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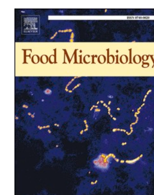

Changes in resistome profile of potential probiotic *Lactiplantibacillus pentosus* in response to edible oil adaptation

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A R T I C L E I N F O

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A B S T R A C T

Despite increasing interest to investigate horizontal gene transfer as a leading cause of antibiotic resistance spread, the resistome is not only influenced by the influx and efflux of genes in different environments. Rather, the expression of existing genes under different stress conditions requires special attention. This study determined whether pre-adapting *Lactiplantibacillus pentosus* strains, isolated from Aloreña green table olives, to vegetable-based edible oils influence their phenotypic and genotypic responses to antibiotics. This has significant diet, food matrix, gut health, and food safety concerns. Pre-adapting *L. pentosus* strains to oils significantly changed their susceptibility profile to antibiotics. However, results generally differed among the three strains; although changes in the Minimum Inhibitory Concentration (MIC) of antibiotics occurred, it depended on the *L. pentosus* strain and the oil used for adaptation. The pre-adaptation of *L. pentosus* strains with olive, sunflower, argan and linseed oils induced gene expressions (e.g., *rpsL, recA* and *uvrB*) in several stress responses. Thus, to analyze this fact in-depth, transcriptional changes were reported in the selected potential probiotic *L. pentosus* CF2-10 adapted with olive or sunflower, rerouting its metabolic pathways to export toxic molecules through efflux pumps and ABC transporters. Pre-adaptation of some lactobacilli with olive or sunflower oils may represent a novel approach for manufacturing probiotic products with improved stability, functionality and robustness.

1. Introduction

Probiotic safety has become a significant criterion and an emerging concern (Toomey et al., 2009). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Food and Agricultural Organization of the United Nations and World Health Organization, 2002; Hill et al., 2014). However, concerning safety criteria, probiotics may have antibiotic resistance, an emerging issue of great responsibility, especially the acquired resistance elements that transfer horizontally into the human and animal microbiomes (Laxminarayan, 2014). They have severe impacts and increased adverse effects on public health and the environment due to the potential spread and the accumulation of antibiotic-resistant bacteria (ABRs) and antibiotic resistance genes (ARGs) in different ecosystems as contaminants (Kraemer et al., 2019; Martínez, 2008; Van Bruggen et al., 2019). The use, abuse and misuse of antibiotics in different sectors (veterinary, agriculture and clinical therapy) generated this issue, thus allowing the continuous cycling of ABRs and ARGs between animals/humans and their environments (Lushniak, 2014; Michael et al., 2014), but this is not the sole mechanism. Biocides, as antiseptics or disinfectants in industrial and sanitary areas, also promote significant challenges to the industry, veterinary and medical settings through their cross-resistance to antibiotics (Braoudaki and Hilton, 2004; Piovesan Merchel Piovesan Pereira et al., 2021; Wand et al., 2017).

The increased emergence of antibiotic resistance (ABRs and ARGs) is not only due to antimicrobial selective pressure exerted by antibiotics and biocides, which have been primarily documented as the leading causes of antibiotic resistance occurrence, spread and persistence (Oz et al., 2014). Instead, other factors can contribute to this issue, such as

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environmental stresses (e.g., extreme temperature or pH, chemicals, and osmotic stress) (Maltas et al., 2020) frequently encountered in clinical, natural environments and food production. Thus, the horizontal gene transfer of multidrug resistance to the pathogens and commensal gut microbiota is a potential threat to public health (Courvalin, 2006); however, antibiotic resistance, either intrinsic or resultant of mutation, does not manifest a safety concern in itself. Furthermore, intrinsic resistant probiotics are helpful during the co-administration of probiotics with antibiotics during antibiotic treatment, allowing gut-microbiota restoration (Gueimonde et al., 2013; Yao et al., 2021).

The fermentation process of table olives is recognized as a stressful environment due to its physicochemical conditions such as high salt concentration, low pH, polyphenols, and some condiments with antimicrobial activity (Borges et al., 2013; Thielmann et al., 2017). In this sense, lactic acid bacteria (LAB), isolated during the natural fermentation process of Aloreña table olives (Abriouel et al., 2012; Casado Muñoz et al., 2014), were resistant to physicochemical conditions and at least three antibiotics. However, these resistances relied on intrinsic mechanisms that were non-transferable or non-acquired determinants; these included chromosomally encoded efflux pumps (NorA, MepA and MdeA). Therefore, according to Qualified Presumption of Safety criteria, they were considered safe for future applications as starter cultures or probiotics.

Among these LAB, some *Lactiplantibacillus pentosus* strains exhibited probiotic potential (Abriouel et al., 2012; Pérez Montoro et al., 2016), and their robustness and functionality were improved after several stresses such as antimicrobials, chemicals and UV light, showing changes in susceptibility patterns to antibiotics and the associated expressed genes (Casado Muñoz et al., 2016a and b). Similarly, the adaptation of *L. pentosus*, isolated from Aloreña table olives, with vegetable-based edible oils showed increased survivability, stress resistance (acid and bile) and functionality through transcriptional network switch to regulate robustness, stability and functionality (Alonso García et al., 2019, 2021). However, no data are available about the changes induced by vegetable-based edible oils on the antibiotic-resistance profile of these bacteria. Taking into consideration the safety of potential probiotic *L. pentosus* under different conditions in the food matrix, in the gut and in the presence of different diet components, the present study focused on the molecular mechanisms underlying oil effects on *L. pentosus* antibiotic resistance at a transcriptomic level and identify the bacterial mechanisms involved. It is noteworthy to highlight that this is the first study dealing with the determination of resistome change of potential probiotic bacteria as a response to vegetable edible-oils present in the diet, probiotic formulation or dietary supplements.

On the other hand, this study will shed light on the potential of other bacteria possessing transferable resistance genes. Exposure to this stress significantly contributes to the spread of antibiotic resistance genes in natural environments. Nevertheless, further studies are required.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Three strains (CF1-6, CF2-10 and MP-10) of *Lactiplantibacillus pentosus* (previously *Lactobacillus pentosus*) isolated by Abriouel et al. (2012) from naturally-fermented Aloreña green table olives by four small-medium enterprises (SMEs) from Malaga (Spain) were selected among the best strains with the probiotic potential to be used in this study. Strains adapted to different oils: almonds, argan, corn, linseed, olive, soy and sunflower, according to Alonso García et al. (2019), were routinely cultured at 30 °C in the Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) for 24 h, and kept in 20% glycerol at −80 °C for long term storage.

2.2. Antibiotic susceptibility of oil-adapted L. pentosus strains

The antibiotic MICs in oil-adapted and non-adapted *L. pentosus* strains were determined as reported by Casado Muñoz et al. (2014) in LSM broth [90% of IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (ISO, 2010). After incubation, the MIC was read, representing the lowest concentration of each antimicrobial agent that inhibited the growth of the strain. All MIC determinations of each antimicrobial against each strain were carried out in triplicate, and reliable results were taken if at least two of the three replicates were in agreement. All antibiotics were purchased from Sigma Aldrich (Madrid, Spain).

