Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

In vitro growth inhibition of *Helicobacter pylori* by lactobacilli belonging to the *Lactobacillus plantarum* group

S. Rokka, A. Pihlanto, H. Korhonen and V. Joutsjoki

MTT Agrifood Research Finland, Food Research, Jokioinen, Finland

Keywords
fermented food, growth inhibition, *Helicobacter*, *Lactobacillus plantarum*.

Correspondence
Susanna Rokka, MTT Agrifood Research Finland, Food Research, Myllytie 1, FIN-31600 Jokioinen, Finland. E-mail: susanna.rokka@mtt.fi

2005/1361: received 15 November 2005, revised 14 March 2006 and accepted 31 May 2006

doi:10.1111/j.1472-765X.2006.01998.x

Abstract

Aims: The aim of this study was to test and locate the *in vitro* anti-*Helicobacter* activity of seven *Lactobacillus* strains belonging to *Lactobacillus plantarum* group.

Methods and Results: Growth inhibition of *H. pylori* was tested using a well-plate assay. Of the strains displaying the strongest growth inhibition, a *L. plantarum* isolated from sauerkraut (MLBPL1) was chosen for further studies. The detected anti-*Helicobacter* activity of MLBPL1 was mainly associated with cell wall, and to a minor extent with the culture supernatant. The active component, which was determined to be between 3 and 10 kDa in size, retained its activity after 10 min treatment at 100°C. The activity was present when MLBPL1 was cultivated in rich laboratory cultivation medium MRS and in different food matrices.

Conclusions: The strains belonging to *L. plantarum* group showed anti-*Helicobacter* activity *in vitro*. The main activity seemed to be associated with cell wall rather than culture supernatant or intracellular fraction.

Significance and Impact of the Study: In view of the rapid spread of resistant *H. pylori* strains caused by antibiotic therapy, addition of a fermented food containing *L. plantarum* to the conventional antibiotic treatment of *Helicobacter* infection could establish a potential complementary means to suppress the infection.

Introduction

Helicobacter pylori is a Gram-negative, spiral-shaped bacterium that lives in gastric mucus. The urease enzyme secreted by the bacterium enables it to survive in the acidic gastric milieu of the host. *Helicobacter pylori* is the major aetiological agent in chronic gastritis, peptic ulcer and gastric cancer (Malaty and Nyrén 2003). The *H. pylori* infection is predominantly acquired in childhood and persists lifelong, often asymptomatic in most infected subjects. The prevalence of *H. pylori* among adults is typically around 80–90% and <40% in developing and developed countries, respectively (Perez-Perez *et al.* 2004).

One-week triple therapy using a proton pump inhibitor or ranitidine bismuth citrate, combined with two antibiotics, is the most frequently recommended treatment for *H. pylori* infection (Malfertheiner *et al.* 2002). Antibiotic treatments are not always effective against *Helicobacter* infection, as antibiotic resistance is a growing problem worldwide. Further, it is not recommended to treat all asymptomatic people infected by *H. pylori* infection with antibiotics. Current anti-*Helicobacter* therapies using antibiotics are expensive for developing countries where most infections occur, in all probability mostly because of poor hygiene. Therefore, the development of alternative methods is deemed necessary.

A traditional and simple way of preserving food is fermentation by lactic acid bacteria (LAB). In addition to preservation, lactic acid fermentation is used to improve the flavour and texture of the product. A number of LAB strains primarily of dairy origin have been shown to inhibit the growth of *H. pylori* (Hamilton-Miller 2003). For instance, cell-free culture supernatant of the commercial

probiotic strain *Lactobacillus johnsonii* La1 (Michetti *et al.* 1999; Felley *et al.* 2001) and *Lactobacillus acidophilus* strain LB (Coconnier *et al.* 1998) decreased *H. pylori* viability *in vitro* and *H. pylori* density in animal models and humans.

The species *L. plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* are genotypically closely related and show highly similar phenotypes (Bringel *et al.* 1996). They are important in the production of a variety of fermented foods of both plant (pickled vegetables, silage, sourdough) and animal (dry fermented sausages, fermented fish, cheese) origin (Rose 1982). Because of their beneficial effect on human health, some strains are considered as potential probiotics (Herias *et al.* 1999). As several other species of LAB, *L. plantarum* is known to produce antimicrobial compounds (Leal *et al.* 1998), which enhance the preservation of a fermented product. *Lactobacillus plantarum*-related LAB strains inhibiting the growth of *H. pylori* would be very interesting in food fermentations, because they could be exploited as potential therapeutic agents to suppress *Helicobacter* infection.

In this study, the effect on the growth of *H. pylori* of seven *Lactobacillus* strains belonging to the *L. plantarum* group was screened *in vitro*. One *L. plantarum* strain showing strong anti-*Helicobacter* activity was chosen for closer examination.

