Antifungal activity of lactobacilli isolated from salami

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Abstract

Sixty-five strains of lactobacilli isolated from salami were tested for their antifungal activity in early and late phases of growth. Ten strains showed inhibitory activity in the early phase of growth towards moulds such as *Aspergillus* and *Penicillium*. The active compounds identified were phenyl-lactate and hydroxy-phenyl-lactate. All strains tested had activity in the late phase, after autolysis. The compounds released were peptidic and showed antifungal activity.

Introduction

It is well known that some lactic acid bacteria produce bacteriocins that inhibit the growth of the same and other species of bacteria (Lindgren & Dobrogosz, 1990; Klaenhammer, 1993). The action of lactic acid bacteria towards moulds has also been demonstrated: *Lactobacillus casei* ssp. *rhamnosus* was shown to produce a low-molecular-weight molecule that can inhibit the growth of *Aspergillus parasiticus* by El-Gendy & Marth (1981). Following this report, several studies showed the peptidic nature of the antifungal compounds produced by *Lactobacillus acidophilus* (Batish *et al.*, 1990), *Lactobacillus coryniformis* ssp. *coryniformis* (Magnusson & Schnürer, 2001) and *Lactobacillus plantarum* (Ström *et al.*, 2002). Moreover, other authors (Gourama & Bullerman, 1995; Corsetti *et al.*, 1998) demonstrated that, in addition to peptides, a mixture of short-chain fatty acids is produced by *Lactobacillus sanfranciscensis*. *Lactobacillus plantarum* synthesizes a number of substances, including benzoic acid, methylhydantoin, and mevalonolactone, that have additive antifungal activity (Niku-Paavola *et al.*, 1999). Recent studies (Lavermicocca *et al.*, 2000) confirmed that the inhibitory activity of *L. plantarum* can be attributed to the organic acids phenyl-lactate and 4-hydroxy-phenyl-lactate.

Moulds play an important, but not univocal, role in the ripening of fermented food by lactic acid bacteria. Their role is generally considered positive in the curing and aging of salami when they develop on the surface; in fact, they:
(1) regulate the flow of water from the inside towards the outside;
(2) bring the pH to a value of 5.6 − 5.7 with a deacidification;
(3) contribute to the formation of aroma and taste trough lipolysis (Selgas *et al.*, 1999) and proteolysis (Martin *et al.*, 2002).

The role of moulds in cheeses is very important for products such as Roquefort, Stilton, Camembert and Gorgonzola. In other cheeses, moulds are considered to be negative because their growth on the surface causes alteration, with the production of mycotoxins, and harms the external aspect of the product (Lund *et al.*, 1995; Filtenborg *et al.*, 1996).

With these considerations in mind, this study was carried out with the aim of understanding whether the inhibitory activity towards mould can be considered as a character for the selection of the lactic acid bacteria used as starter cultures for salami, and whether the inhibition arises from compounds that are formed during the fermentative or postfermentative phases. For this purpose, the most frequently observed species in salami cured naturally, namely *L. plantarum* and *Lactobacillus sakei* (Hugas *et al.*, 1993; Coppola *et al.*, 1998; Papamanoli *et al.*, 2003), and the moulds that are often found on the sausages and cheeses or

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that are employed as starter cultures were examined (Grazia *et al.*, 1986; Filtenborg *et al.*, 1996).

Materials and methods

Microorganisms

A total of 63 strains of *Lactobacillus* isolated from salami with different origins were used. These strains formed part of the collection at DISA, and they are indicated by the letters VLT followed by the registration number. Some type strains from the DSMZ collection were also used, including *L. plantarum* DSMZ 20174^T and *Lactobacillus brevis* DSMZ 20054^T. Phenotypical characterization of *Lactobacillus* strains was carried out with an API 50 CH System (BioMerieux).

The inhibitory activity of *Lactobacillus* species was compared against several species of mould that are often found in association with lactic acid bacteria in fermented foods. Strains of these species were from the authors' collection at DISA and the DSMZ collection.

Lactic acid bacteria were stored at 4 °C fixed in Mann Rogosa Sharpe (MRS) Agar (OXOID) and subcultured monthly; moulds were stored in Sabouraud agar (OXOID) and were also subcultured monthly.