2.3. Molecular response of oil-adapted L. pentosus

2.3.1. RNA extraction

RNA extractions were done from the non-adapted and oil-adapted *L. pentosus* CF1-6, CF2-10 and MP-10 (sunflower, olive, linseed and argan) using Direct-zol™ RNA Miniprep (Zymo Research, California, USA) according to the manufacturer's instructions. Extractions started with the addition of 500 μl of TESL (25 mM Tris, 10 mM EDTA, 20% sucrose, and 20 mg/ml lysozyme; all from Sigma) and 20 μl mutanolysin (20 U) to cell pellets, followed by incubation at 37 °C for 60 min with slight shaking. Then, 300 μl of Tri Reagent was added to the lysates and centrifuged to get the supernatants which were transferred into an RNase-free tube before proceeding to RNA purification using Direct-zol RNA Miniprep (Zymo Research, CA, United States) according to the manufacturer's instructions. Total RNA was isolated from three biological repeats. DNase digestion was performed according to the manufacturer's instructions (Zymo Research, California, USA). RNA quantification and quality assessment were performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and TapeStation. RNAs were adjusted to a 500 ng/ml concentration and frozen at −80 °C until required. RNAs were adjusted to a 2 μg/ml concentration per sample for sequencing and transcriptomic analyses.

2.3.2. Quantitative real-time PCR of stress/resistance genes after adaptation with edible oils

The expressions of stress-resistance genes (*rpsL, recA, srtA* and *uvrB*) were done as reported by Casado Muñoz et al. (2016a). For quantitative real-time PCR (qRT-PCR), mRNAs were subjected to reverse transcription into cDNA and subsequent real-time PCR in a single reaction using the SensiFast SYBR & Fluorescein One-Step kit (Bioline, Barcelona, Spain). The 16S-rRNA gene was used as a housekeeping gene, and a no template control (NTC) was used as the negative control. Primers for the 16S rRNA housekeeping gene and resistance genes were reported by Casado Muñoz et al. (2016a). Quantitative PCRs (qPCRs) were performed in triplicate on an iCycler iQ™ Real-Time PCR Detection System (BioRad, Herts, United Kingdom). PCR-grade water served as a negative control.

2.3.3. Transcriptomic analysis of oil-adapted L. pentosus CF2-10 versus non-adapted strains

2.3.3.1. RNA extraction, library preparation and RNA sequencing. Based on the results obtained in the previous section (2.3.2.), we selected *L. pentosus* CF2-10 adapted with sunflower or olive oils to deepen its molecular response regarding resistance. RNA extraction was done as described above in section 2.3.1. According to the manufacturer's instructions, the Truseq Stranded Total RNA Kit (Illumina, Inc., San Diego, CA, United States) was used to remove ribosomal RNA for library preparation. Sequencing was done on an Illumina Novaseq platform (2 × 150 bp read lengths) at Life sequencing S.L. (Valencia, Spain). First, raw sequencing reads were processed with the *reformat.sh* script from

the *BBTools* suite (Bushnell– sourceforge.net/projects/bbmap/) to remove low-quality bases (Q20) from both ends and short sequences (50 nucleotides). Filtered reads were then scanned for traces of ribosomal RNA with *SortMeRNA* v2.1 (Kopylova et al., 2012), parameters and databases by default.

2.3.3.2. Bioinformatic analysis. The resulting reads were aligned to the protein-coding transcriptome of *Lactobacillus pentosus* BGM48 (GCA_002850015.1) using Salmon v1.1 (Patro et al., 2017), parameters by default. Counts were collapsed with *tximport* v1.12.3 (Soneson et al., 2015) and analyzed with *DESeq2* v1.24.0 (Love et al., 2014) packages on R v3.6. Only genes with ten or more counts across all samples were kept for further comparisons. $P_{adj} < 0.05$ and $|\log_2 FC| > 1.5$ were the criteria for considering a gene as differentially expressed between the studied groups. Gene ontology (GO) terms were obtained from the *Lactobacillus pentosus* BGM48 page of Uniprot. Only terms with two or more counts across differentially expressed genes (DEG) of at least one comparison were used to generate the figures.

2.3.3.3. Validation of differentially expressed genes by qRT-PCR. Five genes were selected for RT-PCR experiments to verify transcriptomic results (Table 1). Total RNA was extracted from *L. pentosus* CF2-10 strains pre-adapted with either sunflower or olive oil and non-adapted strain (control) as described above. RNAs were adjusted to a 500 ng/ml concentration and frozen at −80 °C until required for analysis.

The expression of selected genes (Table 1) was analyzed by quantitative, real-time PCR (qRT-PCR) using SensiFAST™ SYBR & Fluorescein One-Step Kit (BIOLINE). Phenylalanyl-tRNA synthase alpha-subunit (*pheS*) gene was used as a housekeeping gene (Naser et al., 2005), and no-template control (NTC) was used as a negative control. Quantitative PCRs (qPCRs) were performed in triplicate, 20-μl reactions on a CFX96 TouchTM Real-Time PCR Detection System from BioRad. The qPCR conditions were as follows: pre-denaturation at 95 °C for 2 min, denaturation at 95 °C for 30 s, and annealing at 59 °C for 30 s and extension at 72 °C for 30 s; fluorescence signals were collected during annealing and extension, and the whole process was repeated for 40 cycles; final extension for 2 min. Melting-curve analysis included 95 °C for 10 s, 65 °C for 5 s, and 95 °C for 50 s.

2.4. Statistical analysis

Statistical analyses were conducted using Excel 2016 software (Microsoft Corporation, Redmond, WA, United States) to determine averages and standard deviations. All analyses were performed in triplicate. Statistics included the Analysis of Variances (*ANOVA*) by XLStat software (Addinsoft 2020.2.2; New York, United States), Shapiro–Wilk test and Levene test to check data normality, and Tukey HSD test, where $p < 0.05$ was considered statistically significant. Statistical analysis for transcriptome analysis was previously described in Section 2.3.3 "Bioinformatic Analysis".

3. Results

3.1. Phenotypic response of L. pentosus strains to oil adaptation

Phenotypic response to oil adaptation with different edible oils (almonds, argan, corn, linseed, olive, soy and sunflower) was generally different in the three *L. pentosus* strains analyzed (Table 2). An increase, decrease, or no change in the MIC of antibiotics was observed, but it highly depended on the *L. pentosus* strain and the oil used for adaptation. Generally, the MICs of ciprofloxacin, erythromycin, clindamycin, streptomycin and tetracycline increased by 2–8 fold, followed by sulfamethoxazole/trimethoprim, gentamycin, amoxicillin and ampicillin (Table 2). However, the MICs of chloramphenicol and kanamycin exhibited no changes regardless of the strain and oil adaptation (Table 2). On the other hand, we observed a decrease in MICs in a few cases for ciprofloxacin and tetracycline after adapting *L. pentosus* CF1-6 with oil (Table 2).

Concerning oils, linseed, olive, sunflower, and soy were the oils which induced the most changes in antibiotic MICs. In contrast, erythromycin, ciprofloxacin and tetracycline were the antimicrobials that exhibited MIC changes after oil adaptation (Table 1). Regarding L. *pentosus* strains, linseed and argan induced more changes in antibiotic MIC in *L. pentosus* CF1-6; however, olive, linseed and sunflower impacted *L. pentosus* CF2-10; finally, linseed and soy induced changes in antibiotic susceptibility of *L. pentosus* MP-10.

3.2. Genotypic response of stress/resistance genes in oil-adapted L. pentosus

Based on the phenotypic response of *L. pentosus* strains to oil adaptation, linseed, sunflower, olive and argan were selected to test the effect of oil adaptation on the expression of genes involved in tolerance/resistance of three potential probiotic *L. pentosus* (CF1-6, CF2-10 and MP-10) strains. For this, we analyzed the following genes: *rpsL, recA, uvrB* and *srtA*. The results showed that responses differed depending on *L. pentosus* strain, the oil used for adaptation and the gene analyzed (Fig. 1).

