RESEARCH ARTICLE

Quorum sensing in sourdough *Lactobacillus plantarum* DC400: Induction of plantaricin A (PlnA) under co-cultivation with other lactic acid bacteria and effect of PlnA on bacterial and Caco-2 cells

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This work aimed at showing the effect of pheromone plantaricin A (PlnA) by *Lactobacillus plantarum* DC400 towards other sourdough lactic acid bacteria and the potential of PlnA to protect the function of the human intestinal barrier. Growth and survival of sourdough lactic acid bacteria were differently affected by co-cultivation with *L. plantarum* DC400. Compared to mono-cultures, *Lactobacillus sanfranciscensis* DPPMA174 and *Pediococcus pentosaceus* 2XA3 showed growth inhibition and decreased viability when co-cultured with *L. plantarum* DC400. *L. sanfranciscensis* DPPMA174 induced the highest synthesis of PlnA. Survival of strain DPPMA174 only slightly varied by comparing the addition of PlnA to the culture medium and the co-cultivation with *L. plantarum* DC400. Compared to mono-culture, the proteome of *L. sanfranciscensis* DPPMA174 grown in co-culture with *L. plantarum* DC400 showed the variation of expression of 58 proteins (47 over expressed and 11 repressed). Thirty-four of them were also over expressed or repressed during growth of DPPMA174 with PlnA. Fifty-one of the above 58 proteins were identified. They had a central role in stress response, amino acid, energy and nucleotide metabolisms, membrane transport, regulation of transcription, and cell redox homeostasis. PlnA markedly increased the viability of human Caco-2/TC7 cells and the transepithelial electrical resistance.

Keywords:
Cytokines / Lactic acid bacteria / Microbiology / Plantaricin A / Quorum sensing

1 Introduction

Lactic acid bacteria comprise a large group of Gram-positive bacteria which are used in the manufacture of fermented dairy, cereal, meat and vegetable foods. Some of these bacteria are restricted to specific niches and have limited physiological abilities. This is the example of *Lactobacillus sanfranciscensis* which is only found in cereal sourdough products [1]. Other lactic acid bacteria such as *Lactobacillus plantarum* are more versatile and are frequently isolated in fermented foods and plant materials [2, 3], or from the human gastrointestinal (GI) tract [4, 5]. Some of the key factors responsible for the robustness of *L. plantarum* were ascribed to the high metabolic plasticity [6, 7] and multiple

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Abbreviations: AIP, autoinducing peptides; **CDM,** chemically defined medium; **CFS,** partially purified cell-free supernatants; **GI,** human gastrointestinal tract; **IFN-γ,** interferon-γ; **MDLC,** multidimensional HPLC; **QS,** quorum sensing; **TEER,** transepithelial electrical resistance; **VOC,** volatile organic compounds; **WFH,** wheat flour hydrolysate medium

quorum sensing (QS) regulatory networks [8]. In particular, QS allows the cells to respond, in a concerted manner, to changes in the surrounding environment through fine adaptation of the metabolic response [8]. One class of bacterial QS signalling molecules corresponds to autoinducer 2 (AI-2) synthesized through the activity of LuxS enzyme [9–11]. Besides, most Gram-positive bacteria use autoinducing peptides (AIP, or peptide pheromones) which act as species-specific communication signals. The gene for AIP often flanks a two-component regulatory system (QS-TCS) gene cassette [12]. QS-TCS comprises the membrane-located histidine protein kinase (HPK) that monitors one or more environmental factors, and the cytoplasmic response regulator (RR) that modulates the expression of specific genes [13]. The genome of *L. plantarum* WCFS1 contains relatively high numbers of peptide-based QS-TCS, as well as other putative QS genes [8]. Some studies [14] demonstrated that competing microorganisms may activate specific component regulatory systems involved in microbial antagonism such as the plantaricin system which is regulated through the QS pathway [15, 16]. The secreted pheromone plantaricin A (PlnA) serves as the tool to measure the cell density of the synthesizing culture. At certain cell density, PlnA triggers a series of phosphorylation reactions of HPK and RR, resulting in the phosphorylated RR. Phosphorylated RR binds to promoters of the bacteriocin regulon and activates all the genes involved in the bacteriocin biosynthesis (*e.g.*, plantaricins EF, JK, NC8 and J51) [16, 17]. PlnA seems to have strain-specific antimicrobial activity [18]. Upon interaction with membrane lipids, the structure of PlnA changes to α-helical conformation. This enables the nonchiral interaction with the target cell membrane, where PlnA binds to receptor and mediates the pheromone effect [19]. The membrane-interacting mode of action may explain why PlnA also displays antibacterial activity towards sensitive strains. Another interesting activity of PlnA was shown onto rat pituitary cells [20]. PlnA preferentially permeabilized cancerous with respect to normal cells, and it has the capacity to differentiate between inner and outer membrane leaflet of cells [20]. Recently [21, 22], it was shown that the *pln* locus of *L. plantarum* strains has a mosaic-like structure with different modules and reorganizations which contain: (i) highly conserved regions for transport and bacteriocin maturation, and (ii) variable regions for regulation and bacteriocin synthesis. Studies on PlnA and, more in general, on QS under stressful conditions such as co-cultivation with competing bacteria may give new insights on the mechanisms of bacterial adaptation.

L. plantarum is a facultative hetero-fermentative bacterium that dominates in most of the European sourdoughs [23]. Sourdough is a typical example of complex food ecosystem, where bacterial behavior and performance are influenced by interactions between coexisting species [23]. Recently [11, 24], it was shown that the metabolic traits of *L. sanfranciscensis* and *L. plantarum* strains were affected by interactions with other sourdough lactobacilli through LuxS mediated mechanisms of QS.

This work aimed at investigating the AIP mediated molecular mechanisms of QS in *L. plantarum* DC400 when co-cultured with other sourdough lactic acid bacteria. AIP and volatile organic compounds (VOC) were monitored through multidimensional HPLC (MDLC) coupled with electrospray-ionisation (ESI)-ion trap MS (nano-ESI-MS/MS) and gas-chromatography mass spectrometry/solid-phase microextraction (GC-MS/SPME), respectively. The effect of PlnA towards cell viability and protein expression of *L. sanfranciscenis* DPPMA174 was determined through 2-DE coupled with nano-ESI-MS/MS. Viability of Caco-2/TC7 cells (human colon carcinoma) and transepithelial electrical resistance (TEER) were assayed to show the effect of PlnA towards human intestinal mucosa.

2 Materials and methods

2.1 Bacterial strains and culture condition

Lactobacillus plantarum DC400 and DPPMA20, *Lactobacillus paralimentarius* 8D, *Lactobacillus pentosus* 12H5, *Lactobacillus brevis* CR13, *Lactobacillus reuteri* D13, *Lactobacillus sanfranciscensis* DPPMA174, *Pediococcus pentosaceus* 2XA3, and *Weissella cibaria* 10XA16, were identified previously from Italian sourdoughs by 16S rRNA gene sequence analysis. Strains were propagated at 30°C for 24 h in SDB broth [25].

Growth of lactic acid bacteria was assayed in modified chemically defined medium (CDM, Amino Acid Assay Medium, Difco Laboratories, Detroit, USA). The modification was the use of mineral salts at one half concentration with respect to commercial CDM. Twenty-four hours-old cells of lactic acid bacteria were grown in SDB and inoculated (4% v/v) into CDM. *L. plantarum* DC400 was grown at 30°C for 7 (mid-exponential phase of growth), 12 (late-exponential phase of growth) or 18 h (stationary phase of growth) in co-culture with *L. plantarum* DPPMA20, *L. paralimentarius* 8D, *L. pentosus* 12H5, *L. brevis* CR13, *L. reuteri* D13, *L. sanfranciscensis* DPPMA174, *P. pentosaceus* 2XA3 or *W. cibaria* 10XA16. Co-cultivation was carried out into a double culture vessels apparatus separated by a 0.4 μm membrane filter (Millipore Isopore™), under stirring conditions (140 rpm) [11, 24, 26, 27]. Cells grown in mono-culture were used as the control. Mono- and co-cultures of each lactic acid bacterium were also cultivated into wheat flour hydrolysate (WFH) medium [11, 24]. WFH was prepared as described previously [28]. Each fermentation was carried out in triplicate.

2.2 Growth kinetics

Growth data were modelled according to the Gompertz equation as modified by Zwietering *et al.* [29]: $y = k + A \exp - \exp[(\mu_{max}\, e/A)(\lambda\text{-}t) + 1]$; where y is the extent of growth as log CFU/mL at the time t; k is the initial cell density as log

CFU/mL; *A* represents the difference in cell density between inoculation and the stationary phase; μ_{max} is the maximum growth rate as Δlog CFU/mL/h; λ is the length of the latency phase expressed in hours, and *t* is the time. The experimental data were modelled through the non-linear regression procedure of the statistic package Statistica per Windows (Statsoft, Tulsa, Oklahoma, USA).

