Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antimicrobial properties of prunin, a citric flavanone glucoside, and its prunin 6″-O-lauroyl ester

G. Céliz, M.C. Audisio and M. Daz

Instituto de Investigaciones para la Industria Química (INIQUI)-CONICET and Universidad Nacional de Salta, Salta, Argentina

Keywords
antimicrobial substances, grapefruits, *Listeria monocytogenes*, prunin, prunin 6″-O-lauroyl ester.

Correspondence
Mirta Daz, INIQUI-CONICET, Universidad Nacional de Salta, Avenida Bolivia 5150 A4408FVY, Salta, Argentina.
E-mail: mdaz@unsa.edu.ar; audisio@unsa.edu.ar

2010/0351: received 28 February 2010, revised 17 April 2010 and accepted 8 May 2010

doi:10.1111/j.1365-2672.2010.04773.x

Abstract

Aims: To determine the antimicrobial potential of prunin (P), a flavanone glucoside resulting from the hydrolysis of naringin present in grapefruit, and of its prunin 6″-O-lauroyl ester (PL), synthesized by enzymatic catalysis.
Methods and Results: P and its lauroyl ester were tested against Gram-negative and Gram-positive bacteria, yeasts and moulds. P showed no inhibitory effect against the micro-organisms assayed, but stimulated growth of *Pseudomonas aeruginosa* and different *Bacilllus* sp. However, 150 μg ml^{-1} of PL inhibited *Escherichia coli*, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium, many *Bacillus* sp., *Staphylococcus aureus* ATCC29213, *Enterococcus avium* DSMZ17511, and different *Listeria monocytogenes* strains. In the last case, *L. monocytogenes*, sensitive or bacteriocin-resistant cells, lost nearly 4-log reductions after 30 min of contact. A bactericidal mode of action was determined using both scanning and transmission electronic microscopies.
Conclusions: PL could be used as a food additive, because at low concentration (150 μg ml^{-1}) it exhibited antimicrobial activity against important food-borne pathogens. A bactericidal effect was also determined on *L. monocytogenes* sensitive and bacteriocin-resistant mutant strains. P did not show any antimicrobial property at all.
Significance and Impact of the Study: PL is a potential antimicrobial compound with a high anti-*Listeria* property.

Introduction

The consumption of citric fruits appears to be associated with reduced risk of certain chronic diseases (Peterson *et al.* 2006a,b; Tripoli *et al.* 2007) and increased human survival (Fortes *et al.* 2000), and one or more of the citrus flavonoids seem to be responsible for these beneficial effects (Peterson *et al.* 2006a). Citric flavonoids were reported as having antimicrobial, antiviral, anti-allergenic, anti-inflammatory and antioxidant properties (Benavente-Garcia *et al.* 1997; Tirillini 2000; Peterson *et al.* 2006a; Rhodes *et al.* 2006; Fisher and Phillips 2008). Because they can show a great potential as being antimicrobial (Cushnie and Lamb 2005; Rhodes *et al.* 2006; Mandalari *et al.* 2007), they can become interesting alternatives to control different pathogenic bacteria that are becoming antibiotic resistant.

One of the most common flavonoids in citrus is naringin (4′,5,7-trihydroxyflavanone-7-β-D-α-L-rhamnosyl(1 $\rightarrow$ 2)-β-D-glucoside) that is naturally found in grapefruits and sour oranges. The structural modifications of this molecule would alter its hydrophilic/hydrophobic ratio, and it can be expected that the new compound obtained will present properties that differ from the original molecule. In particular, when the modification is the incorporation of an aliphatic chain to the original molecule, it has been reported that this new compound can interact with the cell membrane (Mellou *et al.* 2005, 2006; Torres De Pinedo *et al.* 2007a,b). As a result of these characteristics, it is interesting to re-evaluate the physicochemical and biological properties of new derivative compounds.

The northwest of Argentina is an important producer of citrus fruits, such as lemon, orange and grapefruit, and

Salta province is the main grapefruit producer (Justo and Rivera 2009). The industrialization of grapefruit production generates a large quantity of peel that goes to waste. The extraction of naringin from this waste would allow not only more efficient use of the fruit, but also decrease the solid organic waste volume that needs to be disposed of.

Thus, the aim of this work was to study the antimicrobial properties of prunin (P), a flavanone glucoside resulting from the hydrolysis of naringin present in grapefruit, and of its prunin 6″-O-lauroyl ester (PL), synthesized through enzymatic catalysis. Therefore, their potential antimicrobial spectra against lactic acid bacteria (LAB) and different pathogenic and/or food-spoilage bacteria was analysed. Furthermore, the effect of these compounds assayed was quantified against different, sensitive and bacteriocin-resistant *Listeria monocytogenes* strains.