Pertaining to the *rpsL* gene, up-regulation was observed in *L. pentosus* CF2-10 adapted with olive (9.10 fold), argan (4.75 fold), sunflower (4.23 fold) and linseed (1.68 fold) oils; and in *L. pentosus* MP-10 adapted with argan oil (2.27 fold) (Fig. 1). Similarly, up-regulation of the *uvrB* gene was observed after adaptation of *L. pentosus* CF2-10 with sunflower (6.38 fold) and linseed (1.77 fold) oils; also in *L. pentosus* MP-10 with argan (2.75 fold) and sunflower (1.79 fold) oils (Fig. 1).

Concerning the *recA* gene, the adaptation with sunflower oil

Table 1
Primers and PCR conditions used in this study.

Gene (product)	Primer	Sequence (5′-3′)	Annealing Temperature (°C)	PCR product size (bp)	Reference
mfs (MFS transporter)	*mfs-F*	ATGCAGACAACTGGTCAAAACA	59	200	This study
	mfs-R	ACGACCATCGTCAACATAAAA			
ath (Anthranilate phosphoribosyltransferase)	*ath-F*	ATGGTTATTGCGGTGGCGA	59	213	This study
	ath-R	ACTAAAGGAAGATTGAGCATC			
ABCtr (Peptide ABC transporter substrate-binding protein)	*ABCtr-F*	ATGCAATGGAAATTATTTACAAC	59	212	This study
	ABCtr-R	ATCGTGCCACTAACCACGT			
cas2 (CRISPR-associated endonuclease Cas2)	*cas2-F*	ATGAGTATGTTGGTCGTGGT	59	217	This study
	cas2-R	ATGTCTGGAACTCAAATCCC			
bglA (6-phospho-beta-glucosidase)	*bglA-F*	ATGATAGTGAAAAAACTCACCT	59	212	This study
	bglA-R	ACGGCTTCATGGTTTGGATA			
pheS (Phenylalanyl-tRNA synthase alphasubunit)	*pheS-21F*	CAYCCNGCHCGYGAYATGC	59	411	Naser et al. (2005)
	pheS-23R	GGRTGRACCATVCCNGCHCC			

Table 2
Effect of oil pre-adaptation on antibiotic MIC in three *Lactiplantibacillus pentosus* strains isolated from naturally-fermented Aloreña green table olives.

Oil	*L. pentosus* strains	Fold increase (↗) or decrease (↘) in MIC of different antibiotics										
		AMP	AMX	CHL	CIP	CLI	ERY	GEN	KAN	SMZ/TMP	STR	TET
Almonds	CF1-6	NC	NC	NC	↘2	NC	↗2	NC	NC	↗2	NC	NC
	CF2-10	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	MP-10	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Argan	CF1-6	↗2	NC	NC	↘2	NC	↗2	NC	NC	↗2	NC	↘2
	CF2-10	NC	NC	NC	↗2	NC	↗4	NC	NC	NC	NC	NC
	MP-10	NC	NC	NC	NC	NC	↗2	NC	NC	NC	NC	NC
Corn	CF1-6	NC	NC	NC	↘2	NC	↗2	NC	NC	↗2	↗2	NC
	CF2-10	NC	NC	NC	↗2	↗2	NC	NC	NC	NC	NC	↗2
	MP-10	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Linseed	CF1-6	NC	↗2	NC	NC	NC	↗2	↗8	NC	↗2	↗2	NC
	CF2-10	NC	NC	NC	↗2	↗2	NC	NC	NC	NC	↗2	↗2
	MP-10	NC	NC	NC	NC	↗2	NC	↗2	NC	NC	NC	NC
Olive	CF1-6	↗2	NC	NC	NC	NC	↗2	NC	NC	↗2	NC	NC
	CF2-10	NC	↗2	NC	↗2	↗2	NC	NC	NC	NC	↗2	↗2
	MP-10	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Soy	CF1-6	NC	NC	NC	NC	NC	↗2	NC	NC	NC	↗2	NC
	CF2-10	NC	NC	NC	↗2	↗2	NC	NC	NC	NC	NC	↗2
	MP-10	NC	NC	NC	NC	↗2	NC	↗2	NC	NC	NC	NC
Sunflower	CF1-6	NC	NC	NC	NC	NC	↗2	NC	NC	NC	↗2	NC
	CF2-10	NC	NC	NC	↗2	↗2	NC	NC	NC	NC	↗2	↗2
	MP-10	NC	↗2	NC	NC	NC	NC	NC	NC	NC	NC	NC

Antimicrobials are: AMP, ampicillin; AMX, amoxicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, Clindamycin; ERY, erythromycin; GEN, gentamycin; KAN, kanamycin; SMZ/TMP, sulfametoxazol/Trimethoprim; STR, streptomycin; TET, tetracycline.
(↗and ↘): Strains exhibited fold increase or decrease, respectively in the corresponding antibiotic MICs comparing with controls under standard conditions. (NC): No change in MIC comparing with non-adapted strain to oil.

produced an up-regulation in *L. pentosus* CF2-10 and MP-10 strains. However, this up-regulation was also shown in argan-adapted *L. pentosus* MP-10 and olive-adapted *L. pentosus* CF1-6 (Fig. 1).

Analysis of *srtA* gene expression revealed only up-regulation in sunflower-adapted *L. pentosus* MP-10. In the rest of the cases, we observed no significant changes or down-regulation occurred, especially in *srtA* gene expression of the following cases: olive-adapted *L. pentosus* CF2-10 and MP-10, argan-adapted *L. pentosus* CF1-6, CF2-10 and MP-10, linseed-adapted *L. pentosus* CF1-6 and MP-10, and sunflower-adapted *L. pentosus* CF1-6 (Fig. 1).

3.3. Insights into the molecular response of L. pentosus CF2-10 to oil adaptation

3.3.1. Illumina NovaSeq mRNA sequencing and global transcriptomic analysis of the oil-adapted Lactiplantibacillus pentosus CF2-10

Compared to untreated control, *L. pentosus* CF2-10, adapted with sunflower or olive oil, was subjected to transcriptomic analysis. Raw data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB51051 (https://www.ebi.ac.uk/ena/browser/view/PRJEB51051), and the Pearson correlation coefficient showed a correlation of gene expression patterns among three independent biological replicates. Clean reads of the three libraries were mapped to the *L. pentosus* BGM48 reference genome. About 2735 genes from 3197 (85.55%) were successfully mapped (Tables S1, S2 and S3). Degrees of similarity were evaluated between the different samples using a PCA plot (Principal Component Analysis) (Figs. S1–A) and a heat map (Figs. S1–B); both treatment conditions showed great differences from the “control” condition (Fig. S1). Comparative analysis showed 91 (50 up-regulated and 41 down-regulated) and 78 (49 up-regulated and 29 down-regulated) differentially expressed genes (DEGs) (log_2“fold change” > 1.5 at statistically significance level $P < 0.05$) in olive-adapted (O) and sunflower-adapted (G) *L. pentosus* CF2-10 versus control (C), respectively (Tables S1 and S2). Furthermore, 49 DEGs (19 up-regulated and 30 down-regulated genes) were detected in sunflower-adapted (G) versus olive-adapted *L. pentosus* CF2-10 (Table S3).

