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Lactic acid bacteria: hydrophobicity and strength of attachment to meat surfaces

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The hydrophobicity and strength of attachment of several lactic acid bacteria with antimicrobial activity were studied. Hydrophobicity was determined by bacterial adherence to hydrocarbons (BATH; octane or xylene), adhesion to nitrocellulose filters (NCF), salt aggregation test (SAT) and adherence to phenyl–Sepharose beads (PSB). The relative hydrophobicity of lactic acid bacteria depended markedly on the method used. No correlation between either SAT or BATH (octane) and strength of attachment (Sr value) existed. However, a significant relationship between strength of attachment and BATH (xylene), NCF and PSB, respectively, was observed, showing the highest correlation coefficient ($r = 0{\cdot}778$) for BATH (xylene).

INTRODUCTION

Fresh meats are susceptible to contamination by pathogens and spoilage micro-organisms. Microbial contamination of meat has always been an important issue from the viewpoint of public health and quality characteristics including shelf-life. Since it is not possible to avoid contamination, the development of methods to decontaminate meat has always been a primary economic concern. Several methods have been proposed to reduce initial levels of contaminating bacteria on meat surface; most of them focus on washing and the use of sanitizing agents such as chlorine and organic acids (Dickson 1991; Dickson and Anderson 1992).

It has been observed that lactic acid bacteria (LAB) are capable of inhibiting the growth of a wide variety of spoilage and pathogenic micro-organisms associated with foods (Stiles and Hastings 1991; Ray and Daeschel 1992). The inhibitory effect of these bacteria is related to the fermentative production of acids, hydrogen peroxide, antibiotics and bacteriocins (Schillinger and Lücke 1989; Sobrino *et al.* 1991, 1992; Itoh *et al.* 1995; Jack *et al.* 1995). The potential of LAB as decontamination agents has been studied on meat by several authors (Mattila-Sandholm *et al.* 1991; Winkowski *et al.* 1993; El-Khateib *et al.* 1993).

Cell-surface hydrophobicity has been associated with bacterial attachment to a variety of surfaces. Greater hydrophobicity of cells results in greater attractive forces and higher levels of adhesion (Rijnaarts *et al.* 1993). As LAB could be used as biocontrol agents, it would be interesting to study two important characteristics of attachment of these bacteria: cell-surface hydrophobicity and strength of attachment.

The purpose of this study was to determine cell-surface hydrophobicity and strength of attachment of several LAB with antimicrobial activity to meat surfaces. The ultimate goal is to provide information for a potential use of LAB in meat decontamination procedures.

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MATERIALS AND METHODS

Micro-organisms

Eight strains of LAB with antimicrobial activity were used in this study. Three of them have been previously isolated an identified in our laboratory: *Lactobacillus sake* 148 (Sobrino *et al.* 1991), *Pediococcus acidilactici* 347 (Moreira 1993) and *Ped. acidilactici* L50 (Cintas *et al.* 1995). The others were obtained from different culture collections: *Ped. pentosaceus* FBB63 from the TNO Nutrition and Food Research (Zeist, the Netherlands), *Lactococcus lactis* ATCC 11454 from the American Type Culture Collection (Rockville, MD), *L. cremoris* CNRZ117 from the Institut National de la Recherche Agronomique, Station de Recherches Laitières (Jouy-en-Josas, France), *Lact. curvatus* NCFB2739 from the National Collection of Food Bacteria (Reading, UK) and *Leuconostoc*

cremoris DB1275 from the Facultad de Veterinaria (Madrid, Spain).

Standardized cultures were obtained by growing the micro-organisms at 30°C in MRS broth (Oxoid Ltd, Basingstoke, UK) for two consecutive 24 h periods and an additional 18 h period. The bacterial cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C, washed twice with phosphate buffer (pH 7·0) and adjusted to optical density of 1·0 at 540 nm.