Determination of inhibitory activity

The inhibitory activity of compounds produced during the development phase was determined in plates using the overlay technique (Magnusson & Schnürer, 2001). Lactic acid bacteria were plated on the surface in 10-mm lines on 90-mm plates containing 25 mL of MRS agar. Plates were incubated in anaerobiosis at 30 °C for 48 h. After growth of lactic acid bacteria, 10 mL of Sabouraud that had previously been inoculated with 10^5 CFU mL^{-1} of conidia was added (agar 7 g L^{-1}). Plates were then incubated in aerobic conditions at 25 °C, and, after the growth of fungi, were evaluated for inhibition halos around the areas of growth of lactic acid bacteria. The inhibitory activity of compounds produced during the postfermentative phase was determined using a modified version of the protocol described in Chiavari *et al.* (1998), which utilizes the plate diffusion method described for the determination of the bacteriocine (Tagg *et al.*, 1976). In particular, lactic acid bacteria were allowed to grow at 30 °C and then left alone for 30 days to favour autolysis, confirmed by observation with a scanning electron microscope (SEM). The growth medium was recovered, centrifuged (4500 ***g*** for 10 min) to eliminate cells, and filtered (0.20 μm; Albet Jacs). The supernatants were concentrated by lyophilization and resuspended in phosphate buffer (50 mM, pH 7.0) to 15 times the original concentration. From this solution, 125 μL was placed in 9-mm wells contained in 90-mm plates containing Sabouraud (agar 7 g L^{-1}), which had been previously seeded with *Penicillium nalgiovense*, a mould that is sensitive to inhibitory activity (Chiavari *et al.*, 1998).

Determination of the physicochemical characteristics of the inhibitory substances

The inhibitory capacity of the strains harbouring the greatest activity was characterized in both early and late phases. In the latter case, the activity was compared with a strain that had no early inhibitory activity. Mixtures of compounds, produced by fermentation for 48 h for the early phase and after 30 days for late phase, were characterized. In both cases, cultures were allowed to develop in 250 mL of MRS in 500-mL Erlenmeyer flasks at 30 °C. The supernatant was collected by centrifugation (4500 ***g***, 5 min), sterilized by filtration through 0.20-μm filters, concentrated by lyophilization, and resuspended in phosphate buffer (50 mM, pH 7.0) to 15 times the original concentration. The inhibitory activity of the concentrate was evaluated using 9-mm wells as previously described, both before and after the steps recommended by various authors (Lavermicocca *et al.*, 2000; Magnusson & Schnürer, 2001).

In particular, the following analyses were carried out:

(1) To determine if the compound had a peptidic structure, the concentrated, buffered supernatant (pH 7.0 with 4 N NaOH) was subjected to digestion with the following enzymes: proteinase-K (Sigma), trypsin (Sigma), and protease (Sigma) at 37 °C for 1 h. After the reaction, the concentrate was brought to pH 3.5 with HCl.

(2) Heat resistance was determined by subjecting the supernatant to a number of thermal treatments: 80 and 100 °C for 10 and 60 min.

(3) The influence of pH was examined by assessing the inhibitory activity of the concentrate at various pH values: 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0.

Determination of phenyl-lactic acid and hydroxy-phenyl-lactic acid by HPLC

The concentration of phenyl-lactic and hydroxy-phenyl-lactic acid in supernatants was determined as reported in Valerio *et al.* (2004) with Waters (Milano, Italy) HPLC equipment using a Symmetry column C18 RP (150 × 4.6 mm) (Waters), particle size 5 μm, kept at room temperature.

Image acquisition

Measurement of the inhibition halos was performed after digital acquisition of the plate images using a Bio-Rad Gel-Doc 2000 and elaborated using Adobe Photoshop 6.0. Scanning electron micrographs (SEMs) were taken with a Hitachi 510 S. Samples were prepared using the method described by Bottazzi & Bianchi (1980).