2.3 Cell numbers and viability

Cell numbers were determined by plating on SDB agar at 30°C for 48 h. Cell viability was estimated by using LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Cambridge Bioscience, UK) according to the manufacturer's instruction. Stained bacterial suspensions were observed using a confocal laser scanning microscopy (Leica Microsystems, Milan, Italy) with a 60x objective. Images were analyzed using Image-Pro® Plus image analysis software (Media Cybernetics, Silver Spring, MD).

2.4 Determination of AIP

AIP were determined searching liberated peptides through reverse-phase high pressure liquid chromatography (RP-HPLC) analysis. Cell-free supernatants (CFS) of mono- and co-cultures grown at 30°C for 18 h were used. Aliquots of 1 mL of CFS were added to TFA (0.05% v/v, final concentration) and centrifuged at 10 000 × *g* for 10 min. The supernatant was filtered through a 0.22 μm (pore size) filter. HPLC analysis was carried out with an ÄKTA Purifier system (GE Healthcare, Milan, Italy), equipped with a UV detector operating at 214 nm, and using a reverse-phase C18 XTerra column (Waters, Milford, Massachusetts). Gradient elution was carried out at a flow rate of 1 mL/min, using a mobile phase consisting of water and 2-propanol containing 0.05% TFA. The concentration of 2-propanol was increased linearly from 5 to 46% between 16 and 62 min, and from 46 to 100% between 62 and 72 min. All fractions from this and further step of purification were analyzed by MDLC coupled with ESI-ion trap MS to detect specific fractions containing AIP. The HPLC apparatus consisted of an Ettan MDLC Multidimensional Liquid Chromatography (GE Healthcare) equipped with a Zorbax 300 SD C18 pre-column (5 × 0.3 mm) and a Thermo Electron BioBasic-8 column (150 × 0.18 mm). MDLC was connected to a Finningan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron, Milan, Italy) through the nano-ESI interface (nano-ESI-MS). Aliquots of 10 μL of sample were injected. HPLC separation was carried out at a flow rate of 75 μL/min using gradient elution with (A) water and (B) 84% ACN, both containing 0.1% (v/v) formic acid. The following program was used: 0% eluent B for 30 min; 0 to 100% (v/v) eluent B in 100 min, isocratic elution with 100% eluent B for 100 min, return to 0% eluent B in 5 min, and column reconditioning for 30 min. The flow rate at the nano-ESI source was 2.5 μL/min. The LCQ spectrometer, completely controlled by the Xcalibur software (Thermo Electron), operated in the positive ion mode; MS chromatograms in the total ion current (*m/z* range, 50 to 2,000) and selected ion monitoring modes were recorded for each sample. The sequences deposited on the NCBI-BLAST database were used as references. In addition, chemically synthetised peptides (PlnA, Lys-Ser-Ser-Ala-Tyr-Ser-Leu-Gln-Met-Gly-Ala-Thr-Ala-Ile-Lys-Gln-Val-Lys-Lys-Leu-Phe-Lys-Lys-Trp-Gly-Trp; pltA, Glu-Gln-Leu-Ser-Phe-Thr-Ser-Ile-Gly-Leu-Gln-Leu-Leu-Thr-Ile-Gly-Thr-Arg-Ser-Cys-Trp-Phe-Phe-Tyr-Cys-Arg-Tyr and lp_3089, Met-Val-Gln-Trp-Ala-Lys-Arg-Phe-Ser-Glu-Thr-Lys-Glu-Pro-Val-Val-Leu-Ile-Ser-His-Asn-Gln-Asn-Arg-Cys-Ala-Gly-Lys-Ile-Val-Val-Leu-Met-Met-Ser-Arg-Leu-Glu-Leu-Trp-Gly-Ser) were used as the internal standards [8].

2.5 Purification of PlnA

Mono-culture of *L. plantarum* DC400 and co-cultures of *L. plantarum* DC400 with *L. plantarum* DPPMA20, *L. pentosus* 12H5, *L. sanfranciscensis* DPPMA174 or *P. pentosaceus* 2XA3 were used. Cells were removed by centrifugation at 4000 × *g* for 15 min at 4°C, and 300 g of ammonium sulphate were added *per* liter of CFS. The protein/peptide precipitate was pelleted by centrifugation at 7000 × *g* for 20 min and solubilized in 20 mM of potassium phosphate buffer, pH 7.0. The sample was applied onto a 7 mL S-Sepharose Fast Flow cation exchange column at a flow rate of 1 mL/min. As estimated by MDLC coupled with nano-ESI-MS, the fraction containing PlnA was applied to a FPLC Resource HIC column (GE Healthcare). Proteins/peptides were eluted with a linear gradient of $(NH_4)_2SO_4$ (1.7–0 M), in 0.05 M potassium phosphate buffer, pH 7.5, at a flow rate of 60 mL/h. After desalting, the fraction containing PlnA was further purified using a C2/C18 reverse phase column (PepRPC HR 5/5). Gradient elution was carried out at a flow rate of 1 mL/min, using a mobile phase consisting of water and 2-propanol containing 0.05% TFA. The concentration of 2-propanol was increased linearly from 5 to 46% between 16 and 62 min, and from 46 to 100% between 62 and 72 min. Fraction containing PlnA was re-chromatographed onto MDLC using a Zorbax 300 SD C18 pre-column (5 × 0.3 mm) and a Thermo Electron BioBasic-8 column (150 × 0.18 mm) with a gradient of ACN containing 0.05% TFA. The concentration of PlnA was also determined by the ophthaldialdehyde (OPA) method [30].

2.6 Protein extraction and 2-DE

2-DE analysis was carried out on cells of *L. sanfranciscensis* DPPMA174 grown (18 h at 30°C) in mono-culture, co-culture with *L. plantarum* DC400 or with the purified PlnA added to the culture medium. Cells were washed in 0.05 M Tris-HCl pH 7.5, centrifuged (15 000 × *g* for 15 min at 4°C)

and frozen or directly re-suspended in denaturing buffer composed of 8 M urea, 4% CHAPS, 40 mM Tris base and 65 mM DTT. To extract total proteins, cells were disrupted with a Branson model B15 sonifier by three cycles of sonication (1 min each) [31]. After pelleting of unbroken cells (15 000 × *g* for 15 min at 4°C), the protein concentration of the supernatant was determined by the method of Bradford [32]. Two-DE was carried out using the immobiline/polyacrylamide system, essentially as described by Görg *et al.* [33] and Hochstrasser *et al.* [34], using a Pharmacia 2D-EF system (GE Healthcare). The same amount of total protein (45 μg) was used for each electrophoretic run. Isoelectric focusing was carried out on 18 cm immobiline strips providing a non-linear pH 3-10 gradient (IPG strips, GE Healthcare) by IPG-phore, at 15°C. Voltage was increased from 300 to 5000 V during the first 5 h, then stabilized at 8000 V for 8 h. After electrophoresis, IPG strips were equilibrated as described by De Angelis *et al.* [31]. Following electrophoresis, IPG strips were equilibrated for 12 min against buffer A (6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.05 M Tris-HCl pH 6.8, 2% w/v DTT) and for 5 min against buffer B (6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.05 M Tris-HCl pH 6.8, 2.5% w/v iodoacetamide, 0.5% bromophenol blue). For the second dimension, 12.5% homogeneous SDS-PAGE gels were used. Gels calibration and spot detection were performed as described by De Angelis *et al.* [31]. Gels were stained by using Silver and colloidal Coomassie Blue methods. The protein maps were scanned with a laser densitometer (Molecular Dynamics 300s) and analyzed with the ImageMaster 2D Platinum v6.0 computer software (GE Healthcare). Three gels from three independent experiments were analyzed and spot intensities were normalized as reported by Bini *et al.* [35]. In particular, the spot quantification for each gel was calculated as relative volume (%VOL); the relative VOL was the VOL of each spot divided by the total VOL over the whole image. In this way, differences in color intensities among the gels were eliminated [31, 36]. The induction factor is defined as the ratio between the spot intensity of a protein from cells co-cultivated with *L. plantarum* DC400 or with purified fraction containing PlnA and the spot intensity of the same protein from cells grown in mono-culture. The reduction factor is defined as the ratio between the spot intensity of a protein from cells grown in mono-culture and the spot intensity of the same protein from cells co-cultivated with *L. plantarum* DC400 or with purified fraction containing PlnA. The reduction factor for individual proteins was expressed as the ratio between spot intensity of the same protein in cells grown in mono- *vs.* co-culture. All the induction or reduction factors were calculated based on the average of the spot intensities of each of the nine gels and standard deviation was calculated.