Cell-surface hydrophobicity

Four different methods were used to assess the relative surface hydrophobicity of lactic acid bacteria.

Bacterial adherence to hydrocarbons (BATH). This test was performed as described by Sweet *et al.* (1987). Bacterial cell suspension in phosphate buffer (pH 7·0) was dispensed in 3-ml volumes into test tubes and mixed with 1 ml of either xylene or octane by vortexing vigorously for 30 s. After phase separation (approx. 30 min), the optical density at 540 nm of the aqueous phase was determined. Results are reported as percentage of adherence to xylene or octane.

Bacterial adherence to nitrocellulose filters (NCF). The procedure was carried out by the method of Lachica and Zink (1984). Three millilitres of bacterial cell suspension were filtered through a 8·0-μm nitrocellulose filter (type SC, Millipore, Bedford, MA). The absorbance at 540 nm (A_{540}) of the filtered material was determined. Results are recorded as percentage of adherence to nitrocellulose filter.

Salt aggregation test (SAT). The method of Jonsson and Wadström (1984) was used. Bacterial cell suspensions (25 μl) were mixed with equal volumes of ammonium sulphate of various molarities (0·01–4·0 mol l^{-1}). The lowest concentration of ammonium sulphate giving visible aggregation was scored as the SAT hydrophobicity value.

Adherence to phenyl–Sepharose beads (PSB). The assay was carried out as previously described by Fattom and Shilo (1984). One millilitre of phenyl–Sepharose beads (size 45–165 μm; approximate concentration 40 μmol of gel beads per ml) was added to 5 ml of bacterial cell suspension and thoroughly mixed. The A_{540} of the aqueous phase was measured after 10 min (the time needed for the phenyl–Sepharose beads to settle). Results are reported as percentage of adherence to phenyl–Sepharose.

Attachment experiments

Bovine *Longissimus dorsi* muscle was obtained from a local abattoir. The muscle was cut under sterile conditions into pieces with a total surface area of approximately 20 cm^2. The process was controlled by microbial counts of samples using Plate Count Agar (Oxoid) as culture medium. For all experiments total viable counts were lower than 10^2 cfu cm^{-2}.

Samples of muscle were aseptically placed into a flask containing 20 ml of cell suspension (10^5 cells ml^{-1}). After 20 min at room temperature, samples were removed, drained and placed into another flask containing 99 ml of phosphate buffer (pH 7·0), which was gently inverted 30 times within 30 s to remove 'loosely attached' (to meat) bacteria. Then each sample was removed, drained and transferred to a sterile bag containing 99 ml of phosphate buffer and homogenized for 2 min in a Stomacher 400 (Teckmar Co., Cincinnati, OH). Bacteria remaining on the tissue after rinsing were considered to be 'strongly attached'. Bacteria were enumerated on MRS agar incubated at 30°C for 48 h.

The strength of attachment (Sr value) was calculated as: (strongly attached bacteria)/(loosely + strongly attached bacteria).

Statistical analysis

Regression analysis to determine the correlation between hydrophobicity and strength of attachment (Sr value) of LAB to beef muscle surface was carried out using the Statview™ package (Abacus Concepts, Berkeley, CA). Significance was declared at $P < 0{\cdot}05$. Each test was performed in triplicate and the data presented are the average of at least two experiments.

RESULTS AND DISCUSSION

The cell-surface hydrophobicity of all LAB strains tested is shown in Table 1. Bacterial hydrophobicity depended markedly on the method used. According to Dickson and Koottmaraie (1989), the relative ranking of hydrophobicity obtained with each method of measurement was compared. Although each method resulted in a different ranking, there were no significant differences in ranking with regard to the values measured by BATH, NCF and PSB. There was a good correlation between the NCF and either PSB ($r = 0{\cdot}953$) or BATH (xylene) ($r = 0{\cdot}930$). However, such a correlation was not evident between SAT and the other methods. This is in agreement with the findings of Jones *et al.* (1991), which indicate that the differences may be due to the inaccurate SAT methodology based on the visual estimation of aggregation. The BATH assay showed significant variations depending on the hydrocarbon used. The values obtained with octane were much lower than those obtained with xylene.