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Results and discussion

Spectrum of inhibitory activity during the fermentative phase

Sixty-five strains of *Lactobacillus* were tested for their antifungal capacity during the growth phase using the double-overlay technique with *Aspergillus candidus* DSMZ 814^T and *P. nalgiovense* MF BP3 as the sensitive strains. As shown in Table 1, 54 of the 65 strains had no inhibitory activity. Nine strains had marked inhibitory capacity, particularly intense in VLT01, and produced distinct halos. Two strains had weak inhibitory activity. The typical aspect of the inhibition halos is shown in Fig. 1.

The most active strain, *L. plantarum* VLT01, was chosen for further screening on the sensitivity of various mould species. VLT 73 and VLT 304 were also used as they possessed intermediate activity, and VLT32 as a negative control. As shown in Table 2, VLT01, VLT73 and VLT304 exhibited activity against all the moulds employed, in particular against *Aspergillus*, which produces the most dangerous mycotoxins, and against *Penicillium*, which often colonizes salami casings and cheese crusts. VLT32 did not show any inhibitory activity, as expected.

Inhibitory activity during the late phase

The inhibitory activity during late phases was determined by plating 30-day-old supernatant concentrates in wells using the four strains mentioned previously, which were found to have different inhibitory activities from the early phase. From the results in Table 3, it is evident that even the strains that do not have inhibitory activity in the early phase will, in the late phase, after autolysis, inhibit the growth of mould near the wells. This confirms previous studies (Chiavari *et al.*, 1998) demonstrating that inhibitory activity is caused by compounds that are released following autolysis.

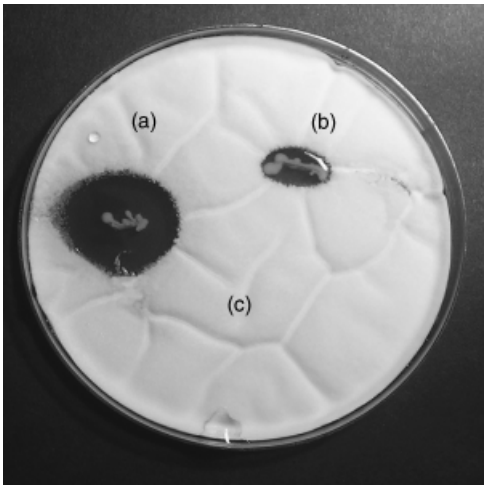

Fig. 1. Plate obtained using the overlay technique. (a) Strong inhibition by VLT01; (b) weak inhibition by VLT304; (c) no inhibition by VLT32.

Table 1. Inhibitory activity of 65 strains of *Lactobacillus* vs. *Aspergillus candidus* and *Penicillium nalgiovense* strains

Species	Strains examined	*Aspergillus candidus* DSM 814^T	*Penicillium nalgiovense* MF BP3
L. plantarum	DSMZ 20174, VLT02, VLT04, VLT31, VLT33, VLT34, VLT35, VLT36, VLT37, VLT38, VLT39, VLT62, VLT71, VLT72, VLT78, VLT 154, VLT 156, VLT154, VLT157, VLT158, VLT160, VLT302, VLT310, VLT452, VLT454, VLT456, VLT457, VLT458, VLT1510, VLT4510,	−	−
L. plantarum	VLT01	+++	+++
L. plantarum	VLT73	+	+
L. plantarum	VLT301	+	++
L. plantarum	VLT304	+	++
L. plantarum	VLT307	++	+
L. plantarum	VLT451	++	+
L. plantarum	VLT452	++	+
L. sakei	VLT32 , VLT74, VLT96, VLT130, VLT148, VLT 159, VLT160, VLT710	−	−
L. pentosus	VLT75, VLT76, VLT77, VLT308, VLT309, VLT310, VLT459	−	−
L. pentosus	VLT308	++	+
L. pentosus	VLT310	++	+
L. pentosus	VLT459	++	+
L. curvatus	VLT152, VLT306, VLT96, VLT166	−	−
L. mali	VLT03, VLT154	−	−
L. mali	VLT112	+	+
L. brevis	DSMZ 20054, VLT118, VLT166	−	−

The inhibitory capacity was scored as follows: −, no inhibition; +, inhibition halo up to 8 mm from the plating line; ++, halo between 9 and 15 mm; +++, halo larger than 15 mm.