Materials and methods

Materials

Molecular sieves (sodium aluminium silicate; 4 Å-pore diameter) were purchased from Sigma (USA) and vinyl laurate (VL) from Fluka (USA). Nozyme 435 (*Candida antarctica* lipase B immobilized on an acrilic resin) was a gift from Novozymes Latin America Limited (Araucaria, Parana, Brazil). The culture media used for antimicrobial assaying were the following: brain–heart infusion (BHI; Britania, Buenos Aires, Argentina), DeMan-Rogose-Sharpe (MRS; Britania) and Mueller-Hinton broth (Britania). When a solid medium was needed, 1·5% w/v agar (Britania) was added. All other reagents were of analytical grade.

Prunin (P) and prunin 6″-O-lauroyl ester (PL) preparation

Prunin (4′,5,7-trihydroxyflavanone-7-β-D-glucoside) was obtained according to the method described by Soria and Ellenrieder (2002). PL was synthesized and characterized as previously reported (Céliz and Daz 2010 under current review). Briefly, a screw cap-closed recipient was filled with 190 ml of acetone, 5 g of molecular sieves, 1·75 g of P and 10 ml of VL. Temperature was set at 50°C prior to starting the reaction with 1 g of Novozym® 435. After 24 h, the solvent was evaporated in a rotary evaporator at room temperature. Residual solids were washed three consecutive times with 12 ml of hexane and centrifuged at 9000 rev min^{-1} for 5 min each time. Then, ethylic ether was added to dissolve the synthesized ester and separate it from the rest (P and other impurities) by centrifugation at 10 000 rev min^{-1} for 10 min. The ethylic ether was evaporated.

Microbial strains and culture conditions

The following strains were used as indicators for antimicrobial testing and were obtained from ATCC (American Type Culture Collection), DMSZ (German Resource Centre for Biological Material), INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina), Dr Carlos Malbrán Microbiology Institute (Buenos Aires, Argentina) and CA (Dr Carina Audisio, Salta, Argentina), as described in Table 1. Bacteria belonging to *Listeria*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Proteus*, *Klebsiella*, *Escherichia, Enterococcus* and *Bacillus* genera were grown in BHI at 37°C for 12 h. *Lactobacillus* spp. were propagated in MRS broth at 37°C for 24 h under a microaerophilic atmosphere (about 7% O_2 and 14% v/v CO_2 pressure).

Antimicrobial assaying of prunin (P) and prunin 6″-O-lauroyl ester (PL)

P, PL and VL compounds were dissolved in 100 μl of dimethylformamide (DMF) because of their low solubility in water. Concentrations were analysed from 50 to 1000 μg ml^{-1}. Two controls were prepared as follows: (i) only culture medium and (ii) 100 μl of DMF (1% v/v). Different aliquots of each compound, previously dissolved in DMF, were added to 10-ml Mueller–Hinton agar, which was mixed well and distributed in sterile Petri dishes. Once the agar surface was dry, plates were sown with 7 μl of active bacteria cultures, and plates were incubated for 12–24 h at 37°C under adequate conditions for each indicator strain. Afterwards, the presence or absence of colonies was determined. All experiments were carried out in duplicate.

Minimum inhibitory concentration (MIC) of PL

The MIC of PL was determined according to the microdilution method published by the Clinical Laboratory and Standars Institute (2003). Different PL concentrations were tested against four different *L. monocytogenes* strains: 99/287S, a bacteriocin-sensitive strain, 99/287R, a bacteriocin-resistant mutant (Audisio *et al.* 2005), 00/360 and 99/287RMori1, after incubation on Mueller-Hinton agar at 37°C for 24 h.

Quantification of the antagonistic effect of P and PL on different cultures of *Listeria monocytogenes*

Listeria monocytogenes 99/287S and 99/287R were studied. Bacterial suspensions in physiological solution (0·85% w/v NaCl) were used, and each cell suspension was supplemented with a determined amount of PL to obtain concentrations of 50, 100 and 150 μg ml^{-1}. P was assayed

Table 1 Antimicrobial spectra of 150 μg ml^{-1} of prunin (P) and 150 μg ml^{-1} of its ester, prunin 6″-O-lauroyl ester (PL), against different micro-organisms