Table 1 Surface hydrophobicity of lactic acid bacteria determined by adherence to hydrocarbons (BATH), adhesion to nitrocellulose filters (NCF), salt aggregation test (SAT) and adherence to phenyl–Sepharose beads (PSB)

	BATH*				
Micro-organisms	Xylene	Octane	NCF*	PSB*	SAT†
Pediococcus acidilactici 347	8·42 ± 0·91 (8)	7·36 ± 0·46 (5)	30·00 ± 2·46 (7)	12·05 ± 1·20 (7)	0·010 (2)
Ped. pentosaceus FBB63	10·12 ± 1·22 (7)	2·16 ± 0·13 (8)	40·16 ± 3·05 (6)	7·90 ± 1·20 (8)	0·010 (2)
Lactococcus lactis ATCC 11454	56·07 ± 0·64 (3)	34·12 ± 1·40 (1)	97·36 ± 3·06 (1)	55·11 ± 2·05 (1)	< 0·010 (1)
Lactobacillus sake 148	60·85 ± 0·73 (1)	21·44 ± 2·01 (2)	96·94 ± 2·88 (2)	50·25 ± 2·60 (2)	4·000 (4)
L. cremoris CNRZ117	59·85 ± 0·81 (2)	13·66 ± 0·54 (4)	63·17 ± 1·50 (4)	41·82 ± 1·30 (3)	2·000 (3)
Lact. curvatus NCFB2739	51·22 ± 1·35 (4)	16·24 ± 0·76 (3)	65·47 ± 1·76 (3)	33·23 ± 2·10 (4)	0·010 (2)
Leuconostoc cremoris DB1275	11·65 ± 0·97 (6)	2·83 ± 0·22 (6)	42·18 ± 1·15 (5)	20·57 ± 1·00 (5)	2·000 (3)
Ped. acidilactici L50	20·87 ± 1·88 (5)	2·59 ± 0·19 (7)	30·00 ± 1·16 (8)	14·14 ± 1·30 (6)	2·000 (3)

* % adherence ± standard error.
† SAT value was scored as the lowest concentration of ammonium sulphate giving visible aggregation.
‡ Numbers in parentheses indicate relative ranking of hydrophobicity.

It has been reported in previous studies with some speculation that the viscosity of the hydrocarbon or size of droplets formed during mixing may be involved (Rosenberg 1984; Dillon *et al*. 1986). As the results of a single method may not be a true representation of the cell-surface hydrophobicity, we adopted the results obtained with BATH (xylene), NCF and PSB for a reliable hydrophobicity assessment. Taking these results together, *L. lactis* ATCC 11454, *Lact. sake* 148 and *L. cremoris* CNRZ117 were the most hydrophobic micro-organisms tested.

The strength of attachment (Sr value) of LAB to beef muscle surface is shown in Table 2. We have previously investigated the effect of bacterial concentration and immersion time in the inoculated medium on the strength of attachment. Most of the strains showed the highest Sr values with bacterial concentrations of 10^5 cells ml^{-1} and immersion times of 20 min (data not shown). Therefore, these conditions were chosen as optimal bacterial concentrations and immersion times for further experiments. The highest Sr values were recorded with *Lact. sake* 148, *L. cremoris* CNRZ117 and *L. lactis* ATCC 11454.

Table 2 Strength of attachment (Sr value) of lactic acid bacteria to beef muscle surface

Micro-organisms*	Sr value†
Pediococcus acidilactici 347	0·361
Ped. pentosaceus FBB63	0·466
Lactococcus lactis ATCC 11454	0·606
Lactobacillus sake 148	0·792
L. cremoris CNRZ117	0·656
Lact. curvatus NCFB2739	0·452
Leuconostoc cremoris DB1275	0·315
Ped. acidilactici L50	0·565

* Bacterial concentration = 10^5 cfu ml^{-1}; immersion time = 20 min.
† Sr value = (strongly attached bacteria)/(loosely + strongly attached bacteria).