2.7 Nano LC-ESI-MS/MS

Protein identification was carried out at the Proteome Factory (Proteome Factory, Berlin, Germany). The MS system consisted of an Agilent 1100 NanoLC system (Agilent, Germany), PicoTip emitter (New Objective, USA) and an Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). Proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, UK) and protein database (National Center for Biotechnology Information, Bethesda, MD, USA). Database searches were also done with the peptide masses against the non-redundant NCBI database using the search program ProFound (http://www.prowl.rockefeller.edu/cgibin/ProFound) from Rockefeller University and ProteoMetrics.

2.8 GC-MS/SPME analysis of VOC

After preconditioning according to the manufacturer's instructions, the carboxen-polydimethylsiloxane coated fiber (85 μm) and the manual SPME holder (Supelco, Bellefonte, PA, USA) were used. Before head space sampling, the fiber was exposed to GC inlet for 5 min for thermal desorption at 250°C. Three grams of each sample were placed into 10 mL glass vials and added of 10 μL of 4-methyl-2-pentanol (final concentration of 4 mg/L), as the internal standard. Samples were then equilibrated for 10 min at 45°C. SPME fiber was exposed to each sample for 40 min. Both phases of equilibration and absorption were carried out under stirring condition. The fiber was then inserted into the injection port of the GC for 5 min of sample desorption. GC-MS analyses were carried out on an Agilent 7890A gas-chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5975C mass selective detector operating in electron impact mode (ionization voltage 70 eV). A Supelcowax 10 capillary column (60 m length, 0.32 mm id) was used (Supelco, Bellefonte, PA, USA). The temperature program was: 50°C for 1 min, 4.5°C/min to 65°C and 10°C/min to 230°C, which was held for 25 min. Injector, interface and ion source temperatures were 250, 250 and 230°C, respectively. The mass-to-charge ratio interval was 30–350 amu at 2.9 scans *per* second. Injections were carried out in splitless mode and helium (1 mL/min) was used as the carrier gas. Identification of molecules was carried out based on comparison of their retention times with those of pure compounds (Sigma-Aldrich, Milan, Italy). Identification was confirmed by searching mass spectra in the available databases (NIST version 2005 and Wiley Vers. 1996) and literature [37, 38]. Quantitative data of the identified compounds were obtained by interpolation of the relative areas versus the internal standard area. All data were obtained at least in triplicates.

2.9 Human cell culture

Human intestinal Caco-2 cells (TC7 clone) [39] were cultured in DMEM, supplemented with 10% heat inactivated FBS (Thermo Scientific HyClone), 1% non-essential amino acids

(NEAA), 50 μg/mL gentamicin/streptomycin, 2 mM L-glutamine and 1% HEPES. Cells were routinely grown in RPMI medium (Hyclon, UK) with the above supplementation and maintained in 25 cm^3 culture flasks at 37°C under humidified atmosphere of 5% CO_2. Experiments were carried out on passages from 65 to 70.

2.10 Human cell proliferation

Cell viability was measured using the Neutral Red (NR) uptake assay [40]. Cells were seeded in 96-well plates at 10^4 cells *per* well and cultivated for 24 h at 37°C. Incubation was carried out with 2.5 μg/mL of PlnA purified from the mono-culture of *L. plantarum* DC400 or from the co-culture with *L. sanfranciscensis* DPPMA174. The fraction purified from the mono-culture of *L. sanfranciscensis* DPPMA174, which eluted under the same chromatographic conditions of PlnA, was used as the negative control. Another negative control was represented by DMEM medium. Chemically synthesized PlnA (2.5 μg/mL) was used as the positive control. All experiments were carried out also using 1000 U/mL of interferon-γ (IFN-γ). After 24, 48 and 72 h incubation, culture media were removed from well plates, and cells were washed with 200 μL PBS and incubated for 4 h at 37°C with 150 μL of freshly prepared NR solution (33 mg/L). Further, cells were washed twice with PBS and 150 μL of lysing solution (50% v/v ethanol in milli-Q water with 1% v/v acetic acid) was added to wells. The 96-well plates were shaken for 10 min and the absorbance was measured at 540 nm using a Novapath microplate reader (Biorad, Hercules, CA).

2.11 Measurement of the TEER

To allow differentiation, Caco-2/TC7 cells were seeded (7.5×10^4 cells/mL) onto 24-well insert plates with polyethylene terepthlate (PET) membrane (pore size of 0.4 μm). Before treatments, growth was allowed for 21 days at 37°C. Treatments for 18, 24 and 48 h were carried out with purified PlnA from the mono-culture of *L. plantarum* DC400 and co-culture with *L. sanfranciscensis* DPPMA174. The integrity of monolayer was monitored by measuring (TEER) through the Millicell-ERS Voltohmmeter (Millipore, Billerica, MA, USA.). DMEM medium was used as the negative control. Chemically synthesized PlnA (2.5 μg/mL) was used as the positive control. All experiments were carried out also using 1000 U/mL INF-γ. Measurements were expressed in Ohms × cm^2, after subtracting mean values of the resistance from cell-free inserts. TEER data were recorded at room temperature.

2.12 Statistical analysis

All data were obtained at least in the three replicates. The percentages were arcsine transformed for data analysis. Analysis of variance (ANOVA) was carried out on transformed data followed by separation of means with Tukey's HSD, using a statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft, Vigonza, Italia).

3 Results

3.1 Kinetics of growth

After 18 h of growth in CDM medium, the mono-culture of *Lactobacillus plantarum* DC400 reached the cell density of 9.27 ± 0.18 log CFU/mL (Table 1). The values of μ_{max} and λ were ca. 0.27 log CFU/mL/h and 3.77 h, respectively. Cell densities of the other lactic acid bacteria ranged from 9.00 ± 0.05 (*Lactobacillus brevis* CR13) to 9.43 ± 0.31 log CFU/mL (*L. plantarum* DPPMA20). The values of μ_{max} varied from 0.11 ± 0.05 (*Lactobacillus pentosus* 12H5) to 0.15 ± 0.04 log CFU/mL/h (*L. plantarum* DPPMA20) as well as λ varied from 0.37 ± 0.07 (*Pediococcus pentosaceus* 2XA3) to 4.20 ± 0.36 h (*Lactobacillus sanfranciscensis* DPPMA174). Compared to mono-culture, the cell density of *L. plantarum* DC400 did not significantly ($p > 0.05$) vary (9.14 ± 0.34–9.39 ± 0.42 log CFU/mL) when co-cultured with the other lactic acid bacteria. Also the cell yield of *L. plantarum* DPPMA20, *Lactobacillus paralimentarius* 8D, *L. pentosus* 12H5, *Lactobacillus reuteri* D13 and *Weissella cibaria* 10XA16 was not affected during co-cultivation with *L. plantarum* DC400. Compared to mono-cultures, cell densities of *L. sanfranciscensis* DPPMA174 and *P. pentosaceus* 2XA3 significantly ($p < 0.05$) decreased (*ca.* 8.08 and 8.39 log CFU/mL, respectively) when co-cultured with *L. plantarum* DC400. Overall, the values of μ_{max} decreased to 0.08–0.09 log CFU/mL/h for all lactic acid bacteria when co-cultured with *L. plantarum* DC400. Except for *L. plantarum* DPPMA20, also the latency phase increased. The highest increase of λ was found for *P. pentosaceus* 2XA3, *ca.* 0.37 (mono-culture) *vs.* 8.35 h (co-culture). Strains of lactic acid bacteria which showed inhibition (*L. sanfranciscensis* DPPMA174 and *P. pentosaceus* 2XA3) or which were not affected (*L. plantarum* DPPMA20 and *L. pentosus* 12H5) under co-cultivation with *L. plantarum* DC400 were selected for further experiments.

