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The Effect of Incubation Temperature, Substrate and Initial pH Value on Plantaricin Activity and the Relative Transcription of *pln* Genes of Six Sourdough Derived *Lactiplantibacillus plantarum* Strains

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Abstract: The aim of the present study was to assess the effect of sourdough related parameters on the growth and plantaricin activity of six *Lactiplantibacillus plantarum* strains against a mixture of 5 *Listeria monocytogenes* strains and to analyze the transcriptomic response of their *pln* genes. Parameters included 3 substrates (MRS broth, mMRS broth, WFE), 3 temperatures (20, 30, 37 °C), 2 initial pH values (5.0, 6.0), 2 NaCl concentrations (0.0, 1.8%) and 12 time points (ranging from 0 to 33 h). The transcriptomic response of the plantaricin genes to the aforementioned parameters was assessed after 21 h of growth. In general, plantaricin activity was strain dependent with that of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516, harboring four *pln* genes, namely, *pln423* (*plαA*), *plαB*, *plαC* and *plαD*, reaching 2560 AU/mL. However, strains LQC 2320 and 2520, in which 18 *pln* genes were detected, namely, *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, exhibited plantaricin activity barely reaching 160 AU/mL. Substrate, temperature, initial pH value and strains significantly affected plantaricin activity, while NaCl had only a marginal effect. Similarly, growth substrate and temperature had a more pronounced effect than initial pH value on gene transcription. A strong correlation between the transcription of the genes belonging to the same locus was observed; however, only a weak correlation, if any, was observed between plantaricin activity and the transcription of the genes assessed.

Keywords: transcriptomic response; *pln* locus; bacteriocins

1. Introduction

Sourdough fermentation is regarded as one of the most ancient and simultaneously ecologically friendly preservation methods. It is a dynamic process, strongly dependent on the metabolites released by highly adapted microorganisms, namely, lactic acid bacteria (LAB), leading to microbiologically stable and safe end products. The ability of sourdough starters to produce bacteriocins has been at the epicenter of intensive study over the past few years, as a clean label technology. Despite the fact that bacteriocin production does not extend the mold free shelf life of sourdough related products, it does positively affect the stability of sourdough, through the prevention of contamination by pathogenic bacteria, and particularly *Listeria monocytogenes*. The latter is particularly important, since extended fermentation times and the diversity of raw materials used in dough making process increases the risk of cross-contamination in the bakery environment.

By definition, bacteriocins are ribosomally synthesized single proteins or protein complexes capable of inhibiting organisms closely related to the producing strains [1,2]. Bacteriocins produced by *Lactiplantibacillus plantarum* strains, namely, plantaricins, have gained much industrial and scientific attention. The stable presence of *Lp. plantarum* in spontaneously fermented foods, ranging from pickled vegetables to wine, attributed to its metabolic versatility and stress adaptation responses, has been extensively reported [3]. In addition, its frequent isolation from type I sourdoughs, with the latter characterized by daily back-sloppings at ambient temperature (<30 °C) and a fermentation time of less than 24 h, has been well documented [4–6].

The genetic organization of the *pln* locus of several *Lp. plantarum* strains has been unraveled up till now [7–13]. The *pln* locus is organized in five or six operons, with variable degrees of conservation [14,15]. The genes encoding the bacteriocin production and associated functions include a structural, an immunity, an ABC transporter and one coding for an accessory protein, while the detection of regulatory genes has been reported as well [16]. The stimulation of gene transcription is performed by a signal transducing network, which includes a secreted induction peptide (IP), a histidine protein kinase (HPK) and a response regulator (RR) [17]. HPK functions as the receptor for IP, with the former transferring the signal to RR through several phosphorylation reactions. Once phosphorylated, RR stimulates the transcription of genes associated with bacteriocin biosynthesis. A common attribute among several *Lp. plantarum* strains, namely, C11, WCFS1 and J51, is the presence of the regulatory operon *plnABCD*, which encodes a quorum sensing system [14]. In more detail, the regulatory operon *plnABCD* encodes an IP, namely, PlnA, an HPK, namely, PlnB and two cytoplasmic RRs, namely, PlnC and PlnD. Instead of *plnABCD*, the presence of the regulatory operon *plNC8-IF-HK-D*, encoding an IP, namely, *plNC8-IF*, an HPK, namely, *plNC8-HK* and an RR, namely, *plNC8-plnD*, was found in strains NC8 and J23. In our previous study [18], the genomic analysis of *Lp. plantarum* strains LQC 2320 and 2520 revealed the presence of the regulatory operon *plNC8-IF-HK-D* and three bacteriocin operons, namely, *plnJKLR*, *plnEFI* and *plnNC8αβc*. In the case of the transport operon, only *plnG*, *plnH* and *plnS* were detected, followed by *plnY*. The presence of *plnM* was detected as well. On the contrary, *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 harbored *pln423* (*plαA*), *plαB*, *plαC* and *plαD* in their *pln* locus.

Although the organization of the *pln* locus has been studied to some extent, the literature is scarce on the transcriptomic response of plantaricin associated genes under fermentation conditions. More accurately, Paramithiotis et al. [19] shed some light by studying the relative transcription of *pln* genes during the lactic acid fermentation of radish (*Raphanus sativus*) roots by an *Lp. plantarum* strain. Regarding sourdough fermentations, no previous study has assessed the relative gene transcription in *Lp. plantarum* at simulated industrial sourdough preparation conditions. Therefore, the aim of this study was to assess the effect of sourdough related parameters applied during type I sourdough fermentations, namely, incubation temperature and time, substrate composition, NaCl concentrations and initial pH values, on the growth and plantaricin activity of the aforementioned six *Lp. plantarum* strains and to further analyze the transcriptomic response of their *pln* genes.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Six Greek wheat sourdough derived *Lp. plantarum* strains, LQC 2320, 2422, 2441, 2485, 2516 and 2520, which previously exhibited antibacterial activity against *L. monocytogenes* [20], were used in the present study. Five *L. monocytogenes* strains, LQC 15186, 15187, 15188, 15189 and 15190, isolated from minced pork meat and assigned to serotype 4b [21], were used as indicator strains. Long term storage of bacterial strains took place at −20 °C in Nutrient Broth (LAB M, Lancashire, UK), supplemented with 50% glycerol. Before experimental use, *Lp. plantarum* and *L. monocytogenes* strains were grown twice in de Mann Rogosa and Sharpe (MRS) broth (LAB M) and brain heart infusion (BHI) broth (LAB M) at 30 °C for 24 h and 37 °C for 24 h, respectively.

2.2. Effect of Parameters Related to Sourdough Preparation on Growth and Plantaricin Activity Kinetics of Lp. plantarum Strains

All plantaricin activity assays were performed by applying a well diffusion assay (WDA) against a mixture of the aforementioned 5 *L. monocytogenes* strains. Growth of all *L. monocytogenes* strains at equal or at least comparable populations was verified as follows: 24 hour individual cultures of the 5 *L. monocytogenes* strains were inoculated into a common BHI broth at 7 log CFU/mL each and incubated at 37 °C for 24 h. Bacterial enumeration was performed by plating serial dilutions on BHI agar (LAB M). All colonies present in the final dilution were selected and further subcultured in BHI broth at 37 °C for 24 h. Then, DNA was extracted according to Paramithiotis et al. [4] and amplification by PCR of two genomic regions belonging to the Listeria Pathogenicity Island 1, which has proven effective in differentiating capacity between these strains [22], took place. The genomic regions were *plcB* gene, and the intergenic region *plcB-orfX*, which were amplified according to Hadjilouka et al. [22]. PCR took place using KAPA SYBR Fast qPCR Master Mix (2×) for ABI Prism (Kapa Biosystems, Wilmington, MA, USA) and StepOnePlus Real-Time PCR System (Applied Biosystems™, Waltham, MA, USA). After amplification, the melting temperature of each amplicon of each isolate was determined by melting curve analysis and compared with the respective of the pure cultures that were also included in the analysis. Melting curve analysis was as follows: 95 °C for 15 s then 60 °C for 1 min and raise to 95 °C at 0.3 °C/s. Two biological replicates were assessed; each PCR reaction was performed with ca. 0.1 μg of DNA.

The substrates used in the present study were MRS broth, MRS broth modified to contain glucose 1.47 g/L, fructose 0.52 g/L, maltose 9.87 g/L and sucrose 8.35 g/L (mMRS), i.e., the carbohydrates of wheat flour at relevant concentrations [23] and wheat flour-water extract (WFE). The latter was prepared according to Gobbetti et al. [24] and Rizzello et al. [25], modified as follows: wheat flour was suspended in tap water, at ratio 1:5 and an 18 h incubation took place under shaking (200 rpm) at 30 °C. Then, the supernatant that was obtained by centrifugation (12,000× *g*; 20 min; 4 °C) was further fortified with wheat flour carbohydrates at their initial concentration and, finally, the wheat flour-water extract was sterilized at 121 °C for 15 min. Each of the aforementioned substrates was adjusted according to the rest sourdough related parameters, namely, two pH values: 5.0 and 6.0, adjusted with NaOH 3 M; two NaCl concentrations, 0.0 and 1.8%; three incubation temperatures 20, 30 and 37 °C and incubation time up to 33 h.