GO terms analysis highlighted the adaptation to each oil-induced

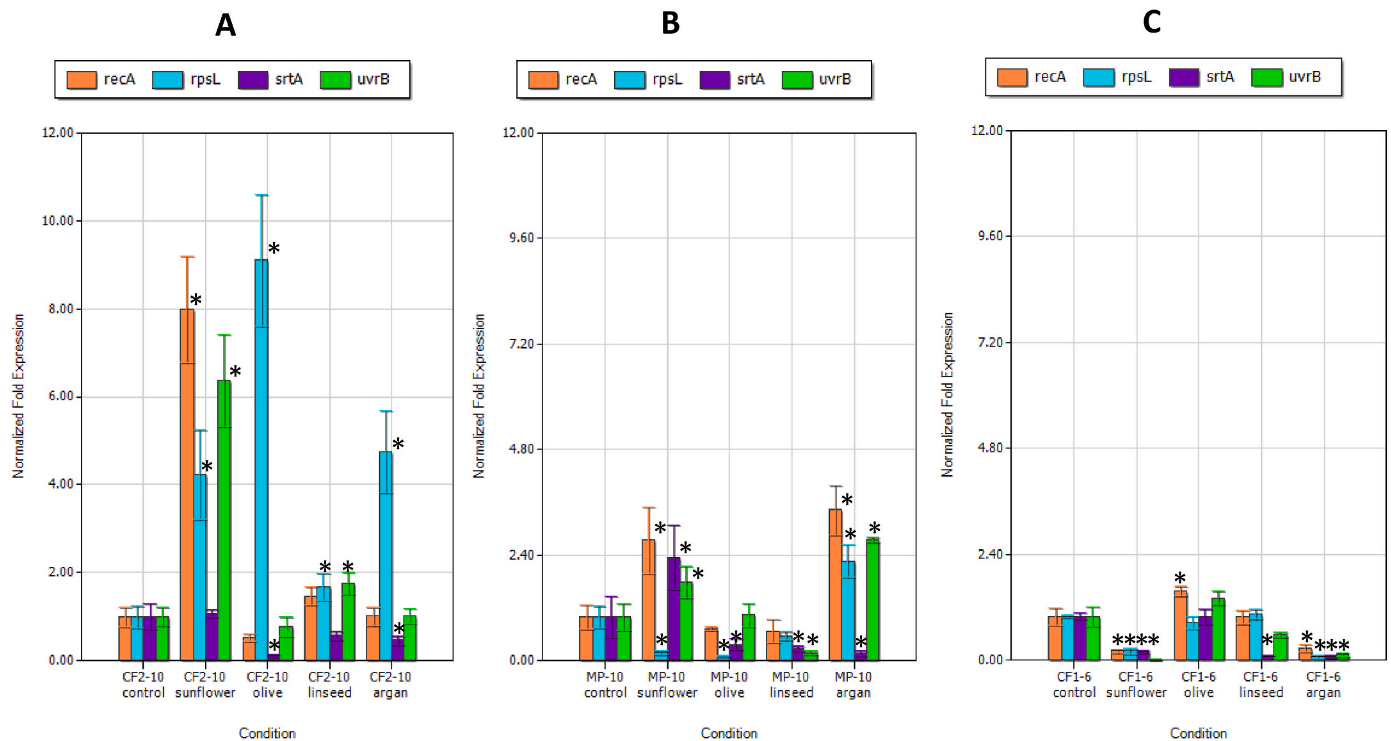

Fig. 1. The effect of oil adaptation on the expression of *rpsL, recA, uvrB* and *srtA* genes in *L. pentosus* CF2-10 (A), *L. pentosus* MP-10 (B) and *L. pentosus* CF1-6 (C). The relative expression levels were normalized to control values for the three *L. pentosus* strains for x-fold expression analysis. Each bar represents the mean value and the standard deviation as the error bar for three independent experiments. * denotes significant differences between controls and oil-adapted strain ($P < 0.05$).

change in gene expression to decipher the biological processes, cellular components, and molecular functions of oil-adapted *L. pentosus* CF2-10 (Fig. 2), i.e., those different from non-adapted controls. Comparative transcriptomics of oil-adapted *L. pentosus* CF2-10 versus non-adapted control indicated that some metabolic pathways were up-regulated in both sunflower and olive-adapted strain versus non-adapted control (Fig. 2). However, others were only up-regulated in one of the oil-adapted strains and thus are exclusively enhanced in sunflower or olive-adapted *L. pentosus* CF2-10 versus control involving the differential expression of different genes. In this sense, GO-terms analysis revealed similar up-regulated metabolic pathways in both sunflower and olive-adapted *L. pentosus* CF2-10 versus control; different DEG levels included: bacterial secretion system; RNA polymerase; glyoxylate and decarboxylase metabolism; valine, leucine and isoleucine degradation; quorum sensing; fatty acid metabolism; aminoacyl t-RNA degradation; biotin metabolism and fatty acid biosynthesis (Fig. 2). Furthermore, up- and down-regulation of the same metabolic pathways were shown for olive and sunflower-adapted *L. pentosus* CF2-10 versus control. These include: protein export; pantothenate and COA biosynthesis; DNA replication; phosphotransferase system (PTS); two-component system, biosynthesis of amino acids; carbon metabolism; microbial metabolism in diverse environments; biosynthesis of secondary metabolites; metabolic pathways; propanoate metabolism; pyruvate metabolism; starch and sucrose metabolism; purine metabolism; oxidative phosphorylation; fructose and mannose metabolism; pentose phosphate pathway; citrate cycle and glycolysis/gluconeogenesis (Fig. 2). On the other hand, in the case of sunflower-adapted *L. pentosus* CF2-10, exclusive up-regulation of nitrogen metabolism, arginine metabolism and glycerophospholipid metabolism was detected (Fig. 2). However, olive-adapted *L. pentosus* CF2-10 exclusively showed other arsenals of up-regulated metabolic pathways such as cationic antimicrobial peptide (CAMP) resistance, sulfur relay system and terpenoid backbone biosynthesis (Fig. 2).

3.3.2. Identification of DEGs involved in resistance after oil adaptation of L. pentosus CF2-10

Fig. 3 clearly shows the up-regulation of DEGs involved in the defense and antimicrobial resistance of *L. pentosus* CF2-10 after oil adaptation (sunflower or olive). In this sense, MFS (major facilitator superfamily) transporter gene was over-expressed in olive-adapted and sunflower-adapted *L. pentosus* CF2-10 by 5.21 (BB562_15125) and 4.17/1.90 (BB562_09955 and BB562_15125, two gene locus) log$_2$FC, respectively (Tables S1 and S2). Furthermore, the anthranilate phosphoribosyltransferase coding gene (BB562_09180) was over-expressed in olive-adapted *L. pentosus* CF2-10 by 4.11 log$_2$FC (Table S1), while in sunflower-adapted *L. pentosus* CF2-10 it was of 4.47 log$_2$FC (Table S2). Also, BB562_09180 gene coding for peptide ABC transporter substrate-binding was up-regulated in olive-adapted and sunflower-adapted *L. pentosus* CF2-10 by 1.77 and 1.52 log$_2$FC, respectively (Tables S1 and S2). On the other hand, CRISPR-associated endonuclease Cas2 (BB562_00540, 2.54 log$_2$FC); hypothetical proteins [(BB562_00880, 1.67 log$_2$FC, ATP binding [GO:0005524], ATPase activity [GO:0016887]); (BB562_01605, 1.54 log$_2$FC, integral component of membrane [GO:0016021]); transcriptional repressor (BB562_12770, 1.69 log$_2$FC); diguanylate cyclase (BB562_13230, 1.61 log$_2$FC); cellulose synthase (BB562_13235, 1.51 log$_2$FC); glycosyl transferase family 2 (BB562_13240, 1.67 log$_2$FC); phosphate ABC transporter substrate-binding protein (BB562_13420, 1.95 log$_2$FC); phosphonoacetaldehyde hydrolase (BB562_13500, 1.97 log$_2$FC); 2-aminoethylphosphonate-pyruvate transaminase (BB562_13505, 1.88 log$_2$FC); metal ABC transporter substrate-binding protein (BB562_15075, 2.49 log$_2$FC); transcriptional regulator Spx (BB562_15285, 1.63 log$_2$FC) were only up-regulated in olive-adapted *L. pentosus* CF2-10 (Table S1). However, in sunflower-adapted *L. pentosus* CF2-10 (Table S2), the following genes coding for hypothetical proteins [(BB562_01300, 2.42 log$_2$FC); (BB562_07910, 1.59 log$_2$FC); (BB562_07915, hydrolase activity [GO:0016787], 1.70 log$_2$FC); (BB562_13245, 1.61 log$_2$FC, integral component of membrane [GO:0016021])], 6-phospho-beta-glucosidase