3.2 Bacterial viability

After 18 h of growth in CDM medium, the number of cultivable cells of *L. plantarum* DC400 was almost the same as that of live cells (Table 1). Live, dead/damaged and cultivable cells of *L. plantarum* DC400 co-cultured with *L. sanfranciscensis* DPPMA174, *L. pentosus* 12H5 or *P. pentosaceus* 2XA3 did not significantly ($p > 0.05$) vary with respect to the mono-culture. No differences were also found between mono-cultures of *L. plantarum* DPPMA20 or *L. pentosus* 12H5 and their respective co-cultures with

Table 1. Kinetics of growth, live, dead/damaged and cultivable cells in mono- and co-culture with *L. plantarum* DC400

Culture conditions	μmax (log CFU/mL/h)	λ (h)	Cultivable cells (log CFU/mL)[a)]	Live cells (log cell/mL)[b)]	Dead/damaged cells (log cell/mL)[b)]
Mono-culture					
L. plantarum DC400	0.27±0.09	3.77±0.15	9.27±0.18	9.31±0.24	6.1±0.4
L. plantarum DPPMA20	0.15±0.04	4.00±0.38	9.43±0.31	9.52±0.17	6.14±0.14
L. sanfranciscensis DPPMA174	0.14±0.09	4.20±0.36	9.18±0.26	9.20±0.28	6.08±0.22
L. pentosus 12H5	0.11±0.05	3.17±0.16	9.25±0.12	9.27±0.05	6.07±0.08
P. pentosaceus 2XA3	0.12±0.01	0.37±0.07	9.10±0.23	9.09±0.36	6.31±0.11
L. brevis CR13	0.13±0.03	2.98±0.24	9.00±0.05	9.15±0.33	6.30±0.05
W. cibaria 10XA16	0.14±0.01	2.77±0.05	9.28±0.16	9.00±0.18	6.18±0.12
L. paralimentarius 8D	0.14±0.08	3.53±0.29	9.25±0.15	9.17±0.05	6.17±0.18
Co-culture					
L. plantarum DC400[c)]–	0.27±0.11	3.97±0.18	9.20±0.24	9.11±0.34	8.17±0.22
L. plantarum DPPMA20	0.08±0.05	6.58±0.41	9.22±0.19	9.34±0.25	6.05±0.14
L. plantarum DC400–	0.21±0.12	3.67±0.11	9.15±0.35	9.37±0.47	6.28±0.08
L. sanfranciscensis DPPMA174	0.08±0.04	7.23±0.25	8.08±0.10	8.32±0.25	9.02±0.24
L. plantarum DC400–	0.26±0.05	3.70±0.23	9.14±0.16	9.30±0.36	6.08±0.18
L. pentosus 12H5	0.09±0.05	4.88±0.31	9.05±0.43	9.10±0.11	6.37±0.23
L. plantarum DC400–	0.33±0.08	3.81±0.15	9.39±0.32	9.52±0.51	6.11±0.17
P. pentosaceus 2XA3	0.08±0.03	8.35±0.26	8.39±0.42	8.55±0.28	9.12±0.28

a) Cell numbers were estimated by planting on SDB agar medium.
b) Cell numbers were estimated using the LIVE/DEAD *Bac*Light Bacterial Viability Kit.
c) Values refer to strains under co-cultivation. Data are the mean of three independent experiments and±standard deviations are shown.

L. plantarum DC400. As previously shown [11], the number of live cells of *L. sanfranciscensis* DPPMA174 decreased from 9.20±0.28 to 8.32±0.25 log cells/mL when co-cultured with *L. plantarum* DC400. No statistical ($p>0.05$) differences were found between live and cultivable cells. Compared to mono-culture, dead/damaged cells of *L. sanfranciscensis* DPPMA174 significantly ($p<0.05$) increased when co-cultured with *L. plantarum* DC400. When co-cultured with *L. plantarum* DC400, also live cells of *P. pentosaceus* 2XA3 significantly ($p>0.05$) decreased (9.09±0.36 to 8.55±0.28 log CFU/mL). Dead/damaged cells increased from *ca.* 6.31 to 9.12 log cells/mL. Almost the same results were found using WFH. This culture medium was used for further experiments.

3.3 Synthesis of PlnA

CFS of mono- and co-cultures were used to identify AIP through MDLC coupled with nano-ESI-MS/MS. Supporting Information Figure S1A shows the MS full-scan chromatogram relevant to partially purified CFS of *L. plantarum* DC400 co-cultured with *L. sanfranciscensis* DPPMA174. Due to the matrix complexity, several genus could be identified but, in several cases, adjacent peaks were not fully resolved. Nevertheless, it was possible to display all co-eluting genus separately through filtration of the signal on particular *m/z* values (data not shown). All genus identified (*e.g.*, Supporting Information Figure S1B) in the preliminary analysis were isolated within the ion trap of the mass spectrometer and fragmented during further chromatographic run. An MS/MS spectrum was collected for each of them. For instance, Supporting Information Figure S1C shows the MS/MS spectrum of the *m/z* 1493.7 ion which was selected from the MS full-scan chromatogram of the co-culture between *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174. All *m/z* ratios of each MS/MS spectrum, together with the *m/z* ratio of the parent ion, were inserted in the NCBInr database. Before searching, the following parameters were specified: species (*Lactobacillus*), *m/z* ratio tolerance for parent and daughter ions recognition (0.2 Da), and, finally, the instrumentation used for MS analysis (ion trap).

Except for PlnA, no evidences were found for other AIP, at least at the minimum detectable concentration (*ca.* 10 μg/L). The minimum concentration was estimated by injecting each sample of CFS with the synthetic analogues of AIP peptides (PlnA, PltA and lp_3089). PlnA was found in the mono-cultures of *L. plantarum* DC400 and DPPMA20, and in all co-cultures of strain DC400 with the other lactic acid bacteria. Based on these results, CFS containing PlnA were subjected to four chromatographic steps. Purified fractions containing PlnA were further analyzed by nano-ESI-MS to exclude the presence of other contaminating peptides. The concentration of PlnA synthesized by *L. plantarum* DC400 was estimated using a reverse-phase C18 XTerra column and the OPA method (Fig. 1). Compared to mono-culture, the synthesis of PlnA markedly increased in the co-cultures with *P. pentosaceus* 2XA3 and, especially, with *L. sanfranciscensis* DPPMA174 (*ca.* 2.5 μg/mL). When the inoculum of *L. sanfranciscensis* DPPMA174 was decreased to *ca.* 6.5 log CFU/mL, the synthesis of PlnA by *L. plantarum* DC400 did

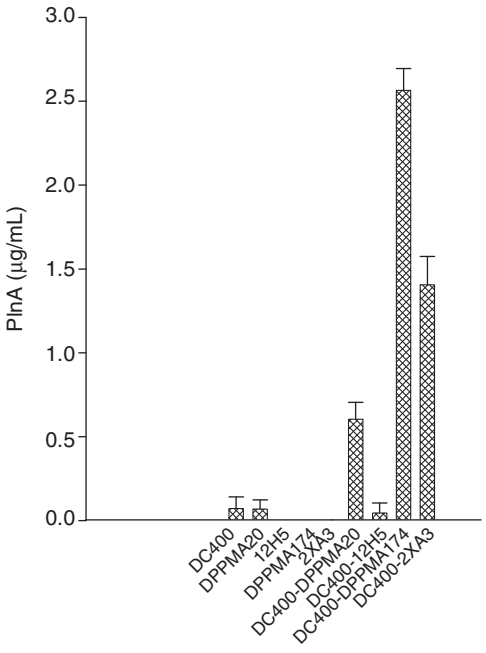

Figure 1. Concentration (µg/mL) of plantaricin A (PlnA) synthesized by mono-culture of *Lactobacillus plantarum* DC400, (DC400); *L. plantarum* DPPMA20 (DPPMA20); *Lactobacillus pentosus* 12H5 (12H5); *Lactobacillus sanfranciscensis* DPPMA174 (DPPMA174); *Pediococcus pentosaceus* 2XA3 (2XA3); and co-culture of *L. plantarum* DC400 with *L. plantarum* DPPMA20 (DC400-DPPMA20); *L. pentosus* 12H5 (DC400-12H5); *L. sanfranciscensis* DPPMA174 (DC400-DPPMA174) or *P. pentosaceus* 2XA3 (DC400-2XA3). Data are the means ± SD of three separate experiments performed in triplicate.

not vary. Almost the same concentration of 2.5 µg/mL was found cultivating *L. plantarum* DC400 with CFS of *L. sanfranciscensis* DPPMA174. The synthesis of PlnA started before the mid-exponential phase (7 h) and increased until the late-exponential phase of growth was reached (12 h).

3.4 Effect of PlnA on the growth of *L. sanfranciscensis* DPPMA174

L. sanfranciscensis DPPMA174 was cultivated in WFH medium supplemented with 2.5 µg/mL of purified or chemically synthesized PlnA (Fig. 2). The addition of purified PlnA caused a decrease of the cell number from *ca.* 9.18 ± 0.26 (mono-culture) to 8.4 ± 0.14 log CFU/mL. Similar results were found using the chemically synthesized PlnA. PlnA (purified or chemically synthesized) had an effect which mirrored that found under co-cultivation with *L. plantarum* DC400. Dead/damaged cells of *L. sanfranciscensis* DPPMA174 cultivated with purified or chemically synthesized PlnA were significantly ($p < 0.05$) higher than those found in the mono-culture (*ca.* 8.80 ± 0.14 *vs.* 6.08 ± 0.22 cells/mL, respectively).