Overnight cultures of the 6 *Lp. plantarum* strains were used to inoculate the aforementioned substrates at final population of ca. 7.5 log CFU/mL. Sampling was performed at regular time intervals (every three hours), the pH value was recorded and bacterial enumeration was performed by plating serial dilutions on MRS agar. For the antilisterial activity assessment, WDA was applied at the same time intervals, using as indicator strains a mixture of the aforementioned 5 *L. monocytogenes* strains, according to Syrokou et al. [20]. In brief, overnight cultures of the indicator strains were inoculated (1% each) into molten BHI agar cooled to 45–47 °C. Then, the agar was dispensed into Petri dishes and allowed to solidify. Wells were then aseptically punched with the aid of Pasteur pipette. In each well, 25 μL of each CFS, which were obtained by centrifugation (12,000× *g*; 10 min; 4 °C) and subsequent neutralization and treatment with catalase (Sigma-Aldrich, St. Louis, MO, USA), were added. Incubation took place at 37 °C for 24 h. The plantaricin activity was quantified by applying the two fold serial dilution approach on the CFS and expressed in AU/mL. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution, exhibiting a clear inhibition zone, multiplied by 40 to obtain AU/mL. All analyses were performed in duplicate and the average values are presented.

2.3. Effect of Parameters Related to Sourdough Preparation on the Transcription of Plantaricin Genes

The high quality draft genome data of the 6 *Lp. plantarum* strains were presented in previous studies [18,26]. Results obtained revealed the presence of 18 plantaricin genes, namely, *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*,

plNC8-HK, *plnD*, *plnI*, *plnM* and *plnG*, in the *pln* loci of two *Lp. plantarum* strains, namely, LQC 2320 and 2520, while the plantaricin genes *pln423* (*plαA*), *plαB*, *plαC* and *plαD* were detected in *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516. Primers were designed to detect these genes (Table 1) and their specificity was verified by PCR and gel electrophoresis using the conditions mentioned in Table 1. In the case of *plαC*, the PCR efficiency of the primer pairs examined was too low (<1.70) and, thus, the relative transcription of this gene was not assessed.

Table 1. Primer sequences and respective amplicon sizes used for the gene transcription assay.

Genes	Primer	Sequence	Amplicon Size (bp)	PCR Efficiency	References
Reference genes					
16S	16SF 16SR	GATGCATAGCCGACCTGAGA CTCCGTCAGACTTTCGTCCA	114	2.05	[27] [28]
IGS	IGSF IGSR	GGCCTATAGCTCAGCTGGTTA GCTGAGCTAAGGCCCCGTAAA	135	2.03	[29]
rpob	rpobF rpobR	CCGCGATGCGAAAACAAT CCWACAGAGATACGGTTATCRAATGC	69	2.04	[30]
Plantaricin genes					
plNC8a	plNC8aF plNC8aR	GGCGGTGATTTAACAACCAAG AATTCCAACGTGCTTTCTTGC	70	2.05	this study
plNC8b	plNC8bF plNC8bR	CGGATCAGTCCCAACTTCAGTA TTTCAATCGTTTTGCGATGCT	80	2.01	this study
plNC8c	plNC8cF plNC8cR	AGCGTAAAAGCAGCAGTGAATA AGTACGTGGCAAATGCCTAAAA	98	2.01	this study
pln423 (plaA)	423F 423R	TGTGGTAAACATTCCTGCTCTG CACTTTCCATGACCGAAGTTAGC	86	2.06	this study
plaB	plaBF plaBR	CGGTGAAAAACCCTGAGGCA TAGCTACCGTTCCAACCTGC	151	1.98	this study
plaD	plaDF plaDR	GCCAAAACAACTGCTGACGG TCCATATCAGCACGCACAGC	95	1.96	this study
plNC8-HK	plNC8-HKF plNC8-HKR	GGTGAAAAACCCTGAGGCAT GCTACCGTTCCAACCTGCT	148	2.00	this study
plNC8-IF	plNC8-IFF plNC8-IFR	ATAAGCTTGATGTCGGGGTTG GATGGCCTCCAAGTGCTTTT	70	2.00	this study
plnD	plnDF plnDR	GTGGTTTTGTTGAGTACATCGAAAT GCATCGGAAAAATTGCGGATAC	126	1.98	this study [31]
plnE	plnEF plnER	TGGTTTTAATCGGGGCGGT ATACCACGAATGCCTGCAAC	87	2.03	this study
plnF	plnFF plnFR	TGCTATTTCAGGTGGCGTTT GCTAATGACCCAATCGGCAG	94	2.08	this study
plnG	plnGF plnGR	TGCGGTTATCAGTATGTCAAAG CCTCGAAACAATTTCCCCC	453	1.96	[31]
plnH	plnHF plnHR	AACTGTTCAACCGACCGGAA ACTCGCGCACCTTCAACTAA	90	2.07	this study
plnI	plnIF plnIR	CTGGCTGCCATTAGTGTCCA GAGCTTCCATTGGCCCGTTA	100	2.09	this study
plnJ	plnJF plnJR	TTGAACGGGGTTGTTGGGG GCCAGCTTCGCCATCATAAA	81	2.03	this study
plnK	plnKF plnKR	GGCCGTCGGAGTCGTAAAAA ATCCCTTGAACCACCAAGCA	90	2.05	this study
plnL	plnLF plnLR	GGGTGCATCGTATTTGCGTG TTTGCAGATCGCCATGAAGC	113	2.01	this study
plnM	plnMF plnMR	AGCAGTGGGAAGATGCTTGA TGCCAACCTGCTTTACCTGT	109	2.02	this study

Table 1. *Cont.*

Genes	Primer	Sequence	Amplicon Size (bp)	PCR Efficiency	References
plnR	plnRF plnRR	GCGCTTATTGTCGTTTTCGC CAGCAGCCCCATCACTAAGC	88	2.01	this study
plnS	plnSF plnSR	TATGGCACCGGCGTATCTTT AACTCGTGCTGTATGCCGAT	121	2.02	this study
plnY	plnYF plnYR	GATTGGGGTACCCACGTCAC AAAGAATCGTCCTAGCCGCA	91	2.07	this study

Thermocycling conditions: initial denaturation at 95 °C for 20 s and then 40× (95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s). Melting curve analysis: 95 °C for 15 s, then 60 °C for 1 min and raise to 95 °C at 0.3 °C/s.

The effects of sourdough associated parameters on bacterial growth, pH value and plantaricin activity of the 6 *Lp. plantarum* strains were assessed every three hours, up to 33 h, as previously described. At the same time intervals, samples were collected, centrifuged (12,000× *g*; 10 min; sample temperature) and the biomass was mixed with 200 μL of RNAlater® solution (Ambion, Waltham, MA, USA). Samples from a specific time point (21 h) were subjected to RT-qPCR analysis as follows: RNA was extracted using the NucleoSpin® RNA Kit (Macherey–Nagel, Duren, Germany) and cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan) according to the instructions of the manufacturer. Real-time qPCR was performed using KAPA SYBR qPCR Kit Master Mix (2×) for ABI Prism (Kapa Biosystems, Boston, MA, USA). Primers and PCR conditions are presented in Table 1. Here, *16S*, *IGS* and *rpob* were evaluated as housekeeping genes; *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM*, *plnG*, *pln423* (*plαA*), *plαB* and *plαD* were selected as target genes. Two biological replicates were analyzed and two RT reactions were performed for each sample, containing ca. 0.1 μg RNA each; each cDNA was used for the assessment of the transcription of the genes under study.