Strain	Origin	P	PL
Bacillus subtilis Mori2	CA	+	–
B. subtilis G2III	CA	+	+
Bacillus subtilis ssp. *niger* C4	CA	+	–
Bacillus spp. AJ3	CA	+	–
Pseudomonas aeruginosa 27 853	ATCC	+	–
Salmonella enterica serovar Enteritidis	INTA	+	–
Salmonella enterica serovar Typhimurium	INTA	+	–
Klebsiella pneumoniae	CA	+	w+
Proteus mirabilis	CA	+	w+
Escherichia coli 21	CA	+	–
Staphylococcus aureus 29213	ATCC	+	–
Lactobacillus johnsonii CRL1647	CA	+	+
Lactobacillus salivarius CRL1384	CA	+	+
Lact. johnsonii IG9	CA	+	+
Enterococcus faecium SM7	CA	+	+
Ent. faecium SM21	CA	+	+
Ent. faecium 1385	CRL	+	+
Ent. faecium CA12	CA	+	+
Enterococcus avium 17511	DSMZ	+	–
Enterococcus spp. AB2MSSC2	CA	+	+
Listeria monocytogenes 99/287S	Malbrán	+	+
L. monocytogenes 99/287R	CA	+	–
L. monocytogenes 99/287RMori1	CA	+	+
L. monocytogenes 00/182RB6	CA	+	–
L. monocytogenes 01/198RB6	CA	+	–
L. monocytogenes 99/320RB6	CA	+	–
L. monocytogenes 00/270RB6	CA	+	–
L. monocytogenes 00/223RB6	CA	+	–
L. monocytogenes 99/267RB6	CA	+	–
L. monocytogenes 01/01RB6	CA	+	–
L. monocytogenes 01/200RB6	CA	+	–
L. monocytogenes 01/155	Malbrán	+	–
L. monocytogenes 99/287RB6	Malbrán	+	–
L. monocytogenes 01/182RB6	Malbrán	+	–
L. monocytogenes 00/360	Malbrán	+	–
L. monocytogenes 99/393	Malbrán	+	–
L. monocytogenes 99/316	Malbrán	+	–
L. monocytogenes 01/00	Malbrán	+	+
L. monocytogenes 003/364	Malbrán	+	+
L. monocytogenes 01/01	Malbrán	+	–
L. monocytogenes 99/316	Malbrán	+	–
L. monocytogenes 03/03	Malbrán	+	–
L. monocytogenes 99/128	Malbrán	+	–
L. monocytogenes 01/182	Malbrán	+	–

+: growth: good colony development.
w+: colony development and its size was smaller than control.
–: inhibition: no colony was detected.

only at a concentration of 150 μg ml^{-1}. Samples were incubated at 37°C, and bacterial viability was assayed by cell counts on Mueller–Hinton plates after 30, 60 and 120 min of contact. All assays were carried out in duplicate.

Listeria cells morphology analysis by scanning and transmission electron microscopies

Listeria monocytogenes 99/287S and the bacteriocin-resistant mutant were morphologically analysed by both scanning and transmission electron microscopies. The cellular pellets from the control cultures and those that were treated for 2 h at 37°C with either 150 μg ml^{-1} of P or PL were obtained by centrifugation at 9000 rev min^{-1} for 10 min at 4°C. They were then resuspended in a mixture of equal volumes of BHI broth and fixation solution (3% glutaraldehyde in phosphate buffer 0·1 mol l^{-1}, pH 7·4). The samples were incubated at 25°C for 30 min. The cells obtained from a later centrifugation in similar conditions were resuspended in 3% glutaraldehyde solution. After postfixation with OsO_4 (1% in phosphate buffer 0·1 mol l^{-1}, pH 7·4), the samples were dehydrated with increasing concentrations of acetone and alcohol. SEM and transmission observation of critical point dried samples were carried out with a Joel JMS 6480 LV and Joel JSM35CF instruments, respectively.

Statistical analyses

All assays were carried out according to the Honest Significant Differences (HSD) Tukey's test and were considered significant at $P < 0{\cdot}05$. The assays were performed in duplicate.

Results

In this work, the antimicrobial ability of P and its ester, PL (Fig. 1), was analysed. Simultaneously, the effect of the 'donor', i.e. the VL, was also determined whether the hydrocarbon chain itself would produce a certain effect on the viability of the strains assayed.

These compounds were dissolved in DMF because of their low solubility in water. Addition of 100 μl of the mentioned dissolutions in 10 ml of Mueller–Hinton broth produced a crystalline solution in the case of P and a liquid–liquid suspension in the cases of PL and VL. It was determined that a control of the solvent [100 μl DMF (1% v/v)], at the maximum concentration that was tested, did not have an inhibitory effect on the micro-organisms assayed. A similar result was found when VL in DMF (maximum 1% v/v) was also tested.

Interestingly, P did not show any activity against the Gram-positive or Gram-negative bacteria assayed with the technique used (Table 1). Its potential antagonistic effect was determined by dissolving P in the agar, and the development of a colony from the indicator micro-organism cells was monitored. Different P concentrations were assayed (from 50 to 1000 μg ml^{-1}), and no inhibitory

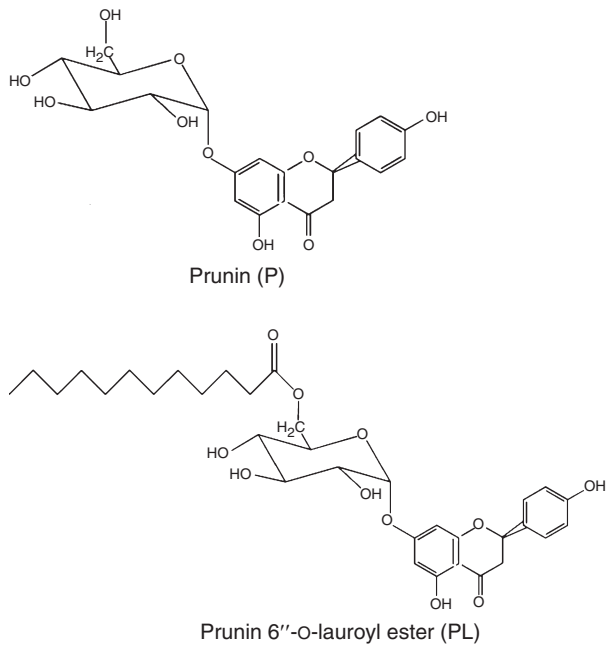

Figure 1 Chemical structure of prunin and prunin 6″-O-lauroyl ester (PL).

effects were detected. In contrast, a certain degree of growth stimulation was observed for *Pseudomonas* and *Bacillus* spp.