The correlation coefficients between hydrophobicity and strength of attachment to beef muscle of LAB are shown in Table 3. No correlation between either SAT or BATH (octane) and strength of attachment (Sr value) existed. However, a linear and significant relationship between strength of attachment and BATH (xylene), NCF and PSB, respectively, was observed, showing the highest correlation coefficient ($r = 0·778$) for BATH (xylene).

The relationship between strength of attachment and the hydrophobicity of bacteria has been studied by several authors. Piette and Idziak (1992) have reported that the strength of adhesion is influenced by the cell-surface charge and hydrophobicity. Furthermore, it has been proposed that

Table 3 Correlation of hydrophobicity to strength of attachment (Sr value) to beef muscle surface of lactic acid bacteria

Hydrophobicity	Correlation coefficient (r)
BATH (xylene)	0·778†
BATH (octane)	0·567
NCF	0·706*
PSB	0·706*
SAT	0·588

* $P < 0·05$.
† $P < 0·025$.
BATH, NCF, PSB and SAT as defined in Table 1.

the cell-surface characteristics have a greater influence on the adhesion strength than on the number of adherent bacteria (van Pelt *et al*. 1995). Dickson and Koottmaraie (1989) found some correlation between relative hydrophobicity of the bacterial cells and the attachment to beef tissue. However, van Loosdrecht *et al*. (1987) have proposed that hydrophobicity is the primary modulator of adhesion for hydrophobic organisms, while adhesion of hydrophilic organisms is dominated by electrokinetic potential. In this sense, Gilbert *et al*. (1991) have also concluded that surface hydrophobicity is correlated to adhesion only for hydrophobic micro-organisms.

In the present study, a correlation between bacterial cell hydrophobicity and strength of attachment to beef muscle was shown by LAB strains tested. Taking into account these two characteristics, the potential use of *L. lactis* ATCC 11454, *Lact. sake* 148 and *L. cremoris* CNRZ117 as biocontrol agents on meat would be very interesting.

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REFERENCES

Cintas, L.M., Rodríguez, J.M., Fernández, M.F., Sletten, K., Ness, I.F., Hernández, P.E. and Holo, H. (1995) Isolation and characterization of Pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Applied and Environmental Microbiology* **61**, 2643–2648.

Dickson, J.S. (1991) Control of *Salmonella typhimurium*, *Listeria monocytogenes*, and *Escherichia coli* O157 :H7 on beef in a model spray chilling system. *Journal of Food Science* **56**, 191–193.

Dickson, J.S. and Anderson, M.E. (1992) Microbiological decontamination of food animal carcasses by washing and sanitizing systems : a review. *Journal of Food Protection* **55**, 133–140.

Dickson, J.S. and Koottmaraie, M. (1989) Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology* **55**, 832–836.

Dillon, J.K., Fnerst, J.A., Hayward, A.C. and Davis, G.H.G. (1986) A comparison of five methods for assaying bacterial hydrophobicity. *Journal of Microbiological Methods* **6**, 13–19.

El-Khateib, T., Yousef, A.E. and Ockerman, H.W. (1993) Inactivation and attachment of *Listeria monocytogenes* on beef muscle treated with lactic acid and selected bacteriocins. *Journal of Food Protection* **56**, 29–33.

Fattom, A. and Shilo, M. (1984) Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. *Applied and Environmental Microbiology* **47**, 135–143.

Gilbert, P., Evans, D.J., Evans, E., Duguid, I.G. and Brown, M.R.W. (1991) Surface characteristics and adhesion of *Escherichia coli* and *Staphylococcus epidermidis*. *Journal of Applied Bacteriology* **71**, 72–77.

