncured) | L. pentosus MP-10C (cured) |
|----------------|-------------|-----------------------------|---------------------------|
| Mercury (Hg)   | 2           | 1                           |                           |
| Cobalt (Co)    | 2048        | 2048                        |                           |
| Copper (Cu)    | 2048        | 2048                        |                           |
| Zinc (Zn)      | 1024        | 1024                        |                           |
| Aluminium (Al) | 2048        | 2048                        |                           |
| Iron (Fe)      | 4096        | 4096                        |                           |
| Cadmium (Cd)   | 8           | 1                           |                           |

Table 8. Tolerance of cured and uncured *L. pentosus* MP-10 isolated from Aloreña Green table olives to heavy metals. ±SD, standard deviations of three independent experiments. *Different lowercase letters represent significant differences according to 2-sided Tukey’s HSD between strains (p < 0.05).*

46.32% and 5 plasmids ranging 29–46 kb (accession numbers FLYG01000001 to FLYG01000006) were annotated using the Prokka annotation pipeline, version 1.11 (Seemann, 2014) as previously reported by Abriouel, *et al.* The predicted CDSs of plasmids were annotated by using BLAST (Basic Local Alignment Search Tool) and the associated GO (Gene Ontology) terms were obtained using Swiss-Prot database.

The general metabolic pathways of *L. pentosus* MP-10 plasmids were reconstructed using BlastKOALA (last updated March 4, 2016) as part of the KEGG (Kyoto Encyclopedia of Genes and Genome) tool in the pathway database (http://www.genome.jp/kegg/pathway.html) for annotating genomes; here, we used the annotated genes predicted in each *L. pentosus* MP-10 plasmid as the input query.

To evaluate the alignment and the synteny of genes between the *L. pentosus* MP-10 and *L. plantarum* WCFS1 plasmid data sets, comparison was done by using Mauve algorithm in Lasergene’s MegAlign Pro software (Lasergene 14).
Effect of plasmid curing on growth, safety and functional properties of *L. pentosus* MP-10.

**Growth properties.** To test whether there is any differences in growth between the uncured and the cured *L. pentosus* MP-10 strains, MRS broth was inoculated (1% v/v) with overnight cultures of each strain and then incubated at 37 °C for 24 h. Growth rates (OD600nm) were measured each hour using Microtiter plate reader (iMark Microplate Absorbance Reader, Bio-Rad instrument). Additionally, we measured pH at different time intervals (following 0, 8 and 24 h of incubation at 37 °C).

To determine the effect of pH on the growth of both strains, MRS broth was adjusted to different pH ranges (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) with phosphate buffer, and they were inoculated (1% v/v) overnight cultures of both strains and then incubated at 37 °C for 24 h, as described above.

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**Figure 3.** SEM (A,C,E,G,I,K,M,O) and EDX (B,D,F,H,J,L,N,P) analysis of uncured *L. pentosus* MP-10 without metal (A,B) and with Al (C,D), Cd (E,F), Co (G,H), Cu (I,J), Fe (K,L), Hg (M,N) and Zn (O,P).
To test whether brine conditions had an effect on the growth of the plasmid-cured versus uncured *L. pentosus* MP-10 strains in MRS broths under the following experimental conditions: unsupplemented vs. those supplemented with either 6.5% (or high concentration of 8%) NaCl or phenolic compounds, or modified MRS broth (without glucose) added with xylan (5 g/l) were inoculated with both strains as described above. Phenolic compounds were obtained from previously pulverized olive leaves using RETSCH laboratory ball mills (Retsh MM 400). The leaf extracts were resuspended in LSM broth, centrifuged and the resulting supernatant was filtered (0.45 µm) and added at different concentrations (0.780 to 200 mg/ml) to MRS broth. The cultures were incubated at 37 °C for 24 h and the OD₆₀₀nm was measured as described above.

In all cases, experiments were done in triplicate.

