ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM SPANISH DRY FERMENTED SAUSAGES

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INTRODUCTION

Although red meat carries a heterogeneous microbial flora, the dry fermented sausages develops a bacterial population comprised mostly of lactic acid bacteria (LAB). These bacteria have the potential to inhibit the growth of pathogenic and spoilage microorganisms and the possibility exists of using them as a safety factor to improve the hygienic quality and to extend the shelf-life of different meat and meat products.

Reduction of pH and removal of carbohydrates are the primary preserving actions exerted by the lactic acid bacteria. It has been also recognized that LAB are capable of producing inhibitory substances other than organic acids that are antagonistic toward other microorganisms (Daeschel, 1989). These substances are produced in smaller amounts and include hydrogen peroxide, diacetyl, bacteriocins, and secondary reaction products. Thus, it was of interest to examamine the ability of a number of LAB isolated from Spanish dry fermented sausages to inhibit a variety of pathogenic and food spoilage microorganisms, try to understand the mechanisms by which these bacteria exert their antimicrobial effect and to study a number of parameters associated to their growth at different temperatures in a complex medium.

MATERIALS AND METHODS

Bacterial strains

A total of 50 LAB isolates were selected among the colonies developed in MRS agar plates (Oxoid) from a number of samples of Spanish dry fermented sausages. Their antimicrobial effect was evaluated against selected indicator bacteria from the Spanish Type Culture Collection (CECT) and from the AFRC Food Research Institute (Langford, Bristol, UK), and included other lactic acid bacteria as well as species of Micrococacceae, Enterococcus, Pseudomonas, Bacillus, Brochothrix thermosphacta and pathogens such a species of Staphylococcus, Salmonella, Shigella, E. coli, Yersinia and Listeria. Other lactic acid bacteria used in this work were from the Federal Center for Meat Research (Kulmbach, FRG).

Assay of the antimicrobial activity by the direct antagonism test

Wells of ca. 7 mm Ø were cut in MRS agar plates (Oxoid). 20 µl of cultures, previously grown overnigth in MRS broth, washed and resuspended in the same volume of medium, were added to each well. The plates were maintained 4 h at 32 ºC and after refilling the wells with MRS agar the indicator microorganisms were overlaid in the plate with about 10^6 cells in 4 ml of soft MRS or BHI agar (0.8% agar). All plates were incubated at 32 ºC and the antimicrobial activity was quantified measuring the clear zones of inhibition around the wells. Experiments were also performed maintaining the LAB cultures at 32 ºC for 24 h, before overlaing the agar plates with the indicator bacteria.

Catalase effect

The effect of catalase on the antimicrobial activity of each selected LAB isolate was evaluated by settling 0.2 ml of a catalase solution (500.000 UI/ml) on MRS plates containing pregrown 24 h LAB isolates as single colonies. The plates were maintained 4 h at 32 ºC and the selected indicator microorganism (L. fermentum) was overlaid in 4 ml of soft MRS agar. All plates were incubated 24 h at 32 ºC and the catalase effect was quantified as reduction in the clear zones of inhibition around the colonies.

Antimicrobial activity of concentrated cell-free supernatants

Cell cultures were centrifuged at 12000 g for 15 min and the supernatants were adjusted to pH 6, filtered through a 0.22 µm pore size filter (Millipore) and lyophilised. The resulting freeze-dried supernatants were resuspended 20-fold concentrated in sterile distilled water and 30 µl deposited onto 6 mm Ø filter paper discs (Whatman Nº 3). After 30 min at room temperature, the discs were deposited in prepoured agar plates overlaid with about 10^6 cells of the indicator strain in 4 ml of soft MRS or BHI agar. All plates were incubated at 32 ºC and the antimicrobial activity was quantified measuring the clear zones of inhibition around the discs.

Effect of heat treatment, proteolytic enzymes and pH on antimicrobial activity

To determine the thermal stability of concentrated cell-free supernatants showing antimicrobial activity against a number of indicator bacteria, 1 ml of the supernatants were boiled for 15 min or autoclaved at 120 ºC for 20 min, cooled and assayed for activity. Supernatants were also assayed by the effect of 1 mg/ml of enzyme solutions of papain, protease II and XIV, typsin and pepsin (Sigma) incubated with samples at 37 ºC for 1 h, and assayed for activity. The activity of the supernatants was also evaluated after maintaining them at 24 ºC for 24 h in a universal buffer solution at different pH values from 2.6 to 12.

Identification and biochemical characterization of the isolates

The LAB isolates of interest were essentially subjected at the growth conditions and cultural and biochemical tests recommended by Schillinger and Lucke (1987), for the rapid and simple identification of lactobacilli from meat and meat products.