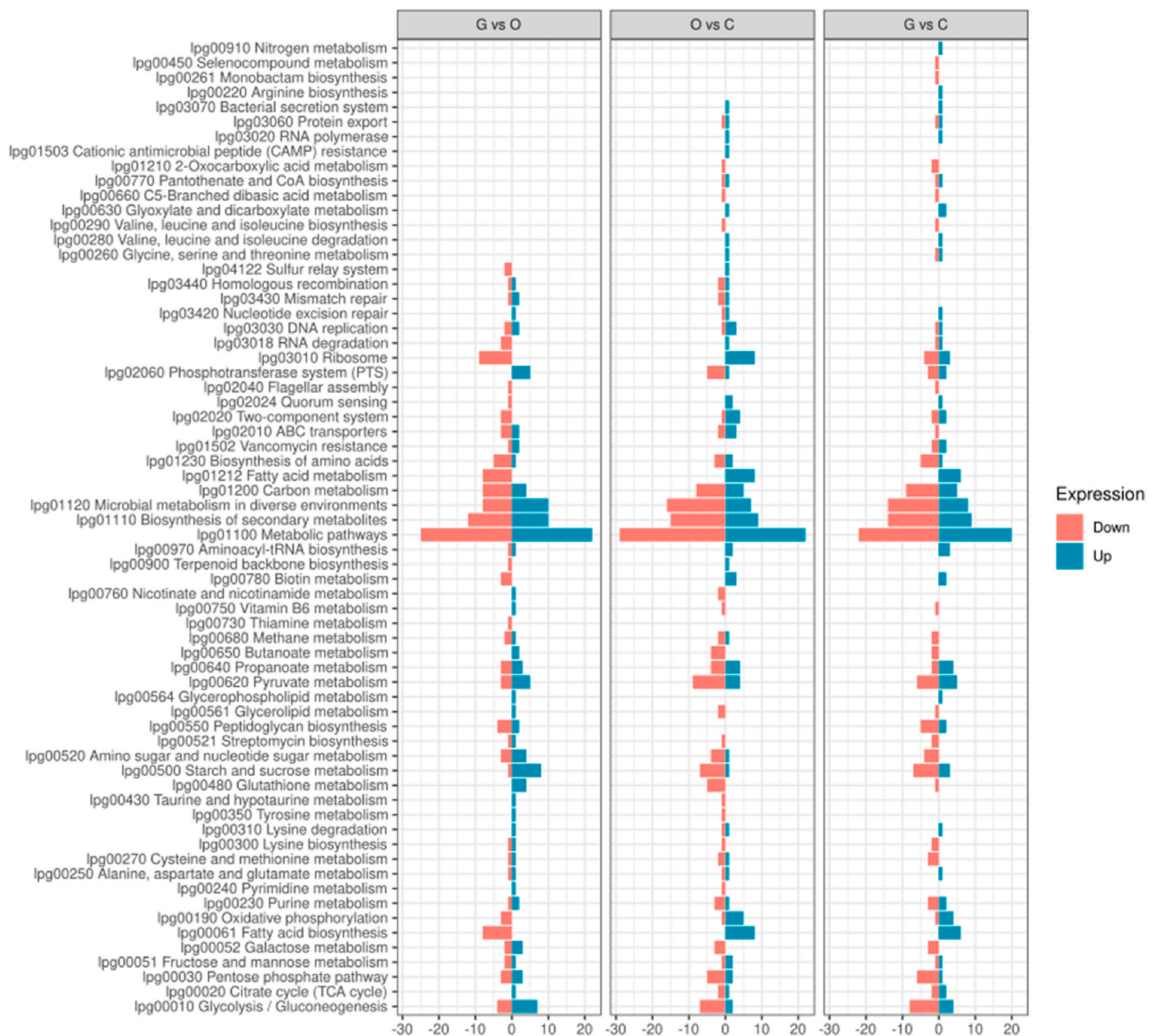

Fig. 2. GO classification of significantly differentially expressed genes (DEGs) in oil-adapted *L. pentosus* CF2-10. Significantly up-regulated (turquoise bars) and down-regulated (brick bars) DEGs (log_2 FoldChange >1.5) between sunflower-adapted (G) and olive-adapted (O) (G vs O); between olive-adapted (O) and non-adapted (C) (O vs C); and between sunflower-adapted (G) and non-adapted (C) (G vs C) *L. pentosus* CF2-10 were annotated into GO terms.

(BB562_01895, 2.13 log_2FC), and short-chain dehydrogenase (BB562_07735, 2.10 log_2FC) were over-expressed.

On the other hand, Circos plots (Fig. 4), displaying percentages of the differentially expressed ARG carrying *L. pentosus* CF2-10, showed that multidrug ABC transporter response to antibiotics and multidrug resistance were the most prevalent resistance mechanisms under different conditions (control and oil-adapted cells) to others. In this sense, both multidrug ABC transporter and multidrug resistance were highly expressed in control (40 and 17.2%, respectively) compared with sunflower- (37 and 12.8%, respectively) and olive- (34 and 15.56%, respectively) adapted *L. pentosus* CF2-10 (Fig. 4). However, genes in response to antibiotics were over-expressed in oil-adapted *L. pentosus* CF2-10 versus control (33.4 and 32% in sunflower and olive-adapted strain versus 29.7% in control). In addition, daunorubicin was over-expressed in sunflower and olive-adapted *L. pentosus* CF2-10 (6.8% and 12.14%, respectively) compared with the control (5.7%). In comparison, aminoglycoside and multidrug MFS ARG were over-expressed in sunflower-adapted *L. pentosus* CF2-10 (4.15 and 3.26%, respectively) versus control (2.8 and 2.9%, respectively) (Fig. 4). Regarding other ARGs such as multidrug DMT transporter and EmrB/QacA family drug, over-expression was detected in oil-adapted *L. pentosus* CF2-10 (0.6–0.8%) versus control (0.4–0.6%). Chloramphenicol, bleomycin and azaleucine resistance genes showed slight differences between conditions (Fig. 4).