2.4. Statistical Analysis

Processing of the Ct values obtained by qPCR was performed according to Hadjilouka et al. [32]. The calculated fold changes were converted to their $\log_2$ values for further processing. The stability values of the reference genes were calculated with NormFinder v0.953 [33]; *IGS* was the most stably transcribed gene and, therefore, it was used for normalization. Regulation of a gene was considered only when the $\log_2$ value of the fold change ($\log_2$FC) was below −1 (downregulation) or above 1 (upregulation), assessed through one-sample *t*-test ($p < 0.05$). The correlation between the transcription of the genes under study was assessed with the Pearson product moment correlation coefficient (r). The contribution of the sourdough related parameters, namely, initial pH value, NaCl content, growth substrate, growth temperature and bacterial strain on plantaricin activity, bacterial population and pH reduction, was evaluated through multifactor analysis of variance. All calculations were performed in Statgraphics Centurion XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results

3.1. The Effect of Parameters Related to Sourdough Preparation on Growth and Plantaricin Activity Kinetics of Lp. plantarum Strains

The melting temperatures of the *plcB* gene and the *plcB-orfX* intergenic region of the *L. monocytogenes* strains LQC 15186, 15187, 15188, 15189 and 15190 were calculated at 80.6093, 80.5373, 80.6099, 80.6114 and 80.4620 °C and at 79.8077, 79.3593, 79.5099, 79.3637 and 79.8074 °C, respectively. A total of 50 colonies present in the final dilution after the incubation of the mixture of the 5 *L. monocytogenes* strains at 37 °C for 24 h were isolated and purified by successive subculturing. Their DNA was extracted and the aforementioned genomic regions were amplified and their melting temperature was assessed. Twelve isolates presented identical melting temperatures for both genomic regions with strain LQC

15186, eight isolates with strain LQC 15190 and ten strains for each of the strains LQC 15187, 15188 and 15189. Therefore, it was indicated that the *L. monocytogenes* strains were grown at equal populations and, thus, the WDA was successfully performed.

The effect of sourdough related parameters, namely, 3 growth substrates (MRS broth, mMRS broth, WFE), 3 incubation temperatures (20, 30, 37 °C), 2 initial pH values (5.0, 6.0), 2 NaCl concentrations (0.0, 1.8%) and 12 time points (spanning from 0 to 33 h), on the plantaricin activity, growth and pH reduction of the six *Lp. plantarum* strains are presented in Figures S1–S6.

Plantaricin activity revealed a strain dependent profile. The strains under study were differentiated into two groups: the first one consisted of strains LQC 2422, 2441, 2485 and 2516 and the second one of strains LQC 2320 and 2520. The strains belonging to the former group were the first to exhibit plantaricin activity (ranging from 80 to 320 AU/mL), after 3 h of incubation, regardless of the treatment applied. In the case of strains LQC 2320 and 2520, detectable levels of plantaricin activity were observed after 6 h of incubation in MRS and mMRS broth and after 9 h in WFE, in all cases quantified as 80 AU/mL. The highest level of plantaricin activity (2560 AU/mL) produced by strains LQC 2422, 2441, 2485 and 2516 was detected after 15 and 18 h of growth in mMRS broth at pH 6, at 30 and 37 °C, respectively, regardless of the NaCl concentration applied. In both cases, the plantaricin activity remained stable until 21 h. In MRS broth with initial pH 6, a slightly decreased maximum plantaricin activity (1920 AU/mL) was observed after the growth of the aforementioned strains both at 30 and 37 °C for 18 h, with the activity remaining stable until 21 h. Growth in WFE revealed substantially decreased maximum plantaricin activity, ranging from 320 to 640 AU/mL. In all cases, lower levels of plantaricin activity were detected towards the end of the monitored period of incubation. Regarding *Lp. plantarum* strains LQC 2320 and 2520, much lower plantaricin activity levels were recorded, varying between 80 and 160 AU/mL, after any treatment was applied. As far as the effect of sourdough related parameters on growth was concerned, good growth rates of all six bacterial strains were demonstrated at any incubation temperature applied, during 33 h of incubation in MRS and mMRS broth. More accurately, the initial bacterial populations were 7.50 log CFU/mL (0 h) and by the end of the monitored period more than 9 log CFU/mL were recorded. During the same period of incubation in WFE, lower population levels were evident (<9 log CFU/mL). Depending on the initial culture pH, the six bacterial strains displayed similar final pH values (<3.5), after growth both at 30 and 37 °C, irrespective of the substrate applied, while growth at 20 °C revealed higher final pH values (>4.0).

Multifactor analysis of variance was applied to estimate the contribution of the sourdough related parameters on the plantaricin activity every 3 h over a period of 33 h. The independent factors, namely, bacterial strains, temperature, substrate and initial pH value, significantly affected the variability of plantaricin activity at the majority of time points tested ($p < 0.05$). A significant contribution of NaCl on plantaricin activity was only detected at 24 h ($p < 0.05$). The mean plantaricin activity obtained either from the single contribution of each significant factor or from the in pair interactions between significant factors, resulted in the substantial interpretation of the effect of sourdough related parameters on plantaricin activity (Table S1). In the case of bacterial strains, in agreement with the biological interpretation of the results, the mean plantaricin activities observed for LQC 2320 and 2520 were similar and were differentiated from the other four bacterial strains, namely, LQC 2422, 2441, 2485 and 2516, at any time point assessed (Table S2). Given that the multifactor analysis of variance for plantaricin activity assessment exhibited similar significant effects among the 12 time points tested, some representative figures illustrating the aforementioned effects are further presented, corresponding to the time point of 21 h. The selection of the aforementioned time point was not random, since the incubation of all six *Lp. plantarum* strains for 21 h revealed the maximum plantaricin activities. Regarding the in pair contribution of strains, namely, LQC 2320 and 2520, and incubation temperatures at the time point of 21 h, the mean plantaricin activities were overlapping, implying a temperature independent production. Regarding strains LQC 2422, 2441, 2485

and 2516, the mean plantaricin activities at 30 and 37 °C were also overlapping but were differentiated from those at 20 °C (Figure 1A1). Regarding the in pair effect of strains and substrates, the mean plantaricin activities observed for strains LQC 2320 and 2520 in MRS and mMRS broth were overlapping but were differentiated from those in WFE. In the case of strains LQC 2422, 2441, 2485 and 2516, a substrate dependent grouping of mean plantaricin activities was demonstrated (Figure 1A2).