Parameters of growth at different temperatures

Growth experiments were done in MRS broth to obtain culture supernatant fluids for lactic acid determination and final pH as well as to determine growth rates (t_d, doubling time) and cell yields. Growth was monitored at 4, 8, 15, 20 and 32 ºC by measuring the absorbance of the cultures at 660 nm. Growth rates were calculated from the linear portion of the log absorbance at 660 nm vs. time plots for each strain. Final pH was recorded in a Radiometer pH meter 28 and cell dry weights were calculated from plots correlating gravimetric determinations vs. absorbance values at 660 nm. Lactic acid was determined by the L-lactic acid enzymatic determination test (Boehringer Mannheim).

RESULTS

The antimicrobial activity of 50 LAB isolates from Spanish dry fermented sausages was evaluated against selected saprophytic and food pathogenic bacteria by the direct antagonism test, where most of them showed a quantifiable antimicrobial effect. Eight isolates were selected for their maximum effect when compared to the others. Their antimicrobial activity was higher against other lactic acid bacteria, but nevertheless, a quantifiable effect was also observed against Gram negative and positive food pathogens (Table I). Their inhibitory effect was greater when the lawn of indicator bateria was deposited in the test plates after 24 h growth of the LAB isolates at 32 ºC.

The eight LAB isolates were further identified by their microscopic appearence, Gram staining, catalase activity and most of the cultural and biochemical tests recommended by Schillinger and Lucke (1987). Using the rapid and simple identification scheme proposed by these investigators, all LAB isolates were tentatively identified as Lactobacillus sake.

The effect of catalase on the antimicrobial activity of the isolates was tested against L. fermentum as the indicator microorganism. The inhibitory activity of L. sake 2 was strongly reduced (90%) by the addition of catalase, whereas the rest of the isolates showed a much lower reduction

TABLE I. Antimicrobial activity of selected LAB isolates onto several indicator bacteria

Indicator	LAB isolate Nº 2 a	2 b	11 a	11 b	20 a	20 b	23 a	23 b	29 a	29 b	38 a	38 b	77 a	77 b	148 a	148 b
L. fermentum	++	+++	+	++	+	+	+	+	+	++	+	+	+	+++	++	+++
L. plantarum	++	+++	+	++	o	+	o	+	+	++	+	++	+	+++	++	+++
L. divergens	+	+++	+	++	++	+++	+	++	o	++	+	+++	+++	+++	+++	+++
M. varians	+	+	o	o	o	+	+	+	o	+	o	+	o	+	o	+
B. thermosphacta	+	+	+	+	+	+	+	+	+	+	+	++	o	+	o	o
S. xylosus	o	+	o	o	o	o	o	+	o	+	o	+	o	+	o	o
S. faecalis	o	++	o	o	o	o	o	o	o	+	o	+	o	+	o	o
E. cloacae	+	+	o	+	o	o	o	+	o	o	o	+	o	+	o	+
E. coli BW545	+	++	o	++	o	o	o	++	+	+	+	+++	o	+	o	+
E. coli K99	+	+	o	o	o	o	o	+	o	o	o	+	o	+	o	o
S. typhimurium	+	+	o	+	+	+	+	+	+	+	+	+	o	++	o	o
S. flexnerii	o	o	o	o	o	o	o	+	o	o	+	+	o	++	o	o
S. aureus	+	+	o	o	o	o	o	+	o	o	+	+	o	+	o	+
Y. enterocolitica	+	+	o	+	o	o	+	++	+	+	+	+	o	+	o	o
Pseudomonas DC7	o	+	o	o	o	+	o	+	o	+	o	o	o	+	o	o
L. monocytogenes	+	+	+	+	+	+	+	+	+	+	+	+	o	+	+	++
B. stearothermophilus	o	o	o	o	o	o	+	+	o	o	+	+	o	o	o	o

a. Indicator strains seeded 4 h later than LAB isolates
b. Indicator strains seeded 24 h later than LAB isolates
- Antimicrobial activity expressed as the area of the halo of inhibition in $mm^2 \times 10^{-2}$ /Klett units:

0 : No activity
\+ : 0-20 ($mm^2 \times 10^{-2}$ /Klett units)
++ : 20-40 (" ")
+++ : 40 (" ")

TABLE II. Antimicrobial activity of concentrated cell-free culture supernatants from L. sake 77 and L. sake 148

LAB isolate	INDICATOR BACTERIA				
	L. fermentum	L. curvatus	L. divergens	L. brevis	L. monocytog
77	++	+	++	++	0
148	++	++	++	+++	+