A different situation was detected when the antimicrobial potential of PL was studied. As can be observed in Table 1, all Gram-positive pathogens were inhibited by 150 μg ml^{-1} of PL. Also, an important effect on *Staphylococcus aureus* and different *L. monocytogenes* strains was remarkable, and in the latter case, the antagonistic effect was strain-dependent. From 12 different *L. monocytogenes* strains tested, only two were not affected by 150 μg ml^{-1} of PL. From ten different mutants spontaneously resistant to enterocins, only one presented certain resistance to 150 μg ml^{-1} of the P ester.

MIC values of the PL are presented in Table 2. It can be observed that the different *Listeria* strains analysed experienced a marked inhibition at concentrations as low as 30·8 μg ml^{-1}. Because of these results, it was decided to quantify the effect of this new compound on *L. monocytogenes* 99/287 and its bacteriocin-resistant variant. The concentration was set at 150 μg ml^{-1}, about four times the MIC value found for this strain, because other *Listeria* strains had been less sensitive to the P ester than *L. monocytogenes* 99/287.

To carry out this experiment, direct contact was established between the compound and the micro-organism in glass tubes. The results showed that P did not just cause inhibition of the pathogens assayed, but also stimulated bacterial development to a certain degree (Figs 2 and 3), which had been observed before with the other detection technique used in this paper. Whereas 50 μg ml^{-1} PL exerted an important inhibitory effect on both *L. monocytogenes* strains. Furthermore, the bacteriocin-resistant clone *L. monocytogenes* 99/287R was the most susceptible: After 30 min of contact, 4-log reduction in the number of viable cells was observed (Fig. 3). The three concentrations assayed showed a similar pattern: rapid loss of viability (within 30 min after contact), a decrease of about 3-log reductions in the number of viable cells (*c.* 5×10^8 CFU ml^{-1} to 5×10^5 CFU ml^{-1}) and increase in contacting times did not increase viability loss (Fig. 2). The *L. monocytogenes* bacteriocin-resistant mutant demonstrated similar behaviour, although being strongly inhibited during the first hour of contact. However, in this case, the concentration of ester was important: 100 or 150 μg ml^{-1} of PL produced a larger antagonistic effect (Fig. 3).

The scanning (data not shown) and transmission electron micrographs revealed that the effect of PL against both sensitive and the bacteriocin-resistant mutant *L. monocytogenes* 99/287 strains was bactericidal but not lytic. Figure 4 presents the results obtained by transmission electron microscopy analyses.

Discussion

Grapefruits are mainly produced in the Salta region of the northwest of Argentina. They are not only used as a

Table 2 *In vitro* activity of prunin 6″-O-lauroyl ester (PL) against different *Listeria monocytogenes* strains (MIC)

	PL (μg ml^{-1})								
Indicator strain	0*	6·2	15·4	30·8	61·6	92·4	154	308	462
Listeria monocytogenes 99/287S	+	+	−	−	−	−	−	−	−
L. monocytogenes 00/360	+	+	+	+	−	−	−	−	−
L. monocytogenes 99/287R	+	+	−	−	−	−	−	−	−
L. monocytogenes 99/287RMori1	+	+	−	−	−	−	−	−	−

+: colony development. −: no growth detected.
MIC, minimum inhibitory concentration.
*Control (no PL added).

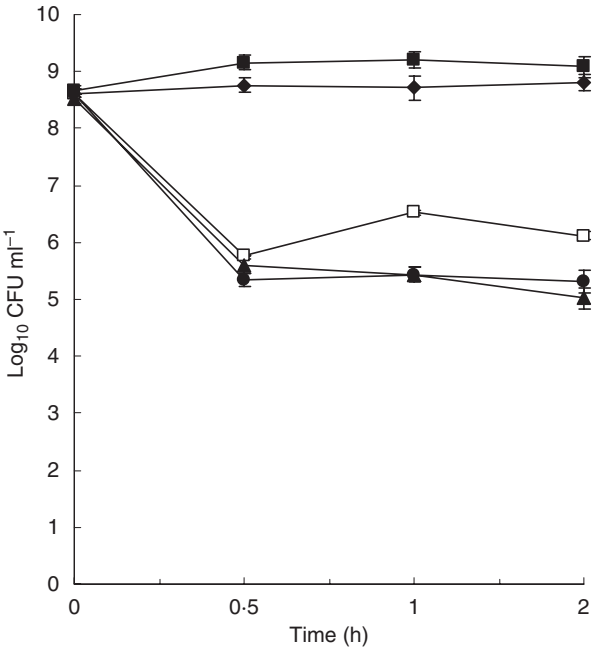

Figure 2 Inactivation curve of *Listeria monocytogenes* 99/287S (bacteriocin-sensitive strain) obtained using initial inocula of 10^8 colony-forming units at 37°C. *Listeria monocytogenes* 99/287S was incubated with prunin (P) (150 μg ml^{-1}) and different concentrations of its ester, prunin 6″-O-lauroyl ester (PL): (◆) Growth control, (■) P, (□) 50 μg ml^{-1} PL, (●) 100 μg ml^{-1} PL, (▲) 150 μg m^{-1} PL. Data are expressed as the mean of duplicate assays.