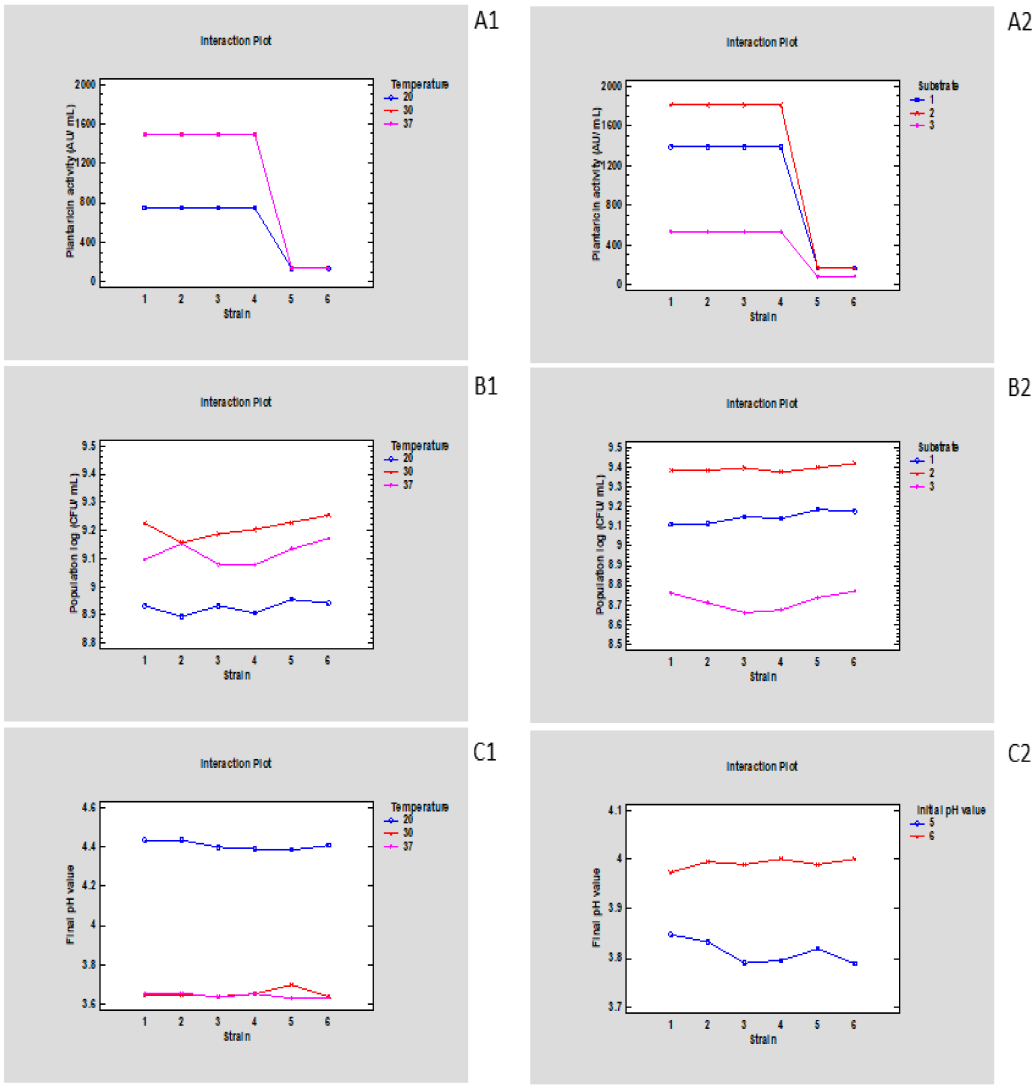

Figure 1. Interaction plots depicting the mean plantaricin activity, population and final pH value, obtained from the combination of two sourdough related parameters. (**A1**,**A2**) The effect on mean plantaricin activity (AU/mL), resulting from the in pair interaction of strains with temperatures and substrates, respectively, after growth for 21 h. (**B1**,**B2**) The effect on mean population, log (CFU/mL), resulting from the in pair interaction of strains with temperatures and substrates, respectively, after growth for 21 h. (**C1**,**C2**) The effect on mean final pH value, resulting from the in pair interaction of strains with temperatures and initial pH value, respectively, after growth for 21 h. Six *Lp. plantarum* strains were included, namely, LQC 2441, 2422, 2516, 2485, 2320 and 2520 corresponding to 1, 2, 3, 4, 5 and 6, three incubation temperatures, namely, 20, 30 and 37 °C, three substrates, namely, MRS broth, mMRS broth and WFE corresponding to 1, 2 and 3 and two initial pH values (5.0 and 6.0).

Regarding the effect of sourdough related conditions on the growth of the six *Lp. plantarum* strains, in most cases a strain independent profile was exhibited. More accurately, multifactor analysis of variance revealed that substrate, temperature and initial pH value had a significant effect on population for the majority of time points tested ($p < 0.05$). Strain was found to significantly affect population only at 6 and 27 h. On the

other hand, NaCl was not a significant factor. Regarding the in pair contribution of strains and temperatures, the 21 h incubation of the six bacterial strains at 30, 37 and 20 °C revealed, in most cases, a temperature dependent mean population. However, the incubation of strain LQC 2422 at 30 and 37 °C, resulted in identical mean populations (Figure 1B1). As far as the in pair effect of strains and substrates on the mean population was concerned, a substrate dependent grouping of means was exhibited after 21 h of incubation, among which no interaction was evident (Figure 1B2). This absence of interaction indicates that the effect of the substrate on the final population is not differentiated among the different strains, which is justified by the presence of parallel lines. In addition, the in pair effect of substrates and temperatures, namely, growth in WFE at 20, 30 and 37 °C, resulted in mean populations different from those obtained in MRS and mMRS broth at the aforementioned temperatures (Table S1). This observation is consistent with the biological interpretation of the effect on population, exerted after incubation of strains in WFE, in which case a decreased growth of bacterial strains (<9 log CFU/mL) was recorded, irrespective of the initial pH value, incubation temperature and NaCl concentration applied.

As far as the effect of the sourdough related parameters on the pH profile was concerned, multifactor analysis of variance demonstrated that substrate, temperature and initial pH were significant factors in a time independent way, while NaCl was also found to significantly affect the final pH value after incubation for 6, 9, 21, 24, 27, 30 and 33 h ($p < 0.05$). In the case of strain, a statistically significant effect on the final pH value was observed for the majority of the time points assessed, except for 21 h. As in the case of plantaricin activity and population, the representative figures depicting the effect of the in pair contribution of significant factors to the final pH values, refer to the time point of 21 h. The incubation of the six *Lp. plantarum* strains, at the three temperatures, revealed that the mean final pH values observed for 30 and 37 °C were overlapping for all strains but LQC 2320 and were differentiated from those at 20 °C (Figure 1C1). Regarding the in pair effect of initial pH values and strains on the final pH values, an initial pH based grouping of means was performed. The aforementioned grouping of means indicated no interaction between the initial pH values and strains, which further demonstrates that the effect of the initial pH on the final pH value is not differentiated among the different strains (Figure 1C2). The mean final pH values obtained from the aforementioned treatments are presented in Table S1.

3.2. The Effect of Parameters Related to Sourdough Preparation on the Transcription of Plantaricin Genes

RT-qPCR analysis was applied to assess the effect of the sourdough related parameters on the transcriptomic response of plantaricin genes in the six *Lp. plantarum* strains. Strains LQC 2320 and 2520 harbored 18 plantaricin genes in their locus, namely, *plNC8a*, *plNC8b, plNC8c*, *plnL*, *plnR*, *plnJ*, *plnK*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, while the presence of only four plantaricin genes, namely, *pln423* (*plαA*), *plαB*, *plαC* and *plαD*, was recorded in *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516. For the reasons described in Section 2.3, the relative transcription of *plαC* was not evaluated in the present study. Given the fact that plantaricin activity assessment was performed at regular time intervals, up to 33 h, a specific time point needed to be selected for further RT-qPCR analysis. The incubation time of 21 h was selected since it was the time point at which the maximum plantaricin activities produced by all six *Lp. plantarum* strains, after any treatment applied, intersect. In addition, it coincides with the incubation time required for artisanal sourdough preparation. Thus, samples obtained from 21 h incubation were selected for RT-qPCR analysis. Regarding the contribution of NaCl to the variability of the plantaricin activity, it was excluded from RT-qPCR analysis, since it was characterized as not significant. The effects of temperature, substrate, pH value and strain on plantaricin gene transcription are exhibited in Figures S7–S17 and are summarized in Tables 2 and 3.

Table 2. Number of samples in which the relative transcription of the genes under study was below, above or within the threshold set under the effect of sourdough related parameters on *Lp. plantarum* strains LQC 2320 and LQC 2520.