- Inhibitory activity as in Table I

TABLE III. Growth rates, final pH, maximum cell dry mass and L-lactic acid production of the L. sake isolates and other lactic acid bacteria at 4 ºC and 32 ºC.

strain	At 4 ºC					At 32 ºC				
	pH	t_d	cdm	L-LA	L-LA Prod.	pH	t_d	cdm	L-LA	L-LA Prod.
2	4.85	138.6	1.13	7.36	0.038	4.00	4.78	1.46	11.68	0.66
11	5.05	210.0	0.96	4.56	0.028	4.05	5.10	1.46	11.44	0.65
20	5.00	150.6	1.04	4.48	0.025	4.05	5.80	1.36	9.44	0.57
23	5.00	187.3	1.03	6.64	0.038	4.10	5.30	1.36	11.36	0.69
29	5.60	182.4	0.91	1.52	0.010	4.05	6.30	1.44	10.72	0.62
38	4.90	138.6	1.11	6.88	0.036	3.95	5.40	1.53	11.76	0.64
77	4.60	117.8	1.06	8.40	0.036	4.20	8.25	1.34	9.54	0.44
148	4.90	111.0	1.00	5.94	0.027	4.50	7.70	1.27	10.72	0.52
(A)	4.70	231.0	1.05	2.54	0.011	4.50	6.89	1.31	11.50	0.73
(B)	5.80	911.8	0.69	Nd	Nd	4.30	6.05	1.42	16.44	0.96
(C)	5.70	911.8	0.69	Nd	Nd	4.60	6.48	1.23	16.02	1.08

(A) L. sake Lb 684
(B) L. curvatus Lb 726
(C) L. plantarum Lb 577

Nd : Not detectable
t_d : Doubling time (h)
cdm : Cell dry weight (mg/ml)
L-LA : L-Lactic acid (mg/ml)
L-LA Prod. : L-Lactic acid (mg L-LA/mg CDW-h)

(10-17%) of their inhibitory effect.

Two isolates, L. sake 77 and L. sake 148, showed in their concentrated cell-free supernatatants an antimicrobial activity against other lactic acid bacteria and a Listeria monocytogenes strain (Table II). The boiling of these extracts for 15 min reduced their activity in a 60 to 80%, whereas the activity was lost by heating to 120 ºC for 20 min. The inhibitory activity was totally lost by incubation of the extracts with 1 mg/ml of papain, proteases II and XIV, trypsin and pepsin. The activity was found to be a maximum at pH 4.6 to 5.6.

The effect of temperature on the growth of the LAB isolates in a complex medium (Table III), showed that most of L. sake isolates grew and acidified the growth medium at 4 ºC, whereas the L. curvatus and L. plantarum strains had only a residual growth at this temperature. At 32 ºC, L.curvatus and L. plantarum showed higher growth rates and L-lactic acid productivities than the L. sake isolates.

CONCLUSIONS

As it has been already acknowledged, the production of lactic acid and the reduction of pH may account for most of the antimicrobial effect shown by the lactic acid bacteria isolated from Spanish dry fermented sausages. The identification of the isolates as L. sake it is not surprising since it has been observed (Lucke, 1986; Schillinger and Lucke, 1987b) that this species may become dominant at the lower ripening temperatures normally used in Europe for most of the dry fermented sausages.

However, it is interesting to note that one of the isolates, L. sake 2, may increase its antimicrobial effect by generation of higher amounts of hydrogen peroxide, surely by several different mechanisms (Gotz et al. 1980; Kandler, 1983). The antimicrobial activity of hydrogen peroxide is well recognized and documented (Daeschel, 1989).

Two more isolates, L. sake 77 and L. sake 148, manifested an antimicrobial activity on their concentrated cell-free supernatatants, which on the basis of their sensitivity to heat, different pH and proteases, have been tentatively assigned as bacteriocins. By definition, bacteriocins are protein-containing macromolecules which exert a bactericidal mode of action on susceptible bacteria (Tagg et al. 1976). Specific bacteriocin receptors on sensitive cells and plasmid-borne determinants of production and immunity are among the secondary criteria for defining bacteriocins (Klaenhammer, 1988). The ability of these bacteriocins to inhibit foodborne pathogens, such as L. monocytogenes, make them attractive as potential preservation agents (Hoover et al. 1988; Pucci et al. 1988)

Most of the L. sake isolates obtained from Spanish dry fermented sausages grow and produce lactic acid at low temperatures, showing promise for being used in a variety of meat and meat products as a safety factor to inhibit psychrotrophic food spoilage microorganisms and pathogens. Some of the isolates synthesize other antimicrobial metabolites such as hydrogen peroxide and bacteriocins, improving their potential as food preservatives.

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