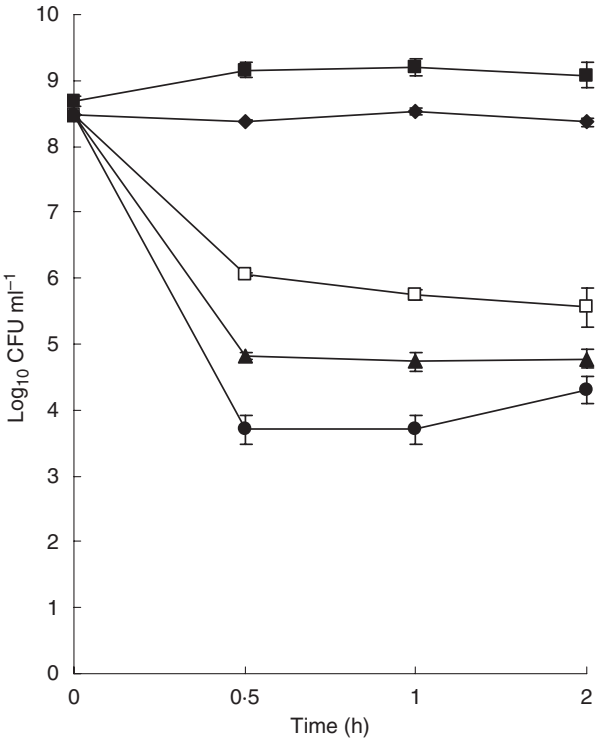

Figure 3 Inactivation curve of *Listeria monocytogenes* 99/287R (bacteriocin-resistant mutant) obtained using initial inocula of 10^8 colony-forming units at 37°C. *Listeria monocytogenes* 99/287R was incubated with prunin (P) (150 μg ml^{-1}) and different concentrations of its ester, prunin 6″-O-lauroyl ester (PL): (◆) Growth control, (■) P, (□) 50 μg ml^{-1} PL, (●) 100 μg ml^{-1} PL, (▲) 150 μg ml^{-1} PL. Data are expressed as the mean of duplicate assays.

fruit or to create juices and concentrates, but can also be used as a naringin source (from its peel) to obtain rhamnose. Production of this valuable compound yields P as a secondary product.

Although P can be used directly by the pharmaceutical and food industries for its biological properties, the modification of the molecule by insertion of a hydrophobic chain changes its hydrophilic/hydrophobic ratio; thus, it would be expected that the new compound will present properties different from the original. The antimicrobial effects can be one of these properties. It is commonly known that certain citrus flavanone glycosides possess antimicrobial properties (Benavente-Garcia *et al.* 1997; Cushnie and Lamb 2005). However, P has not been studied extensively, probably because it is not commercially available.

Even though different P concentrations were assayed (from 50 to 1000 μg ml^{-1}), no inhibitory effects were detected. In contrast, a certain degree of growth stimulation was observed for *Pseudomonas* and *Bacillus* spp. This stimulation on *Pseudomonas* spp. growth may have, at least, two feasible explanations. First, P was not an active compound against this Gram-negative bacterium that is an important food-spoilage micro-organism because of its psychrotrophic, lipolytic and proteolytic properties (Otead 1986; Liu *et al.* 2006). Second, different *Pseudomonas* strains are known to produce rhamnolipids, important biosurfactants with great applications in bioremediation of soils or biodegradation of *n*-alkanes (Zhang and Miller 1994, 1995). As a result of this growth stimulation, P would help with the production of these tensioactive molecules.

However, a different situation was detected when the antimicrobial potential of PL was analysed. In particular, the effect of PL on *L. monocytogenes*, a Gram-positive bacterium that has become an emerging pathogen and affects children, pregnant women and immunocompromized patients, was an important step (Farber and Peterkin 1991; Jurado *et al.* 1993; Hitchins and Whiting 2001). It has been observed that *L. monocytogenes* is developing resistance not only against antibiotics, but also against bacteriocins, like nisin or class II.1 bacteriocins; the latest compounds are natural alternatives that have started losing its efficiency (Davies and Adams 1994; Naghmouchi *et al.* 2007; Ibarguren *et al.* 2010).