Materials and methods

Bacterial strains and culture conditions

Helicobacter pylori strain NCTC 11637, kindly provided by Dr Hilpi Rautelin, University of Helsinki, was cultivated on Brucella agar (BBL, Becton Dickinson, Meyland, France) with 5% defibrinated horse blood supplemented with 1% of Iso Vitalex (Becton Dickinson) in a microaerophilic atmosphere at 37°C for 3 days.

The LAB strains used were *L. plantarum* MLBPL1, isolated from naturally fermented white cabbage (Tamminen *et al.* 2003, 2004), *L. plantarum* DSM 20174 (ATCC 14917), *L. plantarum* Vege-Start 60 (Chr. Hansen A/S, Copenhagen, Denmark), *L. paraplantarum* (Curk *et al.* 1996) DSM 14485, *L. paraplantarum* DSM 10667, *L. paraplantarum* DSM 10641, and *L. pentosus* Vege-Start 60 (Chr. Hansen). These strains were cultivated in MRS broth (BD Diagnostic Systems Sparks, MD, USA) overnight without shaking at 30°C. *Lactobacillus plantarum* MLBPL1 was also cultivated in carrot juice, cucumber juice and whey in cultivation conditions described above. Carrot and cucumber juices were prepared by homogenizing fresh vegetables in a household juice extractor. The extracted juices were pasteurized at 95°C for 30 min before use. To ensure the start of fermentation, 2% glucose (w/v) was added into cucumber juice.

Preparation of cell-free culture supernatant, washed cells, whole cell lysate, cell wall fragments and intracellular fraction

Cell-free culture supernatants were obtained by centrifugation (10 000 ***g***, 5 min) of LAB cultures grown overnight in 1.5 ml of MRS broth. Harvested cells were resuspended into 1 ml of 50 mmol l^{-1} HEPES buffer (Sigma Aldrich Chemie Gmbh, Steinheim, Germany), pH 7 and centrifuged as described above. Whole cell lysate of *L. plantarum* MLBPL1 was obtained by mixing harvested cells, which were washed as explained above, with glass beads (Ø 0.1 mm) and homogenizing by vigorous shaking (Vibrogen Zellmühle, Edmund Bühler, Germany) for 90 s. Cell wall fragments and intracellular fraction were separated from the whole cell lysate by centrifugation (13 000 ***g***, 20 min). All fractions were resuspended into the final volume of 1.5 ml with HEPES.

Growth inhibition on plate

Inhibition of the growth of *H. pylori* by lactobacillar culture supernatants and cellular fractions obtained as described above was screened as in Wendakoon *et al.* (1998). Briefly, *H. pylori* suspension containing *c.* 10^8 bacteria was spread on Brucella agar plates. Wells were cut with a sterile straw and filled with 70 μl of a LAB sample to be tested for anti-*Helicobacter* activity. Amoxicillin trihydricum (Orion Diagnostica, Espoo, Finland) in concentrations of 0.2–1 mg l^{-1} was used as positive control and MRS broth as negative control. Plates were incubated at 37°C under microaerophilic conditions for 3 days, after which the diameters of the inhibitory zones were measured.

Size screening by filtration

The culture supernatant of *L. plantarum* MLBPL1 was filtered using Amicon Microcon YM-3 and Amicon Ultra 10 000 MWCO (Millipore Co., Bedford, MA, USA) to obtain fractions <3, >3, <10 and >10 kDa.

Precipitation with ammonium sulfate

The pH of the cell-free culture supernatant of *L. plantarum* MLBPL1 was adjusted to 6.3 with 1 mol l^{-1} NaOH. The supernatant fluid was then precipitated with ammonium sulfate (40% saturation) overnight at 4°C with gentle stirring. The precipitate was collected by centrifugation

(2000 ***g***, 10 min) and dissolved into 10 mmol l^{-1} phosphate buffer, pH 7.0.

The effect of heating

Samples of the *L. plantarum* MLBPL1 crude and precipitated cell-free culture supernatant at natural pH and pH adjusted to 7 were incubated at 100°C for 10 min in Thermolyne Dri-Bath (Dubuque, IA, USA). The anti-*Helicobacter* activity was tested by measuring inhibition zones on Brucella agar plate as described above.