	$\log_2$(FC)	LQC 2320 Effect of Temperature [1]	LQC 2320 Effect of Substrate [2]	LQC 2320 Effect of pH [3]	LQC 2520 Effect of Temperature	LQC 2520 Effect of Substrate	LQC 2520 Effect of pH	Effect of Strain [4]
plNC8α	<−1	2 (11.1)	2 (11.1)	2 (22.2)	0 (0.0)	4 (22.2)	0 (0.0)	3 (16.7)
	−1 to 1	12 (66.7)	12 (66.7)	7 (77.8)	9 (50.0)	14 (77.8)	8 (88.9)	12 (66.7)
	>1	4 (22.2)	4 (22.2)	0 (0.0)	9 (50.0)	0 (0.0)	1 (11.1)	3 (16.7)
plNC8β	<−1	2 (11.1)	3 (16.7)	1 (11.1)	1 (5.6)	5 (27.8)	0 (0.0)	1 (5.6)
	−1 to 1	9 (50.0)	10 (55.6)	8 (88.9)	8 (44.4)	13 (72.2)	7 (77.8)	15 (83.3)
	>1	7 (38.9)	5 (27.8)	0 (0.0)	9 (50.0)	0 (0.0)	2 (22.2)	2 (11.1)
plnNC8c	<−1	2 (11.1)	2 (11.1)	2 (22.2)	1 (5.6)	5 (27.8)	0 (0.0)	5 (27.8)
	−1 to 1	11 (61.1)	12 (66.7)	7 (77.8)	8 (44.4)	13 (72.2)	8 (88.9)	10 (55.6)
	>1	5 (27.8)	4 (22.2)	0 (0.0)	9 (50.0)	0 (0.0)	1 (11.1)	3 (16.7)
plnR	<−1	4 (22.2)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	−1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	12 (66.7)
	>1	7 (38.9)	3 (16.7)	0 (0.0)	8 (44.4)	0 (0.0)	2 (22.2)	3 (16.7)
plnL	<−1	4 (22.2)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	−1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	12 (66.7)
	>1	7 (38.9)	3 (16.7)	0 (0.0)	2 (11.1)	0 (0.0)	2 (22.2)	3 (16.7)
plnK	<−1	5 (27.8)	7 (38.9)	2 (22.2)	2 (11.1)	7 (38.9)	0 (0.0)	4 (22.2)
	−1 to 1	6 (33.3)	8 (44.4)	7 (77.8)	8 (44.4)	11 (61.1)	7 (77.8)	11 (61.1)
	>1	7 (38.9)	3 (16.7)	0 (0.0)	8 (44.4)	0 (0.0)	2 (22.2)	3 (16.7)
plnJ	<−1	1 (5.6)	7 (38.9)	0 (0.0)	2 (11.1)	7 (38.9)	0 (0.0)	3 (16.7)
	−1 to 1	10 (55.6)	8 (44.4)	8 (88.9)	7 (38.9)	11 (61.1)	7 (77.8)	12 (66.7)
	>1	7 (38.9)	3 (16.7)	1 (11.1)	9 (50.0)	0 (0.0)	2 (22.2)	3 (16.7)
plnE	<−1	3 (16.7)	3 (16.7)	0 (0.0)	3 (16.7)	4 (22.2)	0 (0.0)	1 (5.6)
	−1 to 1	14 (77.8)	11 (61.1)	9 (100.0)	12 (66.7)	13 (72.2)	7 (77.8)	14 (77.8)
	>1	1 (5.6)	4 (22.2)	0 (0.0)	3 (16.7)	1 (5.6)	2 (22.2)	3 (16.7)
plnF	<−1	3 (16.7)	6 (33.3)	0 (0.0)	2 (11.1)	6 (33.3)	0 (0.0)	1 (5.6)
	−1 to 1	8 (44.4)	6 (33.3)	9 (100.0)	9 (50.0)	12 (66.7)	7 (77.8)	14 (77.8)
	>1	7 (38.9)	6 (33.3)	0 (0.0)	7 (38.9)	0 (0.0)	2 (22.2)	3 (16.7)
plnH	<−1	2 (11.1)	8 (44.4)	2 (22.2)	2 (11.1)	6 (33.3)	0 (0.0)	5 (27.8)
	−1 to 1	9 (50.0)	5 (27.8)	7 (77.8)	7 (38.9)	10 (55.6)	7 (77.8)	10 (55.6)
	>1	7 (38.9)	5 (27.8)	0 (0.0)	9 (50.0)	2 (11.1)	2 (22.2)	3 (16.7)
plnS	<−1	3 (16.7)	6 (33.3)	1 (11.1)	2 (11.1)	6 (33.3)	0 (0.0)	3 (16.7)
	−1 to 1	9 (50.0)	7 (38.9)	8 (88.9)	7 (38.9)	12 (66.7)	7 (77.8)	12 (66.7)
	>1	6 (33.3)	5 (27.8)	0 (0.0)	9 (50.0)	0 (0.0)	2 (22.2)	3 (16.7)
plnY	<−1	1 (5.6)	6 (33.3)	1 (11.1)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	−1 to 1	8 (44.4)	9 (50.0)	8 (88.9)	7 (38.9)	12 (66.7)	7 (77.8)	14 (77.8)
	>1	9 (50.0)	3 (16.7)	0 (0.0)	10 (55.6)	0 (0.0)	2 (22.2)	3 (16.7)
plNC8-IF	<−1	4 (22.2)	6 (33.3)	0 (0.0)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	−1 to 1	13 (72.2)	10 (55.6)	9 (100.0)	12 (66.7)	12 (66.7)	7 (77.8)	13 (72.2)
	>1	1 (5.6)	2 (11.1)	0 (0.0)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plNC8-HK	<−1	4 (22.2)	6 (33.3)	0 (0.0)	2 (11.1)	6 (33.3)	0 (0.0)	1 (5.6)
	−1 to 1	13 (72.2)	10 (55.6)	9 (100.0)	11 (61.1)	12 (66.7)	7 (77.8)	13 (72.2)
	>1	1 (5.6)	2 (11.1)	0 (0.0)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plnD	<−1	4 (22.2)	6 (33.3)	0 (0.0)	1 (5.6)	6 (33.3)	0 (0.0)	1 (5.6)
	−1 to 1	13 (72.2)	10 (55.6)	8 (88.9)	12 (66.7)	12 (66.7)	7 (77.8)	13 (72.2)
	>1	1 (5.6)	2 (11.1)	1 (11.1)	5 (27.8)	0 (0.0)	2 (22.2)	4 (22.2)
plnM	<−1	5 (27.8)	7 (38.9)	1 (11.1)	3 (16.7)	6 (33.3)	0 (0.0)	4 (22.2)
	−1 to 1	7 (38.9)	6 (33.3)	7 (77.8)	5 (27.8)	11 (61.1)	6 (66.7)	10 (55.6)
	>1	6 (33.3)	5 (27.8)	1 (11.1)	10 (55.6)	1 (5.6)	3 (33.3)	4 (22.2)
plnI	<−1	3 (16.7)	7 (38.9)	2 (22.2)	2 (11.1)	6 (33.3)	1 (11.1)	3 (16.7)
	−1 to 1	9 (50.0)	6 (33.3)	7 (77.8)	6 (33.3)	12 (66.7)	6 (66.7)	12 (66.7)
	>1	6 (33.3)	5 (27.8)	0 (0.0)	10 (55.6)	0 (0.0)	2 (22.2)	3 (16.7)
plnG	<−1	3 (16.7)	5 (27.8)	1 (11.1)	1 (5.6)	7 (38.9)	1 (11.1)	3 (16.7)
	−1 to 1	7 (38.9)	8 (44.4)	7 (77.8)	6 (33.3)	9 (50.0)	5 (55.6)	12 (66.7)
	>1	8 (44.4)	5 (27.8)	1 (11.1)	11 (61.1)	2 (11.1)	3 (33.3)	3 (16.7)

Table 2. *Cont.*

	$\log_2$(FC)	Effect of Temperature [1]	LQC 2320 Effect of Substrate [2]	Effect of pH [3]	Effect of Temperature	LQC 2520 Effect of Substrate	Effect of pH	Effect of Strain [4]
Total	<−1	55 (17.0)	101 (31.2)	19 (11.7)	30 (9.3)	107 (33.0)	2 (1.2)	46 (14.2)
	−1 to 1	172 (53.1)	154 (47.5)	139 (85.8)	150 (46.3)	211 (65.1)	124 (76.5)	221 (68.2)
	>1	97 (29.9)	69 (21.3)	4 (2.5)	144 (44.4)	6 (1.9)	36 (22.2)	57 (17.6)

Percentage is given in parenthesis. [1] The effect of incubation temperature was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain at 20, 30 and 37 °C pairwise using the lower temperature as control. [2] The effect of substrate was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in MRS, mMRS broth and WFE using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. [3] The effect of pH was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in pH 5 and 6, using the latter as control. [4] The effect of strain was assessed by comparing the transcription of the genes under study after 21 h growth of *Lp. plantarum* strains LQC 2320 and 2520 using the former as control.

Table 3. Number of samples in which the relative transcription of the genes under study was below, above or within the threshold set under the effect of sourdough related parameters on *Lp. plantarum* strains LQC 2441, LQC 2422, LQC 2485 and LQC 2516.

		LQC 2441			LQC 2422			LQC 2485			LQC 2516			
	$\log_2$(FC)	Effect of Temperature [1]	Effect of Substrate [2]	Effect of pH [3]	Effect of Temperature	Effect of Substrate	Effect of pH	Effect of Temperature	Effect of Substrate	Effect of pH	Effect of Temperature	Effect of Substrate	Effect of pH	Effect of Strain [4]
pln423	<−1	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.1)	0 (0.0)	1 (11.1)	3 (16.7)	0 (0.0)	1 (11.1)	2 (11.1)	1 (5.6)	1 (11.1)	5 (4.6)
	−1 to 1	17 (94.4)	10 (55.6)	7 (77.8)	15 (83.3)	7 (38.9)	7 (77.8)	13 (72.2)	8 (44.4)	7 (77.8)	14 (77.8)	6 (33.3)	7 (77.8)	46 (42.6)
	>1	1 (5.6)	8 (44.4)	2 (22.2)	1 (5.6)	11 (61.1)	1 (11.1)	2 (11.1)	10 (55.6)	1 (11.1)	2 (11.1)	11 (61.1)	1 (11.1)	57 (52.8)
plaB	<−1	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.1)	0 (0.0)	1 (11.1)	3 (16.7)	0 (0.0)	1 (11.1)	2 (11.1)	1 (5.6)	1 (11.1)	7 (6.5)
	−1 to 1	17 (94.4)	10 (55.6)	7 (77.8)	15 (83.3)	7 (38.9)	7 (77.8)	13 (72.2)	8 (44.4)	7 (77.8)	14 (77.8)	5 (27.8)	7 (77.8)	47 (43.5)
	>1	1 (5.6)	8 (44.4)	2 (22.2)	1 (5.6)	11 (61.1)	1 (11.1)	2 (11.1)	10 (55.6)	1 (11.1)	2 (11.1)	12 (66.7)	1 (11.1)	54 (50.0)
plaD	<−1	5 (27.8)	8 (44.4)	2 (22.2)	2 (11.1)	6 (33.3)	7 (77.8)	4 (22.2)	7 (38.9)	2 (22.2)	5 (27.8)	3 (16.7)	1 (11.1)	41 (38.0)
	−1 to 1	9 (50.0)	5 (27.8)	3 (33.3)	11 (61.1)	10 (55.6)	2 (22.2)	9 (50.0)	9 (50.0)	4 (44.4)	9 (50.0)	12 (66.7)	5 (55.6)	52 (48.1)
	>1	4 (22.2)	5 (27.8)	4 (44.4)	5 (27.8)	2 (11.1)	0 (0.0)	5 (27.8)	2 (11.1)	3 (33.3)	4 (22.2)	3 (16.7)	3 (33.3)	15 (13.9)
Total	<−1	5 (9.3)	8 (14.8)	2 (7.4)	6 (11.1)	6 (11.1)	9 (33.3)	10 (18.5)	7 (13.0)	4 (14.8)	9 (16.7)	5 (9.3)	3 (11.1)	53 (16.4)
	−1 to 1	43 (79.6)	25 (46.3)	17 (63.0)	41 (75.9)	24 (44.4)	16 (59.3)	35 (64.8)	25 (46.3)	18 (66.7)	37 (68.5)	23 (42.6)	19 (70.4)	145 (44.8)
	>1	6 (11.1)	21 (38.9)	8 (29.6)	7 (13.0)	24 (44.4)	2 (7.4)	9 (16.7)	22 (40.7)	5 (18.5)	8 (14.8)	26 (48.1)	5 (18.5)	126 (38.9)