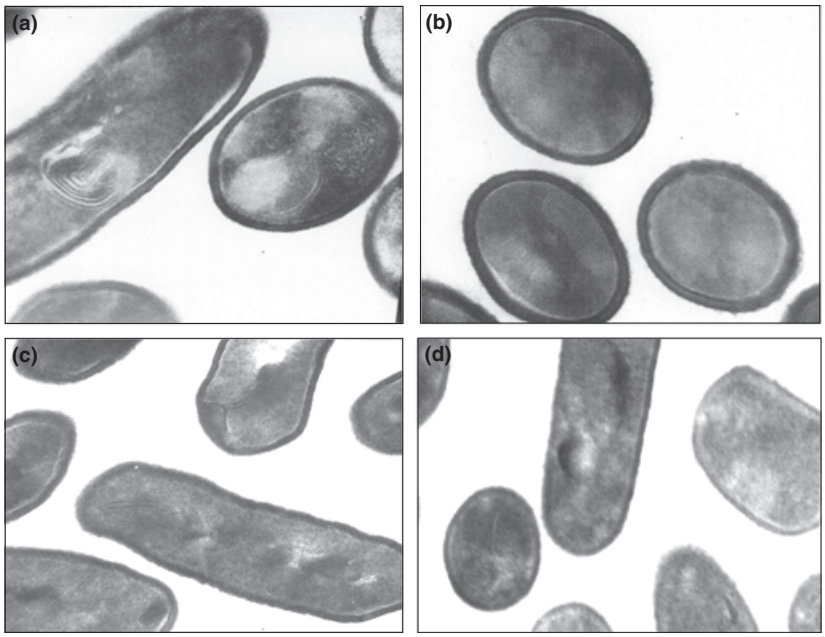

Figure 4 Transmision electronmicrographs of the control cells of *Listeria monocytogenes* 99/287S and *L. monocytogenes* 99/287R (a and c, respectively) and after 2 h of contact with 150 μg ml^{-1} prunin 6″-O-lauroyl ester at 37°C. (b and d, respectively) (×34 000).

The results from this study indicated that PL has an inhibitory effect against various bacteria, especially *L. monocytogenes*. In particular, PL caused a rapid inhibition on both sensitive and the bacteriocin-resistant mutant *L. monocytogenes* 99/287 strains, followed by a tailing effect. Also, the transmission electron microphotographies revealed there was neither cell lysis nor apparent modification of the fine cell structures.

In the case of bacteriocin-sensitive *Listeria* strains, it seems a higher PL concentration will not produce further inhibition. It is important to emphasize these results that suggest PL is a potentially strong antimicrobial agent, and examination of this property is independent of the detection technique. This differs from other research results with flavonoids, of differing origins and purity grades, where the analysis techniques used affected the final result (Cushnie and Lamb 2005). Similarly, from the ten different mutants spontaneously resistant to enterocins, synthesized by different *Enterococcus faecium* strains (Audisio *et al.* 2005; Ibarguren *et al.* 2006), only one presented certain resistance to 150 μg ml^{-1} of the P ester.

It is interesting to mention that few scientific information exists in print regarding the properties of acylated flavonoid derivatives, as these are relatively recently synthesized compounds. Assays carried out with these compounds so far have been more directed towards other biological/pharmaceutical characteristics than to their use as possible antimicrobial agents or additives (Kodelia *et al.* 1994; Mellou *et al.* 2006; Montenegro *et al.* 2007). With respect to the study of antimicrobial activity, Gatto *et al.* (2002) reported that quercetin, one of the most studied flavonoids, and its esters, up to concentrations of 100 μg ml^{-1}, did not show any antimicrobial activity. However, Mellou *et al.* (2005) enzymatically synthesized a lauroyl ester from a flavone glycoside isolated from Greek endemic plants, and they reported that at a concentration of 500 μg ml^{-1} it presented an antagonistic effect on *Bacillus cereus*, and with a concentration of 1000 μg ml^{-1} it inhibited *Staph. aureus*. However, it should be noted these authors detected no effect on Gram-negative bacteria. They also proposed that the antibacterial activity of the acylated flavone glycoside could be attributed to its higher lipophilicity compared to the unmodified flavone glycoside. In this case, P and its ester, PL, were tested and showed a strong inhibition of *L. monocytogenes* strains and antagonistic effects on *Escherichia coli* and *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium, *Staph. aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* with 150 μg ml^{-1} of PL. Furthermore, the effect on viability of *Listeria* cells was quantified, and the fact that whether the strain analysed was sensitive or not to bacteriocins synthesized by LAB was irrelevant for these compounds. This is extremely interesting because one single compound could inhibit micro-organisms with different behaviours to bacteriocins, and importantly it can be applied at low concentrations.

Little is known about the structure–function relationship of natural antimicrobials, but it seems that different substituents within the compounds have a great influence on their biophysical and biological properties. Structural features, such as the introduction of a hydrophobic chain of 12 carbon atoms in the flavanone glucoside P, could

significantly change membrane permeability and subsequent affinity to external and internal binding sites in the bacteria, thus influencing the compound's antimicrobial properties (Fitzgerald *et al.* 2004). So, the antibacterial activity of PL could be attributed to its higher lipophilicity compared to P. The enzymatic addition of a hydrophobic chain, such as the lauroyl moiety to P in this case, is expected to increase their ability to interact with the cell membrane or even to penetrate through the cell membrane (Kodelia *et al.* 1994). Other authors have demonstrated that hydrophobicity and steric properties play important roles in the antibacterial activities of essential oils (Shapiro and Guggenheim 1998).