Enzyme treatments

Samples of *L. plantarum* MLBPL1 cell-free culture supernatant, precipitated with ammonium sulfate, were treated with α-amylase (Sigma, St Louis, MO, USA), pepsin (Sigma), proteinase K (Roche Diagnostics GmbH, Penzberg, Germany), N-glycosidase F (Boehringer Mannheim, Germany) or neuraminidase (Fluka Chemie, Buchs, Switzerland). For α-amylase the incubation time was 15 min and for pepsin 60 min at 37°C with shaking at 200 rev min^{-1}. The enzyme : protein ratio used was 1 : 200. For proteinase K the enzyme : protein ratio used was 1 : 20. Proteinase K incubation was performed at 37°C for 2 days and stopped by adding phenylmethyl-sulfonylfluoride to a final concentration of 1 mmol l^{-1}. Incubation time for N-glycosidase F (5 U mg^{-1}) and neuraminidase (8 mU mg^{-1}) was 20 h at 37°C. Incubation with pepsin was conducted at pH 2 and 4, with other enzymes at pH 7. Negative controls were done by incubating the enzymes alone in MRS broth.

Results

Inhibitory effect of the culture supernatants of different LAB strains on the growth of *H. pylori*

The inhibitory effect of culture supernatants of seven MRS-grown *L. plantarum*-related lactobacilli against *H. pylori* NCTC 11637 was screened by measuring the diameters of inhibitory zones on plate. All members of the *L. plantarum* group showed some anti-*Helicobacter* activity (Fig. 1), but the culture supernatants of the strains *L. plantarum* MLBPL1, isolated from sauerkraut, and *L. pentosus* from a commercial starter Vege-Start 60 showed the clearest inhibition (average diameters of the zones 10 and 11 mm, respectively). The inhibitory activity of MLBPL1 cells cultivated in cucumber juice, carrot juice or whey was similar to the inhibitory activity of MLBPL1 cells cultivated in MRS broth (data not shown). The 10 mm inhibition zone corresponds to amoxicillin trihydricum concentration of 0.5 mg l^{-1}.

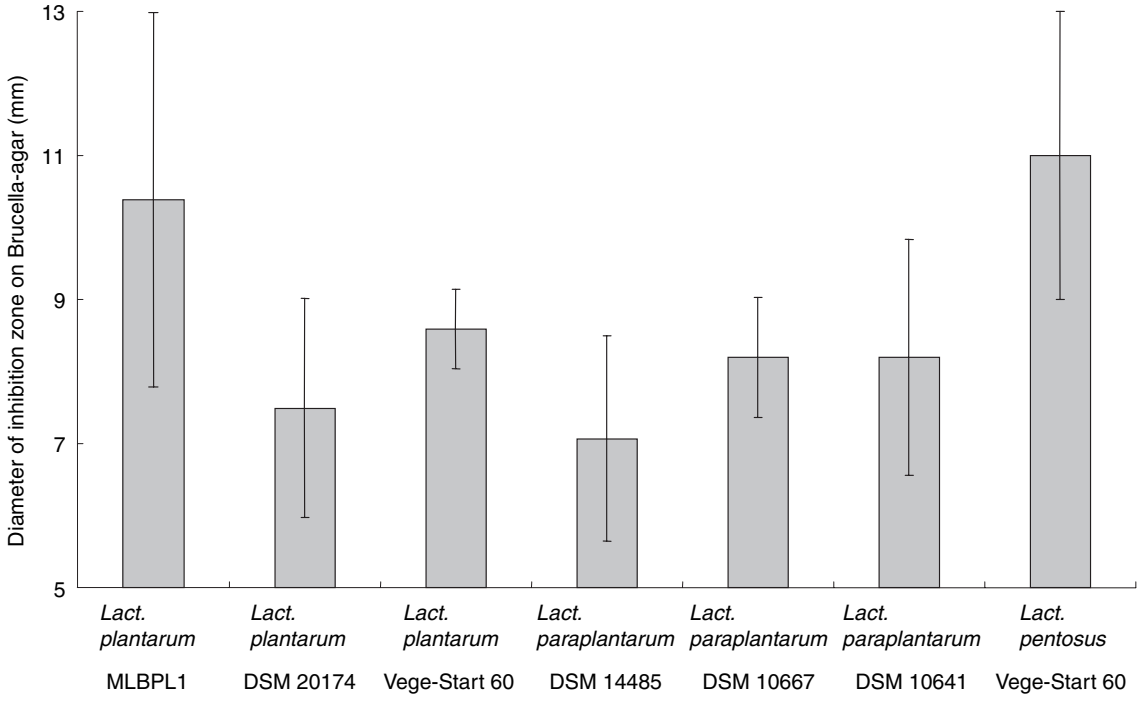

Figure 1 *In vitro* inhibitory action of culture supernatants of MRS-grown cultures of *Lactobacillus plantarum*-related lactobacilli on the growth of *Helicobacter pylori* NCTC 11637. The columns represent the diameter of inhibition zones on Brucella-agar in mm. Arithmetic average values were calculated from five to eight (in the case of MLBPL1 23) independent inhibition test experiments.