Percentage is given in parenthesis. [1] The effect of incubation temperature was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain at 20, 30 and 37 °C pairwise using the lower temperature as control. [2] The effect of substrate was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in MRS, mMRS broth and WFE using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. [3] The effect of pH was assessed by comparing the transcription of the genes under study after 21 h growth of each *Lp. plantarum* strain in pH 5 and 6, using the latter as control. [4] The effect of strain was assessed by comparing the transcription of the genes under study after 21 h growth of the *Lp. plantarum* strains LQC 2441, 2422, 2485 and 2516 pairwise.

Temperature increase revealed a pronounced effect on the relative gene transcription of strain LQC 2520, whereas no such impact was recorded regarding the other strains. More specifically, regarding strain LQC 2520, upregulation was evident in 44.4% of the samples assessed. The most pronounced upregulation was observed for *plnG*, which was upregulated in 61.1% of the cases, followed by *plnY*, *plnI* and *plnM*, which were upregulated in 55.6% of the cases. On the other hand, the genes that were least affected by temperature increase were *plNC8-IF*, *plnD* and *plnE*, the relative transcription of which remained stable in 66.7% of the cases. Regarding strain LQC 2320, the relative transcription of genes *plnY* and *plnG* were most affected by temperature increase, since they were upregulated in the 50% and 44.4% of the samples assessed, respectively. As in the case of strain LQC 2520, the genes that were least affected by temperature increase were *plNC8-IF*, *plNC8-HK*, *plnD* and *plnE*, the relative transcription of which remained stable in 72.2% of the three former cases and 77.8% of the latter one, respectively. Regarding both strains, during growth in MRS and mMRS broth, a temperature increase from 20 to 30 °C, resulted in the upregulation of the majority of the genes under study, irrespective the initial pH value. This was also the case during growth in mMRS broth with an initial pH value of 5, when the temperature increased from 20 to 37 °C. As far as strains LQC 2441, 2422, 2485 and 2516 were concerned, the relative transcription of *plαD* seemed to be more affected than that of *pln423* and *plαB*, since the relative transcription of the former was stable in fewer cases than the respective of the two latter.

The transcription of the genes under study in all strains seemed to be affected by the growth substrate, with the exception of strain LQC 2520, the relative transcription of which remained between −1 and 1 $\log_2$ (FC) in 65.1% of the cases, which in the present study was not considered as regulation. More specifically, in the case of strain LQC 2520, the relative transcription of the genes *plnR*, *plnL*, *plnK*, *plnJ* and *plnG* indicated downregulation in 38.9% of the cases. On the contrary, the relative transcription of *plnNC8a* remained stable in 77.8% of the cases, while the respective of *plnNC8b, plnNC8c* and *plnE* in 72.2% of the samples assessed. As far as strain LQC 2320 was concerned, upregulation was evident when the transcript levels obtained during growth at 37 °C in mMRS broth were compared with the ones in MRS broth, irrespective of the initial pH value. The most affected genes were *plnH*, the relative transcription of which indicated downregulation in 44.4% of the samples assessed, as well as *plnR*, *plnL*, *plnK*, *plnJ*, *plnM* and *plnI*, the relative transcription of which indicated downregulation in 38.9% of the cases. On the other hand, the relative transcription of the genes *plnNC8a* and *plnNC8c* seemed to be the least affected, since in 66.7% of the samples assessed, their relative transcription remained between −1 and 1 $\log_2$ (FC). Similar was the case for *plnE*, which remained unaffected in 61.1% of the samples assessed. Regarding both strains LQC 2320 and 2520, downregulation of the majority of the genes under study was observed during growth at 30 °C in WFE, compared to the respective in MRS and mMRS broth, with an initial pH value of 6. In the case of bacterial strain LQC 2320, a similar transcriptomic response was observed at pH 5, as well. As far as strains LQC 2422, 2485 and 2516 were concerned, the relative transcription of *plαD* seemed to be less affected than that of *pln423* and *plαB*, since the relative transcription of the former was stable in more cases than the respective of the two latter. On the contrary, the opposite was evident in bacterial strain LQC 2441, in which the relative transcription of *pln423* and *plαB* was less affected than the respective of *plαD*.

Decrease in the initial pH value from 6 to 5 had a rather restricted effect on the relative gene transcription, since their $\log_2$ (FC) value ranged between 1 and −1 in the majority of the cases. As far as strain LQC 2320 was concerned, *plnE*, *plnF*, *plNC8-IF* and *plNC8-HK* were completely unaffected by a decrease in the initial pH value. Regarding strain LQC 2520, the least affected genes were *plnNC8a* and *plnNC8c*, the relative transcription of which remained stable in 88.9% of the samples assessed. On the other hand, the relative transcription of *plnM* and *plnG* indicated upregulation in 33.3% of the cases. More specifically, upregulation of the majority of genes under study was recorded upon a decrease in the initial pH value both in mMRS broth at 37 °C and WFE at 30 °C. Regarding strains LQC 2422, 2441, 2485 and 2516, the relative transcription of *plαD* was more affected than that of *pln423* and *plαB*. In brief, regarding strains LQC 2422, 2485 and 2516, a decrease in the initial pH value in MRS broth at 30 °C, resulted in downregulation of *plαD*, while in strains LQC 2441 and 2516, upregulation of the respective in MRS broth at 20 °C, was recorded.

The effect of strain was assessed by comparing the gene transcript levels between the strains included in the present study, pairwise. Regarding strains LQC 2520 and 2320, no statistically significant differences were observed in 68.2% of the samples assessed. The gene that was least affected was *plnNC8b*, since the $\log_2$ (FC) value ranged between 1 and −1 in the 83.3% of the cases. Similar was the case for *plnE*, *plnF* and *plnY*, the relative transcription of which remained stable in 77.8% of the cases. Notably, strain effect during growth in MRS broth at 37 °C, revealed significant differences in the transcription of all genes under study, irrespective of the initial pH value. Regarding the comparison between the gene transcript levels of strains LQC 2422, 2441, 2485 and 2516, significant differences were observed in *pln423*, *plαB* and *plαD*. Strain effect resulted in upregulation of both *pln423* and *plαB* in 52.8 and 50.0% of the samples assessed, respectively. As far as the relative transcription of *plαD* was concerned, downregulation was evident in 38.0% of the cases.

In Table S3, the Pearson product moment (r) correlations between plantaricin activity and relative transcription of the 18 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2320 and 2520 are presented. A total of 18 correlations were evaluated; only in 8 cases, namely, between plantaricin activity and the transcriptomic response of *plnJ*, *plnS*,

plnY, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnM* and *plnI* weak positive correlation ($0.3 \leq r < 0.5$) was detected. In the rest of the cases, no statistically significant correlations were observed. On the contrary, positive correlation was observed between the transcription of the genes under study. More accurately, a total of 153 correlations were evaluated and, in all cases, positive correlation was detected. This was characterized as very strong in 102 cases ($0.8 \leq r < 1$), strong in 18 cases ($0.7 \leq r < 0.8$), moderate in 25 cases ($0.5 \leq r < 0.7$) and weak in 8 cases ($0.3 \leq r < 0.5$). In Table S4, the Pearson product moment (r) correlations between plantaricin activity and relative transcription of the three plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 are presented. Weak negative correlation was recorded between plantaricin activity and *pln423* and *plαB*, respectively. In addition, very strong correlation between the transcription of *pln423* and *plαB* was also evident.