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Table 2. Inhibitory activity of four strains representative of *Lactobacillus* on various species of mould

		Strains with action			
		Strong	Medium	Medium	Absent
Mould	Species	VLT01	VLT304	VLT73	VLT32
DSMZ 814	*Aspergillus candidus*	+++	+	+	–
DSMZ 1240	*Geotrichum candidum*	+++	+++	+++	–
DSMZ 1959	*Aspergillus flavus*	++	+	++	–
MFBP3	*Penicillium nalgiovense*	+++	++	+	–
MF4	*Aspergillus ochraceus*	+++	+	+	–
MF5	*Penicillium camemberti*	+++	++	+++	–
MF11	*Moniliella* spp.	+++	+++	+++	–
MF12	*Aspergillus fumigatus*	++	+	++	–
MF40	*Mucor racemosus*	+	+	+	–
MF80	*Penicillium nalgiovense*	+++	+	++	–
MF117	*Wallemia sebi*	+++	+++	+++	–
MF123	*Penicillium verrucosum*	++	+	+	–
MF128	*Eurotium herbariorum*	+++	+++	+++	–
MF139	*Penicillium chrysogenum*	++	+	++	–

The inhibitory capacity was scored as follows: –, no inhibition; +, inhibition halo up to 8 mm from the plating line; ++, halo between 9 and 15 mm; +++, halo larger than 15 mm.

Table 3. Inhibitory activity of selected strains in the late phase after autolysis

	Aspergillus candidus		*Penicillium nalgiovense*	
After	2 days	30 days	2 days	30 days
Lactobacillus plantarum VLT01	+++	+++	+++	+++
Lactobacillus plantarum VLT304	+	++	++	+++
Lactobacillus plantarum VLT73	+	++	+	++
Lactobacillus sakei VLT32	–	++	–	++

The inhibitory capacity was scored as follows: –, no inhibition; +, inhibition halo up to 8 mm from the plating line; ++, halo between 9 and 15 mm; +++, halo larger than 15 mm.

Physicochemical characteristics of compounds produced in the early phase

The supernatant concentrate from the early phase treated with proteolytic enzymes showed no differences in inhibitory capacity with respect to untreated supernatant, excluding the possibility that the compound has a peptidic nature. Similarly, thermal treatment had no effect on inhibition of growth. The compound was, however, sensitive to changes in pH, and concentrated supernatant showed marked antifungal activity at acidic pH that was reduced at neutral pH (Table 4).

For the VLT01 strain, HPLC analysis indicated a phenyl-lactate and hydroxy-phenyl-lactate concentration of 46.6 and 67.6 mg L^{-1}, respectively. Neither substance was detectable for VLT 32. The values obtained are in agreement with those of other authors (Lavermicocca *et al*., 2000, 2003; Valerio *et al*., 2004), in that phenyl-lactate has been implicated in the inhibitory activity of various species of *Lactobacillus*.

Physicochemical characteristics of the compound produced during the postfermentative phase

The supernatants of lactic acid bacteria cultures were studied after aging for 30 days, which is a sufficient time for autolysis to occur. In strain VLT01, which is active at an early stage, the inhibitory activity was maintained, but showed significant differences with respect to those previously seen in the early phase. In particular, the activity was less sensitive to variations in pH with respect to the early phase, and the products were found to be sensitive to enzymatic treatment. The tests carried out on aged supernatants were sensitive only to the action of proteinase K, which brought about significant changes in the intensity and diameter of the relative inhibition halos.

In the case of thermal treatment (80 °C for 60 min), a residual activity was found similar to that seen in the early phase that was dependent on pH, and thus probably due to phenyl-lactate. These results were confirmed by the presence

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of 7.54 mg L^{-1} of phenyl-lactate. In the case of strain VLT32, the inhibitory activity was completely sensitive to heat; in fact, treatment at 80 °C for 60 min was sufficient completely to inactivate the compound (Fig. 2).