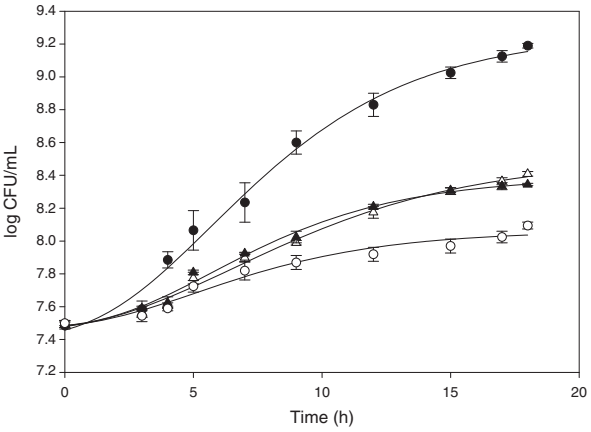

Figure 2. Kinetics of growth of *Lactobacillus sanfranciscensis* DPPMA174. Mono-culture (●); co-culture with *Lactobacillus plantarum* DC400 (○); mono-culture with the purified plantaricin A (PlnA) (2.5 µg/mL) (Δ); and mono-culture with chemically synthesized PlnA (2.5 µg/mL) (▲). Purified PlnA was from the co-culture between *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174. Data are the means ± SD of three separate experiments performed in triplicate.

3.5 2-DE analysis and identification of PlnA induced proteins by nano-LC-ESI-MS/MS

Compared to mono-culture, 2-DE of the cytosolic extracts of *L. sanfranciscensis* DPPMA174 grown (early-stationary phase of growth, 18 h) in co-culture with *L. plantarum* DC400 or with purified PlnA showed an increase of the level of expression (greater than or equal to twofold) of 47 and 29 proteins, respectively (Figs. 3A–C and Table 2). These proteins were distributed over a large range of p*I* (3.9–9.2) and molecular mass (10.0–96.0 kDa). All induced proteins during cultivation with purified PlnA were also over expressed during co-cultivation with *L. plantarum* DC400. Totally, 11 and 5 proteins were repressed in *L. sanfranciscensis* DPPMA174 when co-cultured with *L. plantarum* DC400 or cultivated with PlnA. Also in this case, the five proteins repressed under cultivation with PlnA coincided with those found in the co-culture (Figs. 3A–C and Table 3). Fifty-one of the 58 (47 and 11 over and repressed, respectively) which were differentially expressed during co-cultivation were identified by nano-LC-ESI-MS/MS (Table 2 and Table 3).

3.6 Synthesis of VOC

A range of 25-33 VOC was identified by GC-MS/SPME in the mono-cultures of *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174. Only VOC which showed variations between mono- and co-culture were listed in Table 4. Diacetyl, acetoin, hexadecane and furanone A mainly characterize the mono-culture of *L. plantarum* DC400. Hexadecane, furanone A and, especially, ethyl-acetate were found in the mono-culture of *L. sanfranciscensis* DPPMA174. The identification of furanon A

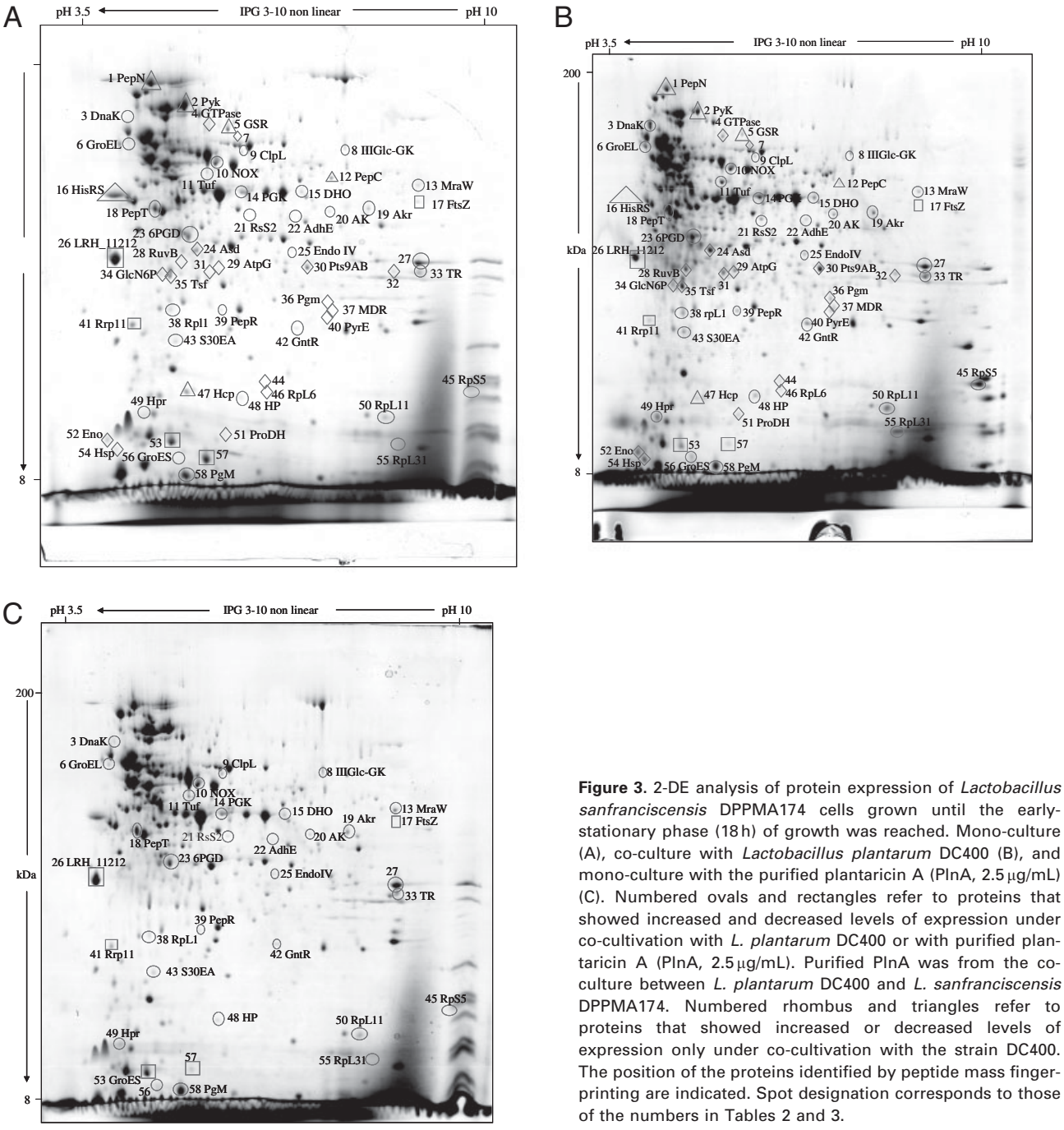

Figure 3. 2-DE analysis of protein expression of *Lactobacillus sanfranciscensis* DPPMA174 cells grown until the early-stationary phase (18 h) of growth was reached. Mono-culture (A), co-culture with *Lactobacillus plantarum* DC400 (B), and mono-culture with the purified plantaricin A (PlnA, 2.5 μg/mL) (C). Numbered ovals and rectangles refer to proteins that showed increased and decreased levels of expression under co-cultivation with *L. plantarum* DC400 or with purified plantaricin A (PlnA, 2.5 μg/mL). Purified PlnA was from the co-culture between *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174. Numbered rhombus and triangles refer to proteins that showed increased or decreased levels of expression only under co-cultivation with the strain DC400. The position of the proteins identified by peptide mass fingerprinting are indicated. Spot designation corresponds to those of the numbers in Tables 2 and 3.

and B was confirmed as described by Ndagijimana *et al.* [37] and Vernocchi *et al.* [38]. Compared to mono-cultures, the co-culture between strains DC400 and DPPMA174 was characterized by a marked decrease of the concentrations of diacetyl, acetoin, ethylacetate and furanone A. On the contrary, the signaling molecule furanon B, and heptadecane and decanoic acid increased or were only synthesized under co-culture condition. Compared to the mono-culture of strain DC400, the concentration of VOC only slightly varied or did not change when co-cultured with *P. pentosaceus* 2XA3, *L. plantarum* DPPMA20 or *L. pentosus* 12H5 (data not shown).