It is important to emphasize that the LAB, all of which were isolated and studied by the group for their antimicrobial or probiotic properties (Audisio *et al.* 2000, 2001; Audisio and Apella 2006), were not affected by these compounds. Therefore, future projects should be directed at analysis of combinations of this flavonoid ester with LABORATORY-synthesized metabolites to determine whether the antimicrobial effect could be enhanced or not.

Conclusions

It was determined that P, without any antimicrobial property at all, became a powerful wide-spectrum antimicrobial agent when a lauroyl chain was incorporated. Thus, 150 μg ml^{-1} of PL inhibited *E. coli*, *Salm.* Enteritidis, *Salm.* Typhimurium, *Staph. aureus*, *Enterococcus avium* and *L. monocytogenes* strains. In particular, PL exhibited a bactericidal effect not only on *L. monocytogenes* but also on a bacteriocin-resistant mutant of this strain. These results clearly demonstrate that the flavonoid ester assayed can be used as an effective antibacterial food additive.

Acknowledgements

G. Céliz thanks CONICET for his fellowship. M.C. Audisio is a member of the Research Career of CONICET. This work was funded by PICTO 36683 (ANPCyT-UNSa), PIP 5057 (CONICET) and PI 1698 and 1725 (CIUNSa). The authors thank Novozymes Latin America Limited (Brazil) for the kind gift of the Novozym 435.

References

Audisio, M.C. and Apella, M.C. (2006) Bacteriocin-like substance produced by *Lactobacillus salivarius* subsp. *salivarius* CRL1384 with anti-*Listeria* and anti-*Salmonella* effects. *Res J Microbiol* **1**, 61–69.

Audisio, M.C., Oliver, G., Apella, M. and Iacute, A.C. (2000) Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected with *Salmonella Pullorum*. *J Food Prot* **63**, 1333–1337.

Audisio, M.C., Oliver, G. and Apella, M.C. (2001) Effect of different complex carbon sources on growth and bacteriocin synthesis of *Enterococcus faecium*. *Int J Food Microbiol* **63**, 235–241.

Audisio, M.C., Terzolo, H.R. and Apella, M.C. (2005) Bacteriocin from honeybee beebread *Enterococcus avium*, active against *Listeria monocytogenes*. *Appl Environ Microbiol* **71**, 3373–3375.

Benavente-Garcia, O., Castillo, J., Marin, F.R., Ortuno, A. and Del Rio, J.A. (1997) Uses and properties of citrus flavonoids. *J Agric Food Chem* **45**, 4505–4515.

Céliz, G. and Daz, M. (2010) Biocatalytic preparation of alkyl esters of citrus flavanone glucoside prunin in organic media. *Process Biochem*, under current review.

Clinical and Laboratory Standards Institute (2003) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard*, 6th edn. M7-A6. Wayne, PA: CLSI.

Cushnie, T.P. and Lamb, A.J. (2005) Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* **26**, 343–356.

Davies, E.A. and Adams, M.R. (1994) Resistance of *Listeria monocytogenes* to the bacteriocin nisin. *Int J Food Microbiol* **21**, 341–347.

Farber, J.M. and Peterkin, P.I. (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* **55**, 476–511.

Fisher, K. and Phillips, C. (2008) Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends Food Sci Technol* **19**, 156–164.

Fitzgerald, D.J., Stratford, M., Gasson, M.J., Ueckert, J., Bos, A. and Narbad, A. (2004) Mode of antimicrobial action of vanillin against *Escherichia coli, Lactobacillus plantarum* and *Listeria innocua*. *J Appl Microbiol* **97**, 104–113.

Fortes, C., Forastiere, F., Farchi, S., Rapiti, E., Pastori, G. and Perucci, C.A. (2000) Diet and overall survival in a cohort of very elderly people. *Epidemiology* **11**, 440–445.

Gatto, M.T., Falcocchio, S., Grippa, E., Mazzanti, G., Battinelli, L., Nicolosi, G., Lambusta, D. and Saso, L. (2002) Antimicrobial and anti-lipase activity of quercetin and its C2-C16 3-O-acyl-esters. *Bioorg Med Chem* **10**, 269–272.

Hitchins, A.D. and Whiting, R.C. (2001) Food-borne *Listeria monocytogenes* risk assessment. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **18**, 1108–1117.

Ibarguren, C., Raya, R.R., Apella, M.C. and Audisio, M.C. (2006) Efecto de bacteriocinas sobre cepas sensibles y resistentes de *Listeria monocytogenes*. In *Actas Simposio Internacional de Bacterias Lácticas y Primer Encuentro red BAL (Bacterias Lácticas)*. Argentina: Tucumán.

Ibarguren, C., Raya, R.R., Apella, M.C. and Audisio, M.C. (2010) *Enterococcus faecium* isolated from honey synthesized bacteriocin-like substances active against different *Listeria monocytogenes* Strains. *J Microbiol* **48**, 44–52.