Table 4. Physicochemical characteristics of compounds produced in the early phase by *Lactobacillus plantarum* VLT01

Treatment	Activity (%)
Concentrate 15-fold	100
pH	
3.5	100
4.0	64
4.5	45
5.0	36
6.0	6
7.0	0
Proteolytic enzymes	
Trypsin	98
Protease	98
Proteinase K	99
Heat treatment	
80 °C × 10 min	100
100 °C × 10 min	98
80 °C × 60 min	98
100 °C × 60 min	98

The above data confirm that the compound released is peptidic, as suggested by previous studies (Chiavari *et al.*, 1998), which in turn demonstrates that autolysis is responsible for the release of active biological compounds that are not apparent in the early phase. In fact, VLT32 was not active in the early growth phases, but produced a supernatant that was capable of inhibition after aging.

Conclusions

The inhibitory activity of lactobacilli against moulds can be realized at different times and is caused by different factors. The early phase is realized during fermentation and arises from the formation of compounds such as phenyl-lactic acid; it is displayed by some strains but not by others. The late phase, realized at the end of cell growth, arises from the release of peptidic compounds. It is a common characteristic of all the strains as the physiological consequence of cellular autolysis.

The inhibitory activity in the fermentative phase, as presented in some strains of *Lactobacillus*, has interesting technological possibilities for a variety of fermented food products, for example dry fermented sausages and cheese. In

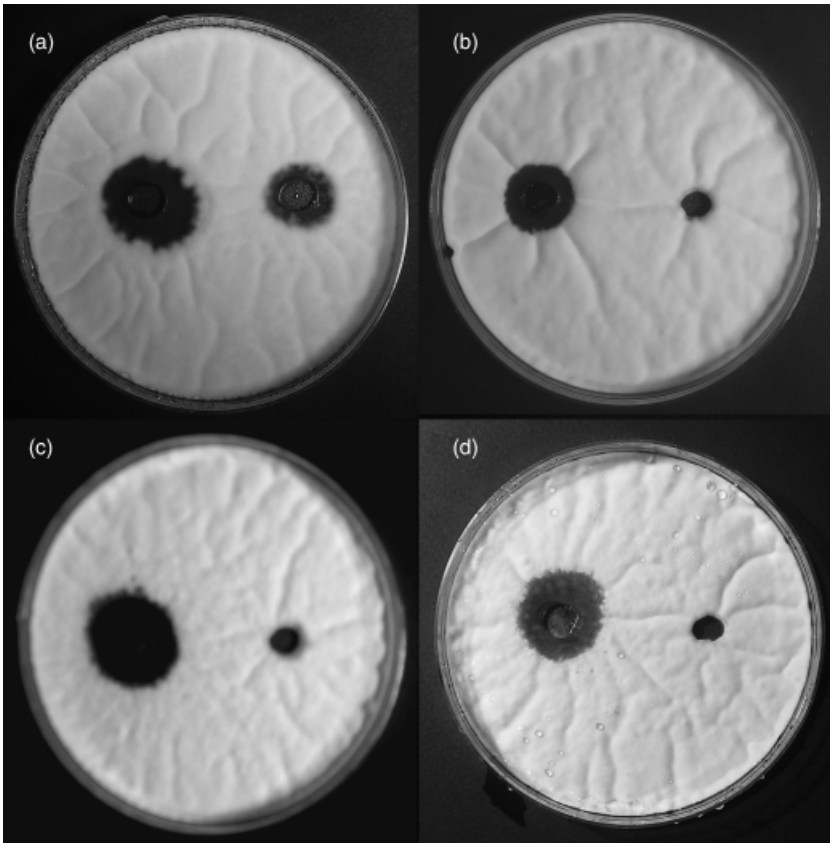

Fig. 2. Plates obtained with supernatants of strains aged for 30 days and thermally treated (80° for 60 min). (a) Strain VLT01, pH 3.5; (b) strain VLT01, pH 7.0; (c) strain VLT32, pH 3.5; (d) strain VLT32, pH 7.0. For each plate, the left wells were untreated, while the right wells were heat-inactivated.

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fact, this lactic acid bacteria is often used as a starter to guide fermentation, and its behaviour towards moulds can be considered to be one of the main selection characteristics. The choice of the strain to use in this regard for fermentation is naturally based on the desired results.

Postfermentative activity is an important phase, especially during the production of salami, and it acts in the late phase with an antagonist action vs. toxygen aspergillis, but also vs. *Penicillium* moulds before their growth become excessive, influencing in a negative way the quality of the products.

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