Itoh, T., Fujimoto, Y., Kawai, Y. and Saito, T. (1995) Inhibition of food-borne pathogenic bacteria by bacteriocins from *Lactobacillus gasseri*. *Letters in Applied Bacteriology* **21**, 137–141.

Jack, R.W., Tagg, J.R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. *Microbiological Reviews* **59**, 171–200.

Jones, D.S., Gorman, S.P., MacCafferty, D.F. and Woolyson, A.D. (1991) The effects of three non-antibiotic antimicrobial agents on the surface hydrophobicity of certain microorganisms evaluated by different methods. *Journal of Applied Bacteriology* **71**, 218–227.

Jonsson, P. and Wadström, T. (1984) Cell surface hydrophobicity of *Staphylococcus aureus* measured by the salt aggregation test (SAT). *Current Microbiology* **10**, 203–210.

Lachica, R.V. and Zink, D.L. (1984) Plasmid associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. *Infection and Immunology* **44**, 540–543.

Mattila-Sandholm, T., Haikra, A. and Skytra, A.E. (1991) The effect of *Pedicoccus damnosus* and *Pediococcus pentosaceus* on the growth of pathogens in minced meat. *International Journal of Food Microbiology* **13**, 87–99.

Moreira, W.L. (1993) Aislamiento y caracterización parcial de una bacteriocina producida por *Pediococcus* sp. 347, de origen cárnico. Doctoral Thesis, Universidad Complutense, Madrid, Spain.

Piette, J.P.G. and Idziak, E.S. (1992) A model study of factors involved in adhesion of *Pseudomonas fluorescens* to meat. *Applied and Environmental Microbiology* **58**, 2783–2791.

Ray, B. and Daeschel, M. (1992) *Food Biopreservatives of Microbiological Origin*. Boca Raton, FL : CRC Press.

Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J. and Zehnder, A.J.B. (1993) Bacterial adhesion under static and dynamic conditions. *Applied and Environmental Microbiology* **59**, 3255–3265.

Rosenberg, M. (1984) Bacterial adherence to hydrocarbons : a useful technique for studying cell surface hydrophobicity. *FEMS Microbiology Letters* **22**, 289–295.

Schillinger, M. and Lücke, F.K. (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology* **55**, 1901–1906.

Sobrino, O.J., Rodríguez, J.M., Moreira, W.L., Fernández, M.F., Sanz, B. and Hernández, P.E. (1991) Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. *International Journal of Food Microbiology* **13**, 1–10.

Sobrino, O.J., Rodríguez, J.M., Moreira, W.L., Cintas, L.M., Fernández, M.F., Sanz, B. and Hernández, P.E. (1992) Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. *International Journal of Food Microbiology* **16**, 215–225.

Stiles, M.E. and Hastings, J.W. (1991) Bacteriocin production by lactic acid bacteria : potential for use in meat preservation. *Trends in Food Science and Technology* **2**, 247–251.

Sweet, S.P., Macfarlane, T.W. and Samarayanake, L.P. (1987) Determination of the cell hydrophobicity of oral bacteria using a modified hydrocarbon adherence method. *FEMS Microbiology Letters* **48**, 130–134.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A.J.B. (1987) Electrophoretic mobility and hydro-

phobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology* **58**, 1898–1901.

van Pelt, A.W., Weerkamp, A.H., Uyen, M.H.W.J.C., Busscher, H.J., de Jong, H.P. and Arends, J. (1985) Adhesion of *Streptococcus sanguis* CH3 to polymers with different surface free energies. *Applied and Environmental Microbiology* **49**, 1270–1275.

Winkowski, K., Crandall, A.D. and Montville, T.J. (1993) Inhibition of *Listeria monocytogenes* by *Lactobacillus bavaricus* MN in beef systems at refrigeration temperatures. *Applied and Environmental Microbiology* **59**, 2552–2557.