3.7 Effect of PlnA on the viability of human Caco-2/TC7 cells

Caco-2/TC7 cell viability was measured as Neutral Red uptake. Compared to DMEM medium (negative control),

Table 2. Properties and putative function of proteins induced (over expressed) in *Lactobacillus sanfranciscensis* DPPMA174 cells when co-cultured with *Lactobacillus plantarum* DC400 or with purified fraction containing plantaricin A (PlnA) until the early-stationary (18 h) phase of growth was reached

Spot[a)]	Estimated Mr (kDa)	Estimated p*I*	Induction factor[b)]		Homologous protein/function	A.N.[c)]	Organism	Number of peptides	Identity score (%)	Sequence coverage (%)
			DC400	PlnA						
3	66	4.45	3±0.125	2±0.068	Chaperone protein DnaK, DnaK	Q8KML6-1	*L. sanfranciscensis* DSM20451	14	100	34.2
4	65	5.3	2±0.099	–	Stress response membrane GTPase, GTPase	gi|116334005	*L. brevis*	11	100	32.2
6	60	4.5	3.5±0.170	2±0.100	GroEL	gi 62754134	*L. sanfranciscensis*	4	100	34.1
7	60	5.5	2±0.100	–	N.D.[d)]	N.D.	N.D.	N.D.	N.D.	N.D.
8	60	5.9	2.5±0.110	2±0.070	Glycerol kinase, IIIGlc-GK	gi|442946	*E. coli*	2	100	
9	59.5	5.55	2±0.078	2±0.097	ATP-dependent Clp proteinase ClpL, ClpL	gi 62719463	*L. sanfranciscensis*	2	100	38.3
10	53	5.4	2.5±0.055	2±0.070	NADH oxidase, NOX	gi 11862874	*L. sanfranciscensis*	11	100	38.7
11	51.5	5.2	3±0.110	2±0.080	Elongation factor Tu, Tuf	gi|62719442	*L. sanfranciscensis*	5	100	38
13	49	8.5	2±0.100	2±0.097	S-adenosyl-methyltransferase mraW, MraW	ZP_03611748.1	*A. minor* 202	9	100	46.2
14	48	5.55	4±0.200	2±0.175	Phosphoglycerate kinase, PGK	gi 81428220	*L. sakei* subsp. *sakei* 23K	8	100	31.4
15	48	5.75	2±0.095	2±0.170	Dihydroorotase, DHO	YP_796023.1	*L. brevis* ATCC 367	11	100	42.5
18	46	4.70	3±0.145	2±0.198	Putative tripeptidase, PepT	emb|CAI30884.2|	*L. sanfranciscensis*	7	100	52.3
19	45.5	6.6	3±0.140	2±0.100	Aldo/keto reductase, Akr	ZP_00384814.1	*E. sibiricum* 255–15	7	100	38.4
20	45	5.83	2.5±0.095	2±0.170	Acetate kinase, AK	dbj|BAB19266.1|	*L. sanfranciscensis*	8	100	32.2
21	43	5.6	3.5±0.195	2±0.198	30S ribosomal protein S2, RsS2	gi 28378686	*L. plantarum* WCFS1	8	100	40
22	42.5	5.75	3.5±0.250	2±0.196	Bifunctional acetaldehyde-CoA/ alcohol dehydrogenase, AdhE	gi 42519642	*L. johnsonii* NCC 533	8	100	42
23	41	5.0	3±0.150	2.5±0.130	6-Phosphogluconate dehydrogenase, 6PGD	gi 125856984	*L. sanfranciscensis*	3	100	25
24	38	5.05	2.5±0.150	–	Aspartate-semialdehyde dehydrogenase, ASD	gb|EEV24529.1|	*A. minor* 202	8	100	46.6
25	37.5	5.71	2.5±0.155	2±0.180	Deoxyribonuclease IV, EndoIV	C2D4H2-1	*L. brevis* subsp. *gravesensis* ATCC 27305	4	100	10
27	35.5	8.5	2.5±0.115	2±0.105	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
28	35	4.85	3±0.150	–	Holliday junction ATP-dependent DNA helicase, RuvB	YP_795367.1	*L. brevis* (strain ATCC 367/JCM 1170)	3	100	17
29	34	5.41	3±0.170	–	ATP synthase gamma chain, AtpG	YP_795416.1	*L. brevis* (strain ATCC 367/JCM 1170)	1	100	7
30	34	5.78	2±0.098	–	Mannose PTS EIIAB, Pts9AB	NP_784348.1	*L. plantarum* WCFS1	2	100	18
31	33	5.3	3±0.145	–	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
32	33	6.85	2±0.098	–	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
33	32.2	8.35	3±0.160	2±0.180	Transcription regulator, TR	Q88Z17-1	*L. plantarum*	3	100	12.3
34	32	4.75	2±0.090	–	Glucosamine-6-phosphate deaminase, GlcN6P	Q88ZS6	*L. plantarum*	2	100	10.9

Table 2. Continued

Spot[a)]	Estimated Mr (kDa)	Estimated p*I*	Induction factor[b)]		Homologous protein/function	A.N.[c)]	Organism	Number of peptides	Identity score (%)	Sequence coverage (%)
			DC400	PlnA						
35	32	4.8	2±0.076	–	Translation elongation factor ts, Tsf	gb|AAX93327.1|	*L. sanfranciscensis*	4	100	32.3
36	26	5.8	2±0.098	–	Phosphoglycerate mutase, Pgm		*L. plantarum* WCFS1	7	100	34.3
37	24.5	5.83	2±0.098	–	Metal-dependent regulator, MDR	Q88Y10	*L. plantarum* WCFS1	6	100	44.2
38	24	4.8	3±0.140	2±0.100	50S ribosomal protein L1, Rpl1	gi|81429283	*L. sakei subsp. sakei 23K*	6	100	38.8
39	23.5	5.45	3±0.145	2±0.175	Putative aminopeptidase R, PepR	gi 56605310	*L. sanfranciscensis*	2	100	18
40	23.5	5.8	2±0.095	–	Orotate phosphoribosyltransferase, PyrE	YP_796309.1	*L. brevis* (strain ATCC 367/JCM 1170)	6	100	50.5
42	23.1	5.75	3.5±0.170	2±0.172	Transcription regulator, GntR	Q03ND6-1	*L. brevis* (strain ATCC 367/JCM 1170)	3	100	11.7
43	22.7	4.9	3±0.150	2±0.128	Ribosomal protein S30EA, S30EA	NP_784490.1	*L. plantarum* WCFS1	6	100	48.9
44	21	5.65	2±0.075	–	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
45	18.5	9.2	4±0.205	2±0.185	30S ribosomal protein S5, RpS5	gi 28377849	*L. plantarum* WCFS1	5	100	40.4
46	19.5	5.65	1.5±0.078	–	50S ribosomal protein L6, RpL6	gbAAX93325.1	*L. sanfranciscensis*	7	100	44.7
48	18.5	5.55	2±0.105	2±0.15	Hypothetical protein, HP	gi 28379164	*L. plantarum* WCFS1	2	100	11.2
49	18	4.6	3±0.150	2±0.170	Phosphocarrier protein Hpr, Hpr	AN_P23534	*S. carnosus*	1	100	10
50	17.5	6.8	3±0.150	2±0.132	Ribosomal protein L11, RpL11	YP_794764.1	*L. brevis* (strain ATCC 367/JCM 1170)	6	100	57.4
51	16	5.45	2±0.105	–	Proline dehydrogenase, ProDH	ZP_00539188.1	*E. sibiricum* 255–15	7	100	34.4
52	15	3.9	2±0.108	–	Enolase, Eno	BAB81005.1	*C. perfrigens* str. 13	1	100	12
54	14	4.4	2.5±0.120	–	Small heat shock protein Hsp1, Hsp	YP_794314.1	*L. brevis* (strain ATCC 367/JCM 1170)	5	100	45
55	14	7.0	2.5±0.098	2±0.170	50S Ribosomal protein L31, RpL31	BAB07499	*B. halodurans* C-125	3	100	44.4
56	10	4.85	2.5±0.100	2±0.15	GroES co-chaperonin, GroES	AJ831551.1	*O. oeni*	1	100	12
58	10	5.0	2±0.100	2±0.115	β-phosphoglucomutase/glucose-1-P phosphodismutase, PgM	ABF06645	*L. reuteri* ATCC 55730	7	100	48.6

a) Spot designation correspond to those of the gels in Fig. 3A, B and C.
b) The induction factor is defined as the ratio between the spot intensity of a protein from cells co-cultivated with *L. plantarum* DC400 or with purified fraction containing plantaricin A (PlnA) and the spot intensity of the same protein from cells grown in mono-culture. All the induction factors were calculated as the average of the spot intensities of nine gels and ±SD are shown.
c) Accession number.
d) Not identified protein.