3.3.3. *Quantitative real-time PCR validation of differential expression*

qRT-PCR was used to compare the expression activity of non-adapted cells (control) and in olive/sunflower-adapted *L. pentosus* CF2-10 and verify the RNA-seq results. In general, gene expressions in the olive and sunflower-adapted bacteria were higher for the following genes: *mfs* (*BB562_15125* gene coding for MFS transporter), *ath* (*BB562_09180* gene coding for anthranilate phosphoribosyltransferase) and *ABCtr*

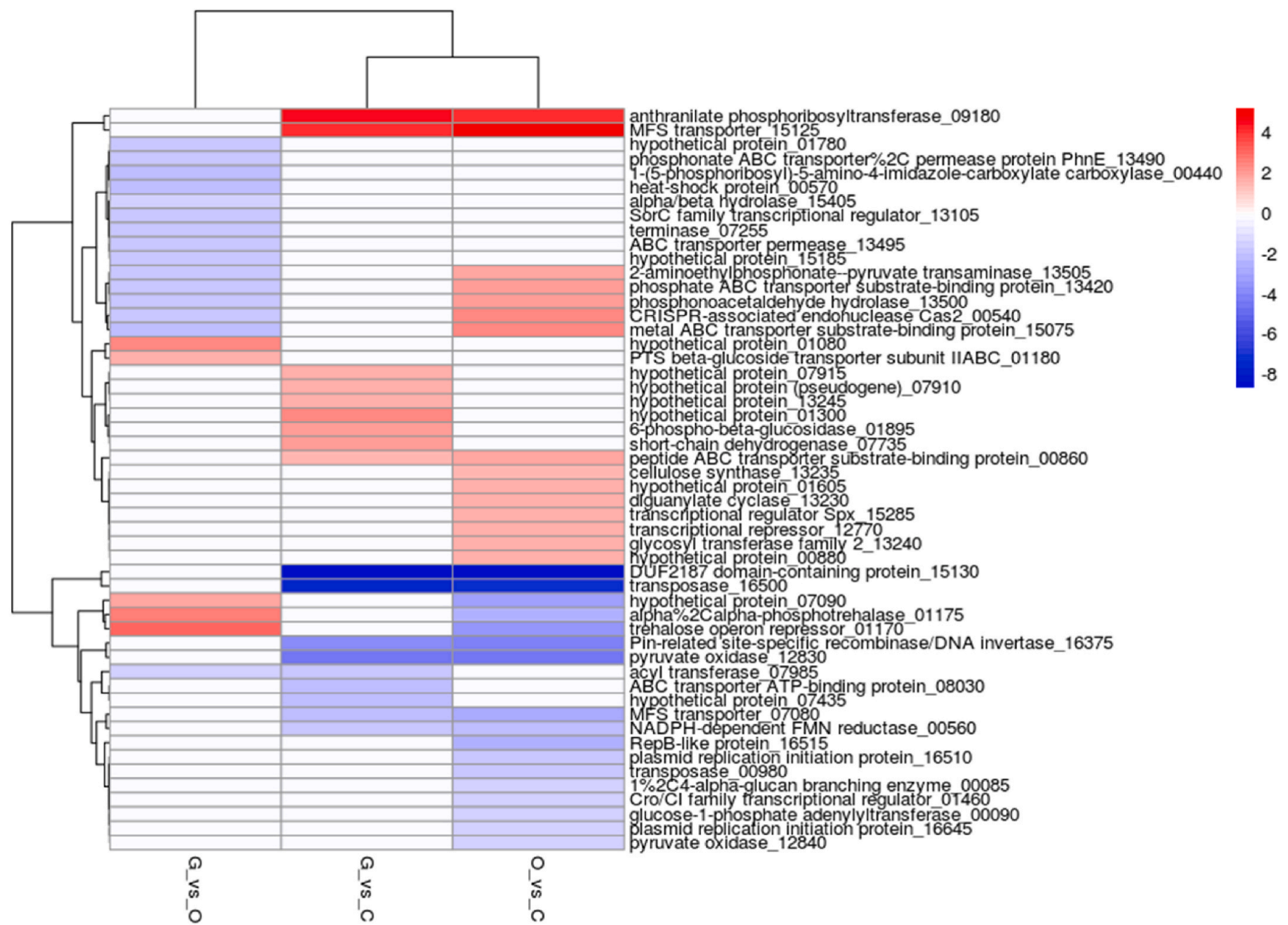

Fig. 3. Expression of differentially expressed genes in the oil-adapted (G or O) *L. pentosus* CF2-10 vs. control (C). Samples in columns and the encoded genes on rows. Differential expression is indicated with the color key. Samples: O vs C, olive-adapted strain vs. control; G vs C, sunflower-adapted strain vs. control; and G vs O, sunflower-adapted strain vs. olive-adapted strain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(*BB562_00860* gene coding for peptide ABC transporter substrate-binding). As determined by qPCR and RNA-seq (Fig. 5), respectively, *mfs* gene was up-expressed in sunflower (5 and 4.17- fold) and olive-adapted *L. pentosus* CF2-10 (5.75 and 5.21- fold), while *ath* gene was over-expressed in sunflower (4.8 and 4.47- fold) and olive-adapted *L. pentosus* CF2-10 (4.5 and 4.11- fold). Furthermore, *ABCtr* gene was over-expressed in sunflower (1.8 and 1.5- fold) and in olive-adapted *L. pentosus* CF2-10 (2 and 1.77- fold) (Fig. 5). However, *cas2* (*BB562_00540* gene coding for CRISPR-associated endonuclease Cas2, 2.8 and 2.54- fold) and *bglA* (*BB562_01895* gene coding for 6-phospho-beta-glucosidase, 2 and 2.13- fold) genes were up-expressed in olive and sunflower-adapted *L. pentosus* CF2-10, respectively. The qRT-PCR validates the differential expression and was consistent with the RNA-Seq (Fig. 5); thus, the transcriptomics results were considered representative.

4. Discussion

The direct consumption of vegetable oils and their use as adjuncts in functional food/dietary supplement formulation have drawn attention from earlier works; however, the impact of these oils on probiotic activities and their effect on defence mechanisms were poorly addressed. Recently, Alonso García et al. (2019, 2021) determined whether pre-adapting *Lactiplantibacillus pentosus* strains, isolated from Aloreña green table olives to vegetable-based edible oils, improved their robustness and functionality (e.g., auto-aggregation, co-aggregation with pathogens, and mucin adhesion). However, results depended on the strain and the oil used for pre-adaptation. To improve functionality, *L. pentosus* strain rerouted its metabolic pathways to balance energy production and storage, cell growth and survivability, host interactions (glycoconjugates) and other physiological features. In this study, we analyzed the effect of edible oils on the defence mechanisms of *L. pentosus* isolated from Aloreña table olives, especially in terms of antibiotic resistance, which is of great relevance not only in probiotic strains but also in other bacteria of health interest such as pathogens. In this way, the transcriptomic analysis may shed light on molecular aspects involved in this adaptation correlated with the increase in resistance.