4. Discussion

The application of bacteriocin producing LAB in food industries has attracted considerable scientific interest, over the past few years, as a clean label technology [34]. However, the suitability of bacteriocinogenic LAB for more complex ecosystems, such as sourdough, has been studied only to some extent [35–37]. Bacteriocin production counteracts food contamination during processing and further contributes to the microbial stability and safety of the end products. *Lp. plantarum* has been consistently reported to prevail spontaneous sourdough fermentations and its bacteriocinogenic potential has been documented as well [38,39]. However, the suitability of *Lp. plantarum* in terms of cell growth and bacteriocin production, under sourdough related conditions, has not been evaluated so far.

The WDA for plantaricin activity assessment revealed that the six *Lp. plantarum* strains were clustered into two groups, most likely based on the plantaricin associated genes present in their *pln* plocus. More accurately, *Lp. plantarum* strains LQC 2320 and 2520, harboring 18 *pln* genes, exhibited maximum plantaricin activity, barely reaching 160 AU/mL, while maximum plantaricin activity produced by strains LQC 2422, 2441, 2485 and 2516, in which four *pln* genes were present, was quantified as 2560 AU/mL. Among the 18 genes harbored in strains LQC 2320 and 2520, the presence of *plNC8-IF, plNC8-HK* and *plnD*, constituting the regulatory operon *plNC8-IF-HK-D*, which induces the transcription of the other operons and its own operon as well, was evident. On the other hand, the remaining four bacterial strains harbored the genes encoding plantaricin 423, a structural protein (*PlαA*), a putative immunity one (*PlαB*) and two putative translocation proteins (*PlαC*, *PlαD*) [40]. Plasmid encoded plantaricin 423 belongs to the class IIa or pediocin like bacteriocins, with strong antilisterial activity.

Temperature constitutes a crucial factor in cell growth and bacteriocin production. Several authors have suggested that maximum plantaricin activity levels are recorded at temperature values below the optimum one for cell growth [41,42]. However, this is not a rule, since there are cases of maximum bacteriocin activity levels recorded at temperature values close to the optimum for cell growth [43]. In this study, temperatures applied for the preparation of the so-called type I sourdoughs, namely, 20 and 30 °C, were used as sourdough related parameters. In addition, 37 °C, corresponding to the temperature at which the highest counts of *Lp. plantarum* strains were obtained [39,44], was included in the present study as well. Results obtained in the present study revealed that, for strains LQC 2441, 2422, 2485 and 2516, growth at 30 and 37 °C, and not at 20 °C, facilitated bacteriocin production. In the case of bacterial strains LQC 2320 and 2520, growth temperature did not play a crucial role. Consistent with our results, Parlindungan et al. [39] reported that both 30 and 37 °C were the optimal temperatures for bacteriocin production by *Lp. plantarum* B21. In addition, Todorov et al. [45] reported similar bacteriocin ST26MS and ST28MS production by *Lp. plantarum* strains, both at 30 and 37 °C.

Initial pH values have a pronounced effect on bacteriocin activity. According to the literature data, commonly recorded maximum bacteriocin activity levels correspond to initial pH values ranging from 5.5 to 6.0. [43]. In the present study, two pH values,

namely, 5.0 and 6.0, close to the pH of a wheat flour and water mixture, were included. The data obtained revealed lower levels of plantaricin activity by the four aforementioned *Lp. plantarum* strains after growth in a medium adjusted to an initial pH of 5, compared to that at pH 6. This is consistent with the previous data from Todorov [46,47], according to which higher levels of bacteriocin activity were obtained in MRS broth adjusted to pH 6, compared to that at pH 5.

Regarding growth medium, there is a consensus that it constitutes one of the most significant factors in bacteriocin production. In the present study, three substrates, namely, MRS broth, mMRS broth and WFE, were included. Decreased population levels and plantaricin activity were recorded after the growth of the six bacterial strains in WFE, compared to the respective in MRS and mMRS broth. This can be assigned to the presence of specific nutrients in MRS and mMRS broth, which are missing from WFE. More specifically, yeast extract that serves as a nitrogen source for cell growth with an abundance of different amino acids, elements such as magnesium and phosphorus and even Tween 80 have been reported to positively interfere with bacteriocin production in a strain dependent way [45]. In agreement with our results, Paramithiotis et al. [19] reported a decreased plantaricin activity during radish fermentation, compared to growth in MRS broth. In addition, Todorov et al. [46] reported decreased activity levels as well, after the growth of bacteriocin producing *Lp. plantarum* strains in BHI broth, M17 broth, 10%, *w*/*v* soy flour or 10%, *w*/*v* molasses. The aforementioned observation was probably attributed to the absence of specific nutrients from the medium. As far as the effect of sugars on bacteriocin activity was concerned, Leroy et al. [37] reported that supplementing a sourdough simulation medium with a combination of three (6.67 g/L of glucose, fructose, maltose, respectively) or four sugars (5 g/L of glucose, 5 g/L of fructose, 10 g/L of maltose, 2.5 g/L of sucrose) resulted in higher bacteriocin activity levels by *Lactobacillus amylovorus* DCE 471, compared to single energy sources. However, a direct comparison with MRS broth would be interesting. Other studies by Todorov et al. [46,47] revealed that carbohydrates, applied as sole energy sources, affect bacteriocin production, in a concentration and strain dependent way. More accurately, in some cases, the fortification of MRS broth with glucose, sucrose or maltose at 20 g/L yielded higher bacteriocin activity by *Lp. plantarum* strains, compared to the control, while in other cases supplementation of the medium with 20 or 50 g/L did not improve bacteriocin activity. However lower concentrations of glucose, such as 5 or 10 g/L, resulted in decreased bacteriocin activity, probably due to rapid consumption of the carbon source. Similar was the case when bacterial strains grew in the presence of 20 g/L of fructose.

Medium components, such as NaCl, constitute stress factors that, at specific concentrations, could be beneficial for bacteriocin production [48]. NaCl has a significant role in dough preparation, since it affects the sensory characteristics of the dough and strengthens its structure as well. In the present study, two NaCl concentrations were applied, namely, 0 and 1.8%, with the latter content retrieved from traditional bread recipes of Greece. In the majority of the cases, the different NaCl concentrations had no statistically significant effect on neither bacteriocin activity nor the growth of the six *Lp. plantarum* strains. Regarding the former, the effect seems to be, at least, species dependent. Indeed, the enhancement of lacticin 481 production by *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30 [49] and amylovorin L471 by *Lb. amylovorus* DCE 471 [36] were reported with the addition of 0.2 M and 10 g L^{-1} NaCl, respectively, in the growth medium. Similarly, Leal-Sanchez et al. [50] reported that the best conditions for plantaricin S production by *Lp. plantarum* LPCO10 included the addition of 2.3–2.5% NaCl. On the contrary, the addition of 1% NaCl negatively affected the production of sakacin K by *Latilactobacillus sakei* CTC94 [51] and the addition of 2% NaCl negatively affected the production of carnobacteriocin B2 by *Carnobacterium piscicola* A9b [52] and curvacin A by *Lt. curvatus* L471 [53]. Consistent with our results, Drosinos et al. [54] reported similar maximum bacteriocin titres by *Leuconostoc mesenteroides* E131 both at 0 and 2.5% NaCl. The exact mechanism by which NaCl affects bacteriocin production is yet to be fully elucidated; however, in some cases, interaction with induction factors has been exhibited [55–57]. Similarly, the effect of NaCl on bacterial growth can also

be species or strain dependent [49–53]. Notably, bacterial growth is not always correlated with bacteriocin activity [58,59].

Regarding the relative transcription of the 18 *pln* genes under sourdough simulated conditions, temperature and growth substrate had a pronounced effect on the transcript levels of genes, harbored in bacterial strains LQC 2520 and 2320, respectively. In the case of bacterial strains LQC 2422, 2441, 2485 and 2516, substrate and strain were found to significantly affect the relative transcription of the three *pln* genes. In a previous study, Paramithiotis et al. [19] reported that the *pln* gene transcription was not affected by the experimental conditions, namely, radish fermentation vs. growth in MRS broth. Earlier studies by Hurtado et al. [60] and Doulgeraki et al. [61] assessed the effect of different NaCl concentrations on the expression of *pln* genes, a parameter which was excluded from RT-qPCR analysis in our study, since only a marginal proportion of the variance observed could be attributed to NaCl addition.