Table 3. Properties and putative function of proteins reduced (repressed) in *Lactobacillus sanfranciscensis* DPPMA174 cells when co-cultured with *Lactobacillus plantarum* DC400 or with purified fraction containing plantaricin A (PlnA) until the early-stationary (18 h) phase of growth was reached

Spot[a)]	Estimated Mr (kDa)	Estimated *pI*	Induction factor[b)]		Homologous protein/ function	A.N.[c)]	Organism	Number of peptides	Identity score (%)	Sequence coverage (%)
			DC400	PlnA						
1	96	4.65	2±0.100	–	Putative aminopeptidase N, PepN	emb|CAI30886.1|	*L. sanfranciscensis*	4	100	12
2	68	4.9	2±0.105	–	Pyruvate kinase, Pyk	C2D4J2-1	*L. brevis* subsp. *gravesensis* ATCC 27305	2	100	10
5	50	5.50	1.5±0.076	–	Glutathione reductase, GSR	Q03NW4-1	*L. brevis* ATCC 367/JCM 1170	3	100	21
12	49	5.85	3±0.155	–	Putative cysteine aminopeptidase, PepC	emb|CAI30820.1|	*L. sanfranciscensis*	2	100	13
16	47.5	4.0	4±0.255	–	Histidyl-tRNA synthetase, HisRS	YP_794909.1	*L. brevis* ATCC 367/JCM 1170	4	100	24
17	47	8.5	2±0.110	1.5±0.075	Cell division protein FtsZ, FtsZ	gi|184155053	*L. fermentum* IFO 3956	3	100	32
26	36	4.0	3±0.148	2±0.100	Glutathione reductase, LRH_11212	B5QLW7	*L. rhamnosus* HN001	1	98	15
41	23.45	4.55	2.5±0.118	1.5±0.076	Response regulator Rrp11, Rrp11	NP_786469.1	*L. plantarum* WCFS1	3	95	10.6
47	19	5.0	3±0.145	–	Hypothetical cytosolic protein, Hcp	C2FIP5	*L. plantarum* ATCC 14917	2	100	11.1
53	15	4.55	2.5±0.120	1.5±0.076	N.D.[d)]	N.D.	N.D.	N.D.	N.D.	N.D.
57	13	5.25	3.5±0.170	2.5±0.125	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a) Spot designation correspond to those of the gels in Fig. 3A, B and C.
b) The reduction factor is defined as the ratio between the spot intensity of a protein from cells grown in mono-culture and the spot intensity of the same protein from cells co-cultivated with *L. plantarum* DC400 or with purified fraction containing plantaricin A (PlnA). All the reduction factors were calculated as the average of the spot intensities of nine gels and ±SD are shown.
c) Accession number.
d) Not identified protein.

incubation (24–72 h) with purified PlnA from the mono-culture of *L. plantarum* DC400 or from the co-culture with *L. sanfranciscensis* DPPMA174 significantly ($p<0.05$) increased the cell viability (Fig. 4). The same level of induction was found when 2.5 μg/mL of chemically synthesized PlnA were used. No induction was found for the fraction purified from the mono-culture of *L. sanfranciscensis* DPPMA174 which eluted under the same chromatographic conditions of PlnA. As expected, exposure to IFN-γ for 24, 48 and 72 h caused a significant decrease of the viability of Caco-2/TC7 cells (Fig. 5). On the contrary, the negative effects IFN-γ were abolished under simultaneous treatment with purified or chemically synthesized PlnA.

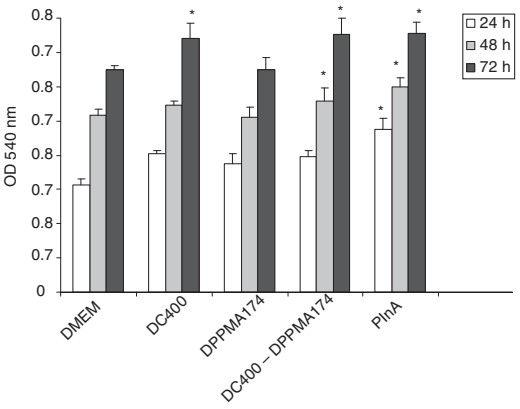

Figure 4. Viability of Caco-2/TC7 cells measured as Neutral Red uptake after 24, 48 and 72 h of incubation with purified plantaricin A (PlnA, 2.5 μg/mL) from the mono-culture of *Lactobacillus plantarum* DC400 (DC400) or the co-culture with *Lactobacillus sanfranciscensis* DPPMA174 (DC400-DPPMA174). The fraction purified from the mono-culture of *L. sanfranciscensis* DPPMA174, which eluted under the same chromatographic conditions of PlnA, was used as the negative control (DPPMA174). Another negative control was DMEM medium (DMEM). Chemically synthesized PlnA (2.5 μg/mL) was used as the positive control (PlnA). Asterisk indicates a significant difference ($p<0.01$) with respect to negative controls.

3.8 Effect of PlnA on the TEER

Addition of purified PlnA either from the mono-culture of *L. plantarum* DC400 or from the co-culture between strains DC400 and DPPMA174 significantly ($p<0.05$) induced TEER (Fig. 6). The same was found by adding 2.5 μg/mL of chemically synthesized PlnA. Compared to DMEM medium, the addition of IFN-γ significantly ($p<0.05$) decrease TEER. Nevertheless, the addition of purified or chemically synthesized PlnA decreased the negative effect of IFN-γ towards TEER of Caco-2/TC7 cells.

Table 4. Concentration (ppm) of some[a)] VOC found in the mono-cultures[b)] of *Lactobacillus plantarum* DC400 and *Lactobacillus sanfranciscensis* DPPMA174, and in the co-cultures[b)] between strains DC400 and DPPMA174

Chemical class	DC400	DPPMA174	DC400-DPPMA174
Ketones			
Diacetyl	11.34[c)]	0.00[e)]	1.08[d)]
Acetoin	17.20[c)]	0.00[d)]	0.00[d)]
Aldehydes			
Nonanal	0.00[e)]	0.12[d)]	1.71[c)]
2-Butyl-2-octenal	0.00[d)]	5.49[c)]	0.00[d)]
Esters			
Ethyl-acetate	14.5[e)]	166.68[c)]	25.94[d)]
Ethyl hexanoate	0.00	2.49[c)]	0.00
Sulfur compounds			
2-Hexyl thiophene	8.67[c)]	6.87[d)]	2.81[e)]
Alkanes			
Hexadecane	75.4[c)]	42.14[d)]	10.86[e)]
Heptadecane	0.00[d)]	0.00[d)]	0.90[c)]
Lactones			
Furanone A	26.44[c)]	18.06[d)]	13.88[e)]
Furanone B	0.362[d)]	0.00[e)]	21.25[c)]
Free fatty acids			
Decanoic acid	0.00[d)]	0.00[d)]	14.48[c)]

a) Only VOC which showed variation from the mono-cultures to co-culture were reported.
b) Cells were grown until the late-exponential phase of growth (18 h) was reached.
c–e) Data are the mean of three independent experiments and values in the same raw with different superscript letters differ significantly (≤ 0.06).

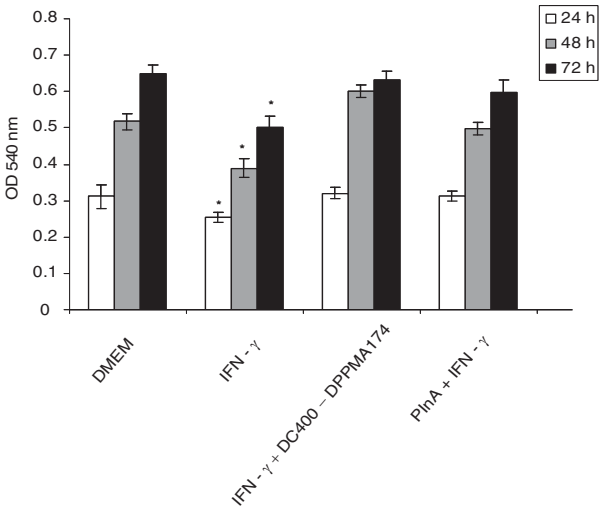

Figure 5. Viability of Caco-2/TC7 cells measured as Neutral Red uptake after 24, 48 and 72 h of incubation with interferon-γ alone (IFN-γ, 1000 U/mL) and IFN-γ with purified plantaricin A (PlnA, 2.5 μg/mL) (IFN-γ+DC400-DPPMA174). DMEM medium was used as the negative control (DMEM). Chemically synthesized PlnA (2.5 μg/mL) together with IFN-γ (PlnA+IFN-γ) was used as the positive control. Data are the means ±SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t*-test. Asterisk indicates a significant difference ($p<0.01$) with respect to the negative control.

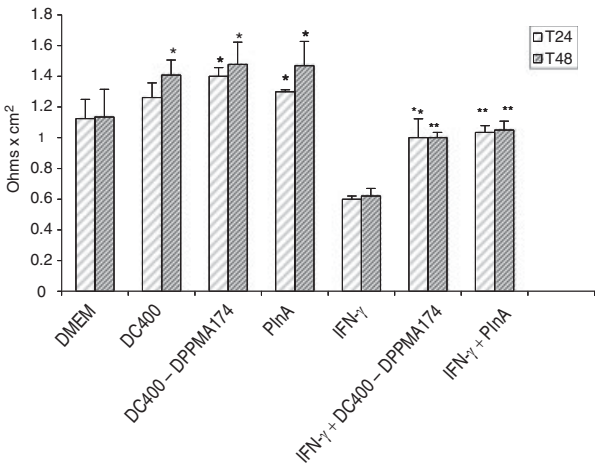

Figure 6. Transepithelial electric resistance (TEER) (Ohms x cm^2) of Caco-2/TC7 cells after 24 and 48 h. Incubation was with: purified plantaricin A (PlnA, 2.5 μg/mL) from the mono-culture of *Lactobacillus plantarum* DC400 (DC400); purified PlnA (2.5 μg/mL) from the co-culture between *L. plantarum* DC400 and *Lactobacillus sanfranciscensis* DPPMA174 (DC400-DPPMA174); chemically synthesized PlnA (PlnA, 2.5 μg/mL); interferon-γ (IFN-γ, 1000 U/mL); IFN-γ and purified PlnA from the co-culture between *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174 (IFN-γ+DC400-DPPMA174); and IFN-γ and chemically synthesized PlnA (IFN-γ+PlnA). DMEM medium was used as the negative control (DMEM). Data are the means ±SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Student's *t*-test. Asterisk indicates a significant difference ($p<0.01$) with respect to the negative control. Two asterisks indicate a significant difference ($p<0.01$) with respect to IFN-γ.