**Evaluation of metal tolerance.** The sensitivity of both *L. pentosus* strains (MP-10 and MP-10C (cured)) towards metals: cadmium (CdSO₄·8H₂O), cobalt (CoCl₂), copper (CuCl₂·2H₂O), iron (FeSO₄·7H₂O), mercury (HgCl₂), aluminium (Al₂O₃), or zinc (ZnCl₂) was tested in LSM broth supplemented with 0 to 10 mg/ml of each metal and then inoculated with 2% (v/v) of an overnight culture of each strain. After 24 h of incubation at 37 °C, the MIC that completely inhibited visible growth.

To analyse the removal of metals by cured and uncured *L. pentosus* MP-10, MRS broth supplemented with ½MIC of each metal was inoculated with 2% (v/v) of an overnight culture of each strain. After 24 h at 37 °C, the MIC added: Fe at 2 mg/ml; Al, Co and Cu at 1 mg/ml; Zn at 0.5 mg/ml; Cd at 4 µg/ml and Hg at 1 µg/ml and 0.5 µg/ml were considered “100%” baselines to calculate relative metal removal rates (as a percentage).

Metal concentrations were measured using 7900 ICP-Mass Spectrometer (Agilent, USA) with graphite tube atomizer and autosampler, a superior matrix tolerance and advanced collision/reaction cell (CRC) technology to remove the polyatomic interferences that can affect some of the trace elements. The spectrometer software was Agilent ICP-MS MassHunter Work Station, which provides simple autotuning functions, and a Method Wizard automates the method setup process.

Biosorption of metals by *L. pentosus* MP-10 was further examined using scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy before and after metal uptake. For this, a drop of the bacterial pellet, which had been previously exposed to a metals (as previously described), were disposed into microporous capsules (ANAME, Spain), dried and then dehydrated in a series of 20, 40, 60, 80, and 100% ethanol solutions (15 min each) before suspension in acetone for 1 h. After this, the capsules were subjected to critical-point drying before examination by SEM (FESEM, MERLIN de Carl Zeiss, Oxford).

**Safety and probiotic properties.** To determine differences in antimicrobial (antibiotic and biocide) susceptibility of *L. pentosus* MP-10C versus wild strain, we determined the MIC of several antimicrobials following the method previously described by Casado Muñoz, et al. using LSM broth (Oxoid).

To determine if plasmids further play a role in several probiotic properties, we analyzed acid- and bile- tolerances, auto-aggregation, co-aggregation with pathogens (*L. innocua* CECT 910, *S. aureus* CECT 4468, *E. coli* CCUG 47553, and *S. Enteritidis* UJ3449) and mucin adhesion in both *L. pentosus* strains (MP-10 and MP-10C) according to the methods reported by Pérez Montoro et al. 35.

**Gene expression analysis.** To analyse the role of plasmid in several metabolic and probiotic properties, both the uncured and cured *L. pentosus* strains were subjected to RNA extraction using Direct-zol™ RNA Miniprep (Zymo Research, California, USA) according to the manufacturer’s instructions. RNA quantification and quality assessment were carried out by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNAs were adjusted to a concentration of 500 ng/ml and frozen at −80 °C until required for analysis.

The expression of selected genes (Table S1) was determined by quantitative, real-time PCR (qRT-PCR) using SensiFAST™ SYBR & Fluorescein One-Step Kit (BIOLINE) as reported in Pérez Montoro et al. 35.

**Statistical analysis.** All analyses were performed in triplicate. Statistical descriptors were calculated using Excel 2007 (Microsoft Corporation, Redmond, Washington, US), e.g., determining averages and standard deviations. Statistical comparison of growth and probiotic properties assays were conducted by analysis of variance (ANOVA) using Statgraphics Centurion XVI software (Statpoint Technologie, Warrenton, Virginia, US). The same software was used to perform Shapiro–Wilk and the Levene tests to check data normality and to perform 2-sided Tukey’s multiple contrast to determine the pair-wise differences between strains. Level of significance was set at *P < 0.05.*

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Author Contributions
H.A. and N.B. designed the experiments. H.A., N.B. and C.K. wrote the main manuscript text. H.A., B.P.M., J.F.O. and L.L.L. did the experiments and prepared figures and tables. All authors reviewed the manuscript.

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