In our study, *plnE*, *plNC8-IF*, *plNC8-HK*, *plnD* were some of the least affected genes for the majority of treatments applied and were further characterized by a very strong correlation between their transcriptomic responses. The fact that *plNC8-IF*, *plNC8-HK* and *plnD* encode an induction peptide, a histidine protein kinase and a response regulator, respectively, of the regulatory operon, probably justifies their similar transcriptomic profiles. On the other hand, *plnG* harbored in bacterial strains LQC 2320 and 2520 and *plαD* found in strains LQC 2422, 2441, 2485 and 2516, were the most affected genes by culture conditions. Strong and very strong correlations were recorded between the transcriptomic response of genes corresponding to the same operons, namely, bacteriocin operons (*plnEFI*, *plnRLKJ*, *plNC8αβc*), truncated transport operon, (*plnGHS*), followed by *plnY*. However, in the gene cluster *pln423*, *plαB* and *plαD*, only the correlation between the transcriptomic responses of the two former genes was very strong. As far as the correlation between plantaricin activity and gene transcription was concerned, a weak correlation was evident only in some cases. This observation is consistent with Hurtado et al. [60] and Paramithiotis et al. [19], which attributed the inability to correlate bacteriocin activity with gene expression, to post-transcriptional, post-translational modifications and physicochemical parameters interfering in the WDA, as well. Although an obvious correlation between WDA and relative gene transcription was not established, the status of unregulated *plNC8-IF*, *plNC8-HK* and *plnD* (constituting the regulatory operon) upon temperature increase and pH decrease, was in agreement with the temperature and initial pH independent plantaricin activity exhibited by both strains LQC 2320 and 2520. Similar was the case for the downregulation of *pln* genes after growth of bacterial strain LQC 2320 in WFE at 30 and 37 °C at pH 6 was consistent with the phenotypically determined decreased plantaricin activity of the strain, after growth in WFE.

Summarizing the results of the present study, there are two major findings that need to be taken into consideration in future studies. The first refers to the specificity of the bacteriocins produced. It seems that the specificity of Pln423 against *L. monocytogenes* played a decisive role in the increased antilisterial activity of the cell free supernatants of the strains that carry the *pln423* gene. Thus, this gene may serve as a target in genetic screening approaches. In addition, antimicrobial activity in general may result from a mixture of bacteriocins, with possibly variable levels of specificity to the indicator microorganism used. In the present study, the physical clustering of the *pln* genes into a single cluster is probably the reason for their comparable transcriptomic response, which was observed in many cases, to the environmental stimuli examined. However, there were also cases in which differences in the transcriptomic responses were observed. These cases probably arise from differences in the transcriptional organization between the different loci, which needs to be further assessed. The second finding refers to the lack of correlation between gene transcription and plantaricin activity. This may be attributed to post-transcriptional or post-translational modifications, to the compromised stability of mRNA and the resulting proteins [62] as a result of the environmental stimuli examined, or to physicochemical

parameters interfering with WDA. Such effects need to be fully elucidated, especially in food systems, in order to enable optimization of bacteriocin activity.

5. Conclusions

Plantaricin activity is a strain dependent property; the six strains included in the present study were differentiated into two groups on the basis of their phenotype and this was supported by the organization of their *pln* locus. Growth substrate, temperature, initial pH value and bacterial strains were found to have a significant effect on plantaricin activity; on the contrary, NaCl had only a marginal effect. Similarly, growth substrate and temperature seemed to have a more pronounced effect than initial pH value, on the transcription of the genes under study, always in a strain dependent manner. Only weak correlation was observed between plantaricin activity and the transcription of some of the genes assessed. However, a strong correlation between the transcription of the genes belonging to the same locus was observed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7040320/s1, Figure S1: Growth expressed in log (CFU/ mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in MRS broth adjusted to initial pH 6, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum strains*, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S2: Growth expressed in log (CFU/ mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in MRS broth adjusted to initial pH 5, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S3: Growth expressed in log (CFU/ mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in mMRS broth adjusted to initial pH 6, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S4: Growth expressed in log (CFU/mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in mMRS broth adjusted to initial pH 5, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S5: Growth expressed in log (CFU/ mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in WFE broth adjusted to initial pH 6, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S6: Growth expressed in log (CFU/ mL) (■), pH reduction (•) and plantaricin activity expressed in AU/ mL (bars) of 6 *Lp. plantarum* strains, after incubation in WFE broth adjusted to initial pH 5, at 30 (A), 37 (B) and 20 °C (C), over a period of 33 h. The effect of NaCl concentration (0 and 1.8%) is indicated by numbers, namely 1 and 2, respectively. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to blue, red, green, pink, black and orange color, respectively. Figure S7: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B1) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of the aforementioned genes, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at initial pH 6, for 21 h. The lowest temperature was used as control. (B2) Effect of temperature increase on the relative gene transcription, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE, at initial pH 5, for 21 h. The lowest temperature was used as control. If visible, blue, yellow, red,

light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S8: (C1) Effect of substrate on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2320 in MRS, mMRS broth and WFE at initial pH 6, at 30, 37 and 20 °C, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. (C2) Effect of substrate on the relative transcription of the plantaricin genes at the aforementioned conditions, at initial pH 5. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S9: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B1) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of the aforementioned genes, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at initial pH 6, for 21 h. The lowest temperature was used as control. (B2) Effect of temperature increase on the relative gene transcription, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE, at initial pH 5, for 21 h. The lowest temperature was used as control. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S10: (C1) Effect of substrate on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, during growth of *Lp. plantarum* strain LQC 2520 in MRS, mMRS broth and WFE at initial pH 6, at 30, 37 and 20 °C, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. (C2) Effect of substrate on the relative transcription of the plantaricin genes at the aforementioned conditions, at initial pH 5. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S11: Effect of strain on the relative transcription of *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, was obtained by comparing the transcription of the aforementioned genes after growth of *Lp. plantarum* strains LQC 2320 and 2520 in MRS, mMRS broth and WFE, both at pH 5 and pH 6, at 30, 37 and 20 °C, for 21 h, using LQC 2320 as control. If visible, blue, yellow, red, light green, fuchsia, orange, green, dark blue, khaki, light gray, purple, tyrquoise, gold, gray, dark red, dark green, pink and black bars correspond to *plNC8a*, *plNC8b*, *plNC8c*, *plnL*, *plnR*, *plnK*, *plnJ*, *plnE*, *plnF*, *plnH*, *plnS*, *plnY*, *plNC8-IF*, *plNC8-HK*, *plnD*, *plnI*, *plnM* and *plnG*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S12: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2441 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2441 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2441 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, at 30, 37 and 20 °C,

for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below −1 (the values that were used as threshold) at $p < 0.05$. Figure S13: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2422 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2422 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2422 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, at 30, 37 and 20 °C, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S14: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2516 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2516 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, at 30, 37 and 20 °C, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S15: (A) Effect of pH decrease, namely from 6 to 5, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of *Lp. plantarum* strain LQC 2485 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Growth at pH 6 was used as control. (B) Effect of temperature increase, namely from 20 to 30 and 37 °C, on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2485 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, for 21 h. The lowest temperature was used as control. (C) Effect of substrate on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD*, during growth of strain LQC 2485 in MRS, mMRS broth and WFE, both at initial pH 6 and pH 5, at 30, 37 and 20 °C, for 21 h, using MRS broth as control. In the comparison between WFE and mMRS broth, the latter was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Figure S16: (A) Effect of strain on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD* during growth of the *Lp. plantarum* strains LQC 2441, 2422, 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Strain LQC 2441 was used as control. (B) Effect of strain on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD* during growth of the *Lp. plantarum* strains LQC 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Strain LQC 2422 was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $P < 0.05$. Figure S17: Effect of strain on the relative transcription of *pln423* (*plαA*), *plαB* and *plαD* during growth of the *Lp. plantarum* strains LQC 2485 and 2516 in MRS, mMRS broth and WFE, at 30, 37 and 20 °C, for 21 h. Strain LQC 2485 was used as control. If visible, lime, coral and aquamarine bars correspond to *pln423* (*plαA*), *plαB* and *plαD*, respectively. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below -1 (the values that were used as threshold) at $p < 0.05$. Table S1: Mean plantaricin activity (AU/ mL), population (log CFU/ mL) and final pH value, with 95.0% confidence intervals. The means were obtained from the in-pair interactions of sourdough related parameters. Independent factors included: 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 corresponding to 1, 2, 3, 4, 5 and 6, three incubation temperatures, namely 20, 30

and 37 °C, three substrates, namely MRS broth, mMRS broth and WFE corresponding to 1, 2, 3 and two initial pH values, namely 5 and 6. Table S2: Mean plantaricin activity for each strain, with 95.0% confidence intervals. The treatment was applied at regular time intervals of 3 h, over a period of 33 h. The 6 *Lp. plantarum* strains, namely LQC 2441, 2422, 2516, 2485, 2320 and 2520 correspond to 1, 2, 3, 4, 5 and 6, respectively. Table S3: Pearson product moment (r) correlations between the relative transcription of the 18 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2320 and 2520 and plantaricin activity. Table S4: Pearson product moment (r) correlations between the relative transcription of the 3 plantaricin genes present in the genome of *Lp. plantarum* strains LQC 2422, 2441, 2485 and 2516 and plantaricin activity.

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