Jurado, R.L., Farley, M.M., Pereira, E., Harvey, C.R., Schuchat, A., Wenger, J.D. and Stephens, D.S. (1993) Increased risk of meningitis and bacteremia due to *Listeria monocytogenes* in patients with human immunodeficiency virus Infection. *Clin Infect Dis* **17**, 224–227.

Justo, A.M. and Rivera, I. (2009) Inserción de Argentina en el mercado mundial de cítricos. *Estudios Socioeconómicos de los Sistemas Agroalimentarios y Agroindustriales* **1**, 1–52. http://www.inta.gov.ar/ies/docs/docs_area/doc_area_1_2743.htm..

Kodelia, G., Athanasiou, K. and Kolisis, F. (1994) Enzymatic synthesis of butyryl-rutin ester in organic solvents and its cytogenetic effects in mammalian cells in culture. *Appl Biochem Biotechnol* **44**, 205–212.

Liu, M., Gray, J.M. and Griffiths, M.W. (2006) Occurrence of proteolytic activity and N-acyl-homoserine lactone signals in the spoilage of aerobically chill-stored proteinaceous raw foods. *J Food Prot* **69**, 2729–2737.

Mandalari, G., Bennett, R.N., Bisignano, G., Trombetta, D., Saija, A., Faulds, C.B., Gasson, M.J. and Narbad, A. (2007) Antimicrobial activity of flavonoids extracted from bergamot *Citrus bergamia* Risso peel, a byproduct of the essential oil industry. *J Appl Microbiol* **103**, 2056–2064.

Mellou, F., Lazari, D., Skaltsa, H., Tselepis, A.D., Kolisis, F.N. and Stamatis, H. (2005) Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *J Biotechnol* **116**, 295–304.

Mellou, F., Loutrari, H., Stamatis, H., Roussos, C. and Kolisis, F.N. (2006) Enzymatic esterification of flavonoids with unsaturated fatty acids: effect of the novel esters on vascular endothelial growth factor release from K562 cells. *Process Biochem* **41**, 2029–2034.

Montenegro, L., Carbone, C., Maniscalco, C., Lambusta, D., Nicolosi, G., Ventura, C.A. and Puglisi, G. (2007) In vitro evaluation of quercetin-3-O-acyl esters as topical prodrugs. *Int J Pharm* **336**, 257–262.

Naghmouchi, K., Kheadr, E., Lacroix, C. and Fliss, I. (2007) Class I/Class IIa bacteriocin cross-resistance phenomenon in *Listeria monocytogenes*. *Food Microbiol* **24**, 718–727.

Otead, D. (1986) Microbial lipases: their characteristics, role in food spoilage and industrial uses. *J Dairy Res* **53**, 481–505.

Peterson, J.J., Beecher, G.R., Bhagwat, S.A., Dwyer, J.T., Gebhardt, S.E., Haytowitz, D.B. and Holden, J.M. (2006a) Flavanones in grapefruit, lemons, and limes: a compilation and review of the data from the analytical literature. *J Food Compost Anal* **19**, S74–S80.

Peterson, J.J., Dwyer, J.T., Beecher, G.R., Bhagwat, S.A., Gebhardt, S.E., Haytowitz, D.B. and Holden, J.M. (2006b) Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature. *J Food Compost Anal* **19**, S66–S73.

Rhodes, P.L., Mitchell, J.W., Wilson, M.W. and Melton, L.D. (2006) Antilisterial activity of grape juice and grape extracts derived from Vitis vinifera variety Ribier. *Int J Food Microbiol* **107**, 281–286.

Shapiro, S. and Guggenheim, B. (1998) Inhibition of oral bacteria by phenolic compounds. Part 1. QSAR analysis using molecular connectivity. *Quant Struct-Act Rel* **17**, 327–337.

Soria, F. and Ellenrieder, G. (2002) Thermal inactivation and product inhibition of *Aspergillus terreus* CECT 2663 alpha-rhamnosidase and their role on hydrolysis of naringin solutions. *Biosci Biotechnol Biochem* **66**, 1442–1449.

Tirillini, B. (2000) Grapefruit: the last decade acquisitions. *Fitoterapia*, **71**, S29–S37.

Torres De Pinedo, A., Peñalver, P. and Morales, J.C. (2007a) Synthesis and evaluation of new phenolic-based antioxidants: structure-activity relationship. *Food Chem* **103**, 55–61.

Torres De Pinedo, A., Peñalver, P., Pérez-Victoria, I., Rondón, D. and Morales, J.C. (2007b) Synthesis of new phenolic fatty acid esters and their evaluation as lipophilic antioxidants in an oil matrix. *Food Chem* **105**, 657–665.

Tripoli, E., Guardia, M.L., Giammanco, S., Majo, D.D. and Giammanco, M. (2007) Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. *Food Chem* **104**, 466–479.

Zhang, Y. and Miller, R.M. (1994) Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Appl Environ Microbiol* **60**, 2101–2106.

Zhang, Y. and Miller, R. (1995) Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of n-alkanes. *Appl Environ Microbiol* **61**, 2247–2251.