4 Discussion

Systems of bacterial quorum sensing (QS) are very complex and they may markedly differ in terms of: (i) specific sensory components; (ii) biochemistry and transport of signalling molecules; (iii) target functions; (iv) surrounding conditions stimulating QS; and (v) interactions between signaling molecules and eukaryotic cells [41, 42]. To date, only a few studies [11, 24, 43] considered the mechanisms of cell-cell communication and competition in sourdough lactic acid bacteria. This study aimed at describing the mechanism of inter-species competition of *L. plantarum* DC400, mainly based on the biosynthesis of AIP. Interactions with human Caco-2/TC7 cells were also investigated. Assays for bacterial cell-cell communication were carried out on chemically defined (CDM) and, especially, undefined (WFH) media. WFH as the culture medium and long-time fermentation were used to resemble the chemical composition of wheat flour and the most widely used protocol of sourdough propagation [23]. According to previous studies [11, 24], co-cultivation of *L. plantarum* DC400 with other lactic acid bacteria did not affect growth and survival of strain DC400. On the contrary, some lactic acid bacteria such as *L. sanfranciscensis* DPPMA174 [11] and *P. pentosaceus* 2XA3 were markedly affected by co-cultivation with *L. plantarum* DC400. Compared to mono-culture, the number of dead/damaged cells markedly increased and those of cultivable cells decreased. Therefore, co-cultivation with strain DC400 might be considered as a stressing condition, especially, for *L. sanfranciscensis* DPPMA174. As previously shown for other strains of *L. plantarum* isolated from different food ecosystems [15, 18, 21], strain DC400 synthesized the pheromone PlnA either under mono- or co-culture conditions. The biosynthesis of PlnA was induced at different extents depending on microbial partner. Except for *L. pentosus* 12H5, co-cultivation of *L. plantarum* DC400 with several species of sourdough lactic acid bacteria leads to the induction of the synthesis of PlnA. The partner *L. sanfranciscensis* DPPMA174 induced the highest biosynthesis of PlnA which, in turn, determined lethal conditions for it. The survival of *L. sanfranciscensis* DPPMA174 only slightly varied by comparing the addition of purified or chemically synthesized PlnA to the culture medium and the co-cultivation with *L. plantarum* DC400. PlnA represents an induction factor for gene regulation (pheromone behaviour) and it acts as an antimicrobial peptide [15]. PlnA is an atypical bacteriocin. It should be included in the bacteriocin class IIc, meaning a non-pediocin-like peptide which does not show post-translational modifications. Again, PlnA lacks a dedicated immunity protein and its biological activity resembles that of eukaryotic antimicrobial peptides [20, 44].

The presence of PlnA determined a proteomic response in *L. sanfranciscensis* DPPMA174. All proteins induced by cultivation with purified PlnA were also over-expressed during co-cultivation with *L. plantarum* DC400. Nevertheless, the number of over-expressed proteins was markedly higher under co-culture conditions [11, 24]. As response to PlnA, *L. sanfranciscensis* DPPMA174 increased the level of expression of proteins involved in stress response (chaperones: DnaK, GroEL, ClpL, GroES, 30 ribosomal proteins S2, S5 and S30EA, 50 ribosomal proteins L1, L11 and L31), amino acid metabolism (S-adenosyl-methyltransferase, MraW; putative tripeptidase, PepT and putative aminopeptidase R, PepR), energy metabolism (glycerol kinase, IIIGlc-GK; phosphoglycerate kinase, PGK; aldo/keto reductase, Akr; acetate kinase, AK; bifunctional acetaldehyde-CoA/alcohol dehydrogenase, AdhE; 6-phosphogluconate dehydrogenase, 6PGD; β-phosphoglucomutase/glucose-1-phosphate phosphodismutase, PgM), membrane transport (phosphocarrier protein Hpr, Hpr), nucleotide metabolism (dihydroorotase, DHO and deoxyribonuclease IV, EndoIV), regulation of transcription (elongation factor Tu, Tuf; transcription regulator, TR; transcription regulator, GntR) and cell redox homeostasis (NADH oxidase, NOX). At the same time, other proteins such as cell division protein (FtsZ), glutathione reductase (GSR) and response regulator (Rrp11) were repressed. Although bacteriocin activity has the cell membrane as the main target, PlnA seemed also to interfere with the global cell metabolism of *L. sanfranciscensis* DPPMA174. Under stressing conditions or as a mechanism of response to QS, most of the above proteins were found to be over-expressed also in other lactic acid bacteria, including *L. plantarum* DC400 [11, 24, 45]. According to previous studies [24], the co-cultivation between *L. sanfranciscensis* DPPMA174 and *L. plantarum* DC400 involved also mechanisms different from those based on the PlnA activity. The over expression of proteins related to stress response (stress response membrane GTPase, GTPase; holliday junction ATP-dependent DNA helicase, RuvB, 50 ribosomal proteins L6, RpL6 and small heat shock protein, Hsp), amino acid metabolism (aspartate-semialdehyde dehydrogenase, Asd; proline dehydrogenase, ProDH), energy metabolism (ATP synthase gamma chain, AtpG; glucosamine-6-phosphate deaminase, GlcN6P; phosphoglycerate mutase, Pgm; enolase, Eno), membrane transport (mannose PTS EIIAB, Pts9AB), nucleotide metabolism (orotate phosphoribosyltransferase, PyrE), regulation of transcription (translation elongation factor Ts, Tsf and metal dependent regulation, MDR) was shown.

The synthesis of VOC, also responsible for the sensory properties of sourdough baked goods [23], was influenced by the microbial association. Compared to mono-cultures, the stressful co-culture between *L. plantarum* DC400 and *L. sanfranciscensis* DPPMA174 influenced the synthesis and the concentration of specific VOC (e.g., furanone B and decanoic acid) [42]. It was suggested that the evolved biological function of a number of furanone analogues is to act as inter-species signal molecules in several ecosystems [46]. Decanoic acid was also induced in *Lactobacillus helveticus* under stressing conditions [37].

Potential probiotic effects and high survival during gastrointestinal (GI) transit were attributed to strains of *L. plantarum*, making this species a promising candidate for delivering functional molecules for human health [15]. Although the phenomenon of cell-cell communication between prokaryotic and eukaryotic cells is already known [47], limited attention was paid to interactions between QS molecules (e.g., peptide pheromones) and human intestinal mucosa. A competence and sporulation factor (CSF), QS pentapeptide, of probiotic *Bacillus subtilis* activated key survival pathways, including p38 MAP kinase and protein kinase B (Akt), and induced cytoprotective heat shock proteins which prevented oxidative intestinal cell injury and loss of the barrier function [47]. This study showed that PlnA increased the viability of Caco-2/TC7 cells (human colon carcinoma). Caco-2/TC7 cells are one of the *in vitro* systems most largely used to mimic the intestinal mucosa. Despite their neoplastic origin, these cells have the capacity to spontaneously differentiate to mature enterocytes and to express brush border enzymes. Under culture conditions, Caco-2/TC7 cells develop the morphological and functional characteristics of enterocytes, including intercellular tight junctions, the integrity of which is measured by TEER [48]. Compared to negative control (medium alone), purified and chemically synthesized PlnA markedly increased the level of TEER. Probiotics and/or commensal bacteria may prevent epithelial damage during inflammatory disorders [49, 50]. Nevertheless, the mechanism of epithelial protection was not completely elucidated [15]. This study also showed that PlnA eliminated the negative effect of cytokines (IFN-γ) towards viability of Caco-2/TC7 cells and integrity of the tight junctions.

Robustness of *L. plantarum* is already known under different food ecosystems. This species well adapts and strongly competes during sourdough fermentation [11, 23]. Notwithstanding other regulatory factors such as acidity, nutrient competition, synthesis of diacetyl and LuxS mediated compounds, pheromone PlnA could play a central role in the regulation of the microbial interactions in food ecosystems. Under GI conditions, PlnA could contribute to prevention of intestinal cell damage and protection of barrier functions.

5 References

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