It was reported that essential oils were used as alternative antimicrobials due to their ability to decrease or inhibit pathogen growth and biofilm formation (El-Tarabily et al., 2021; Millezi et al., 2016; Reda et al., 2020). However, some oils (e.g., manuka, marjoram and oregano) at sub-inhibitory concentration induced antibiotic resistance in *Staphylococcus aureus* (Turchi et al., 2019). In this study, we first analyzed the effect of the adaptation to different vegetable edible oils on the phenotypic and genotypic responses of three potential probiotic *L. pentosus* strains to antibiotics, thus providing new insights on how they become resistant in different environments (e.g., in a food matrix, gut or other

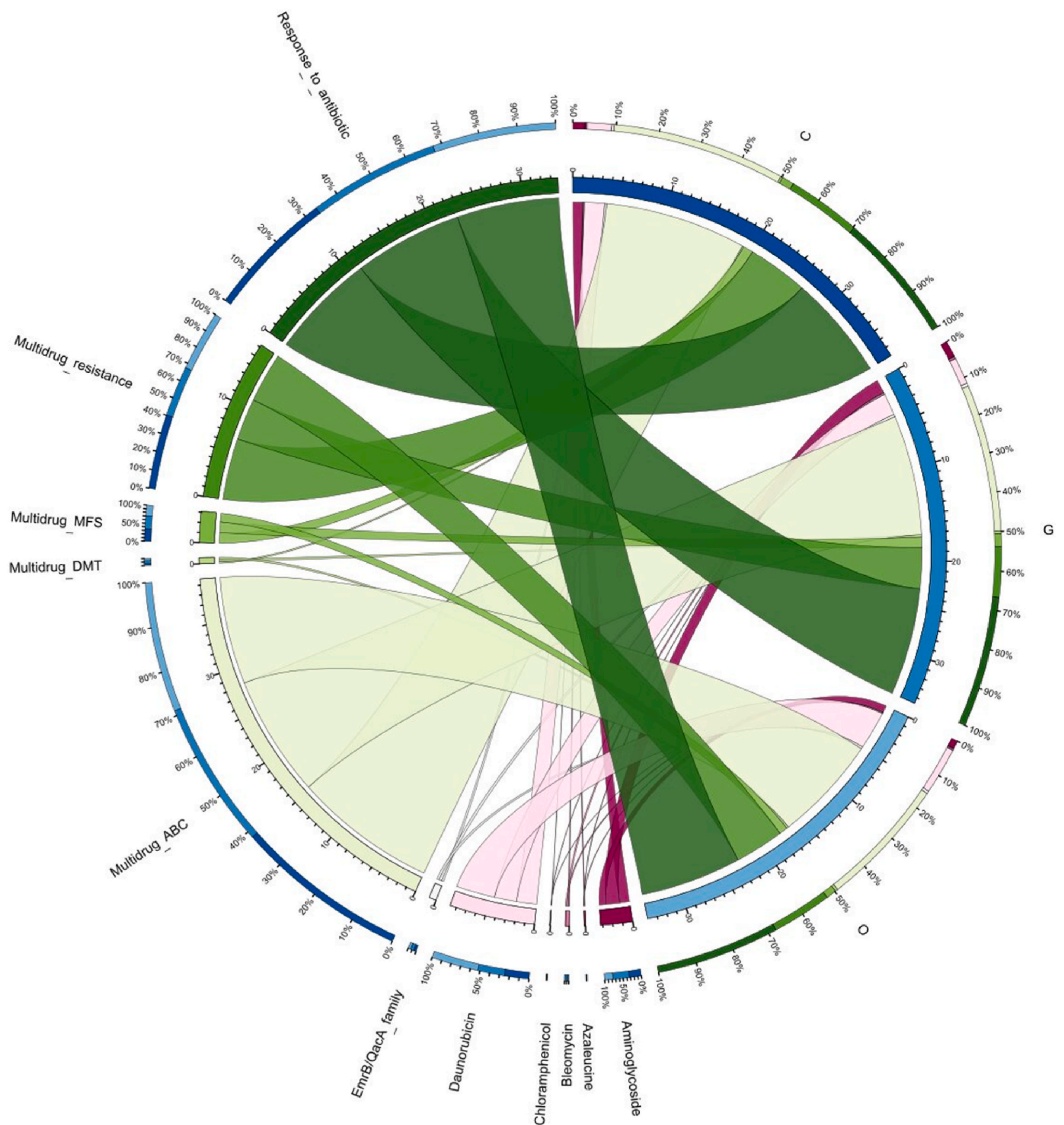

Fig. 4. Circos plot illustrating abundance of differentially expressed antibiotic resistance genes (ARGs) in oil-adapted (G or O) *L. pentosus* CF2-10 vs. control (C). The width of each connecting ribbon is directly proportional to the percentage of expressed ARG in each transcriptome sample. A specific ribbon color represented each ARG, and the width of each ribbon demonstrates the abundance of each differentially expressed ARG. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

condition involving edible oils). In this sense, previous studies demonstrated that physicochemical stresses, including antimicrobials, chemicals and UV light, changed the susceptibility patterns to antibiotics by increasing MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, and decreasing the MICs for clindamycin, erythromycin, streptomycin and trimethoprim in most *L. pentosus* strains isolated from Aloreña table olives (Casado Muñoz et al., 2016a).

The results obtained in the current study indicated that oil adaptation induced the increase of antibiotic MICs in a similar way as for physicochemical stresses (e.g., antimicrobials, chemicals and UV light) as reported by Casado Muñoz et al. (2016a) for ciprofloxacin and tetracycline. However, the MIC of other antibiotics (e.g., erythromycin, clindamycin, streptomycin, trimethoprim, and ampicillin) was increased after oil adaptation, most of which was decreased after

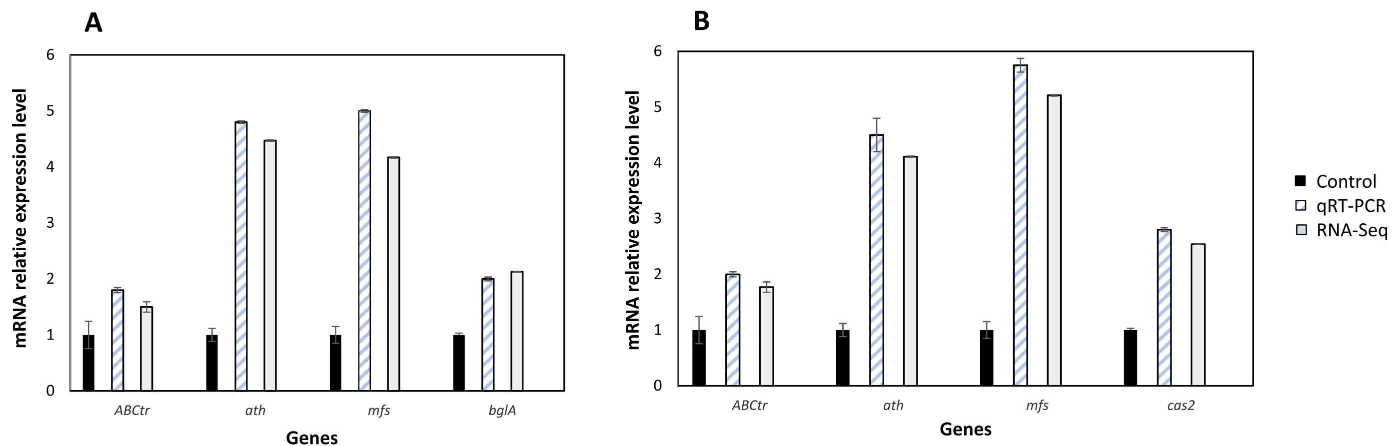

Fig. 5. Validation of relative RNA sequence results of five selected genes in the non-adapted cells (control) and sunflower (A) and olive (B) -adapted *L. pentosus* CF2-10, by comparing with qRT-PCR results. All values were normalized to "control" values.

physicochemical stress exposure (Casado Muñoz et al., 2016a). This finding indicates that the susceptibility pattern of antibiotics may rely mainly on the stress applied and the strains tested. Furthermore, we observed that linseed, olive, sunflower and soy were the main oils that induced more changes in antibiotic MICs.

Expression profiles of selected genes involved in stress/resistance response (*rpsL, recA, uvrB* and *srtA*) differed depending on the oil used for adaptation, *L. pentosus* strain, and the target gene. Despite the uniform phenotypic antibiotic susceptibility response to oil adaptation observed in most cases, the repertoire of induced and repressed genes differs. In this sense, we observed the over-expression *rpsL* gene in limited cases; however, its expression may be responsible for the increased MIC of tetracycline and streptomycin in *L. pentosus* CF2-10. A 30S-ribosomal protein S12 with polyspecific effects may act as an RNA chaperone (Coetzee et al., 1994) to protect the ribosome structure and function under stress.

On the other hand, the *uvrB* is related to the nucleotide excision repair (NER) system (*uvrA, uvrB, uvrC* and *uvrD* genes). Notably, the *uvrB* and *recA* genes had paired expression patterns involved in the same repair mechanism, which were activated in some cases of strain adaptation with some oils, similarly to what occurred with *L. pentosus* strains exposed to antibiotics (Casado Muñoz et al., 2016a). In this sense, the SOS response as an inducible pathway (Van der Veen and Abee, 2011) may partly be responsible for some antibiotics' increased MICs after oil adaptation.

Regarding the *srtA* gene, which encodes an enzyme that anchors surface proteins to the cell wall (Mazmanian et al., 1999), up-regulation was only detected in a single case, suggesting that the *srtA* gene was not involved in the increased antibiotic MIC after oil adaptation.

Oil adaptation as a stress condition may induce changes in the phenotypic and genotypic response of potential probiotic *L. pentosus* strains, highlighted by their increased robustness and functionality and their resistance to antimicrobials involving a repertoire of genes. However, to ensure survival and improve stress tolerance of these bacteria as necessary as starter/protective cultures or probiotics, this will rely on the individual screening of each strain under each stress condition. We selected a potential probiotic *L. pentosus* CF2-10, which exhibited more changes at phenotypic and genotypic levels after oil adaptation. In this sense, *L. pentosus* CF2-10 was phenotypically susceptible to tetracycline (Casado Muñoz et al., 2014). However, it was shown to be resistant to this antibiotic after oil adaptation, while in the other cases, increased antibiotic MIC was observed, thus increasing their resistance. To investigate the molecular mechanisms behind the increased antibiotic resistance, comparative transcriptomic analysis under stressing (oil adaptation) and non-stressing conditions (control) revealed that olive or sunflower oils impacted *L. pentosus* CF2-10, showing significant differences and also between stress conditions. Overall, *L. pentosus* CF2-10 switched the expression of several genes involved in different metabolic pathways to survive oil adaptation being; some mechanisms shared by both oils used in adaptation (sunflower and olive) include bacterial secretion system, nucleotide metabolism, fatty acid metabolism, amino acid metabolism, quorum sensing and stress response. Some of these mechanisms are involved in growth and survival and also in probiotic activity, as reported by Alonso García et al. (2021) for olive-adapted *L. pentosus* AP2-16, which responded by rerouting its metabolic pathways to balance energy production and storage, cell growth and survivability, host interactions (glycoconjugates), and other physiological features involved in robustness and functionality. In the current study, the pre-adaptation with olive or sunflower oils switches the transcriptional network of *L. pentosus* CF2-10 to regulate its functionality and robustness; antibiotic resistance is one of the affected features. In this sense, over-expression of the MFS transporter gene involved in antibacterial drug resistance (Andersen et al., 2015; Ranaweera et al., 2015) was observed in both olive and sunflower-adapted *L. pentosus* CF2-10. Besides antimicrobials, MFS efflux pumps play an essential role in the extrusion of toxic metabolites, Krebs cycle intermediates, quorum sensing signals and other unknown molecules, being quorum sensing molecules involved in the expression of the efflux gene (Evans et al., 1998; Martinez et al., 2009). According to phenotypic susceptibility tests, olive and sunflower-adapted *L. pentosus* CF2-10 showed increased MICs for ciprofloxacin, clindamycin, streptomycin and tetracycline, thus over-expressed MFS gene may be involved in the resistance to these antibiotics. Independent of the horizontal gene transfer of ARGs, MFS efflux pumps non-horizontally transferred in different bacteria such as probiotics or pathogens could be induced under some stress conditions (environment, diet, and digestive conditions), leading to different effects. Vegetable edible oils are widely consumed as a component of the diet. Some oils that are extensively used in the Mediterranean diet, such as olive oil, could represent a promising approach toward the design and manufacture of probiotic products with improved stability, functionality and robustness and also, in some cases, to inhibit pathogens (Dang Xuan et al., 2018; Tabassum and Vidyasagar, 2014).

On the other hand, the anthranilate-phosphoribosyltransferase gene is involved in the aromatic amino acid biosynthesis pathway, specifically the tryptophan synthesis. This gene's expression may enhance the growth and resistance of *L. pentosus* since the tryptophan pathway is essential for bacterial growth. The pre-adaptation of probiotics with oils represents a new approach to increasing tryptophan biosynthesis in food and the gut. Tryptophan has a vital role in host-microbiota cross-talk underlying regulation of gut functions in health conditions and during disease states such as irritable bowel syndrome (IBS) and inflammatory

bowel disease (IBD) (Bosi et al., 2020).

Additionally, the gene coding for peptide ABC transporter substrate-binding is involved in self-immunity and substrate export, conferring resistance against different antimicrobials, including antibiotics (Smits et al., 2020). In this sense, other transporters such as phosphate ABC transporter substrate-binding protein and metal ABC transporter substrate-binding protein or other proteins (transcriptional regulator Spx and hypothetical protein with ATPase activity) were only up-regulated in olive-adapted *L. pentosus* CF2-10 being involved in the influx of essential nutrients and efflux of toxic molecules such as antibiotics (Jousselin et al., 2013; Murina et al., 2019).

Finally, the overall analysis of DEGs abundance in sunflower or olive-adapted *L. pentosus* MP-10 confirmed the effect of oil adaptation on the increased response to antibiotics by increasing resistance against some antibiotics utilizing multidrug transporters. Furthermore, it is noteworthy to highlight the increased resistance to the chemotherapeutic agent daunorubicin. Oil-adapted probiotics with high daunorubicin-resistance is a good strategy to administer to a patient exposed to this compound, since it inhibits commensal anaerobes such as *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Clostridium* among others (van Vliet et al., 2009).

5. Conclusions

Vegetable oils are primarily consumed and used as adjuncts in functional food/dietary supplement formulation. Thus, understanding the molecular mechanisms behind the impact of oil adaptation on potential probiotic lactobacilli is crucial to designing oil adaptation as a strategy to increase their robustness and functionality under several stresses. However, the safety aspects must be analyzed to resist changes after oil adaptation to avoid resistant bacteria. In the present study, pre-adaptation of probiotic *L. pentosus* strains with oils induced changes in their phenotypic and genotypic profile related to antibiotic susceptibility and defence mechanisms. Thus, transcriptional changes were reported in the potential probiotic *L. pentosus* CF2-10 adapted with olive or sunflower, which exhibited increased antibiotic MIC and over-expression of stress genes (*rpsL, recA* and *uvrB*), rerouting its metabolic pathways to the export of toxic molecules such as efflux pumps and ABC transporters. Pre-adaptation of some lactobacilli with olive or sunflower oils may represent a novel approach for manufacturing probiotic products with improved stability, functionality and robustness. The control of resistance spread must include horizontal gene transfer and expression of genes induced by stress and are involved in antimicrobial resistance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data shared are published in the paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104148.

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