The other tested strains displayed only a minor inhibitory effect (average diameters of inhibition zones <9 mm). MRS medium, cucumber juice, carrot juice and whey used as control samples did not inhibit the growth of *H. pylori*. Of the strains displaying the strongest growth inhibition of *H. pylori* (*L. plantarum* MLBPL1 and *L. pentosus* Vege-Start 60), *L. plantarum* MLBPL1 was chosen for further studies because of the more extensive utilization of *L. plantarum* in food and feed applications.

Inhibitory effect of whole MLBPL1 cells and cellular fractions

In addition to the culture supernatant, whole cells as well as cell lysate and cell wall fragments of *L. plantarum* MLBPL1 contained anti-*Helicobacter* activity (Fig. 2). In this test the volume of all samples was equivalent to the volume of the original MLBPL1 cultivation and culture supernatant. Thus, the concentration of washed cells and cellular fractions was equivalent to the concentration of corresponding compounds in the original MLBPL1 culture, and so the anti-*Helicobacter* activity of the samples tested can be compared with each other. The anti-*Helicobacter* activity was present in MRS-grown and washed *L. plantarum* MLBPL1 cells (7×10^8 cells ml^{-1}), in the whole cell lysate and in cell wall fragments with average diameters of the inhibition zones 22, 15 and 12 mm, respectively. The intracellular fraction did not significantly inhibit the growth of *Helicobacter*.

Fractionation, ammonium sulfate precipitation and enzyme treatment of the anti-*Helicobacter* culture supernatant

The substance with anti-*Helicobacter* activity in the cell-free culture supernatant of an MRS-grown *L. plantarum* MLBPL1 was found to be between 3000 and 10 000 Da in size (Table 1). This active substance could be concentrated from culture supernatant by ammonium sulfate precipitation (40% saturation). Both the precipitate and crude culture supernatant retained anti-*Helicobacter* activity after 10 min of treatment at 100°C, but unlike crude culture supernatant, the ammonium sulfate precipitate retained anti-*Helicobacter* activity also at neutral pH (Table 1).

The ammonium sulfate precipitate of the *L. plantarum* MLBPL1 culture supernatant was treated with different enzymes. It was not possible to remove the anti-*Helicobacter* activity of MLBPL1 culture supernatant by incubation with any of the enzymes tested (data not shown). On the contrary, incubation with N-glycosidase F slightly increased the anti-*Helicobacter* activity of MLBPL1.

Discussion

In this study, the anti-*Helicobacter* activity of seven LAB strains belonging to the *L. plantarum* group was screened *in vitro*. All members of the *L. plantarum* group showed some anti-*Helicobacter* activity, but the culture

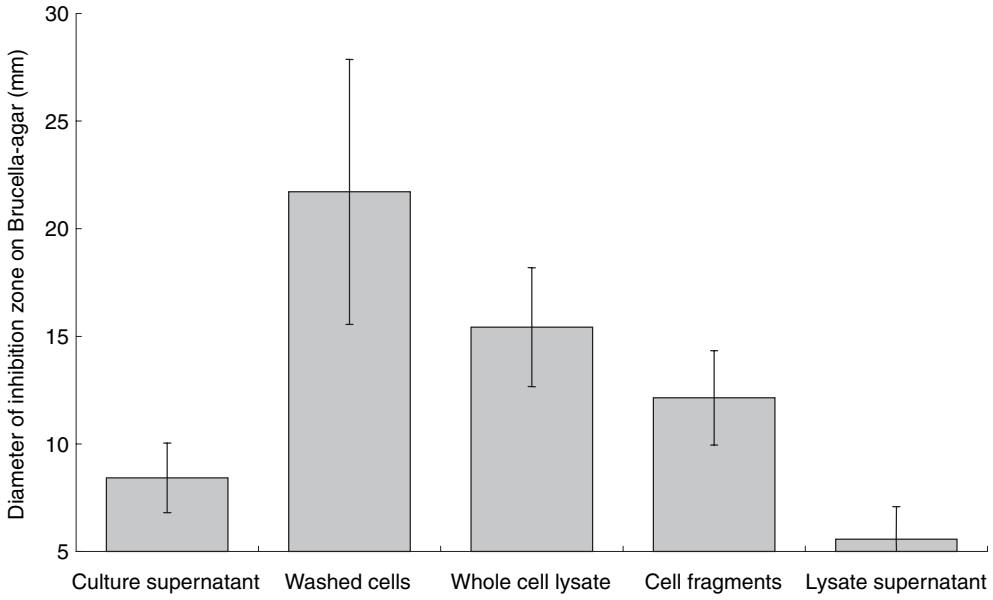

Figure 2 *In vitro* inhibitory action of different fractions of an MRS-grown *Lactobacillus plantarum* MLBPL1 culture on the growth of *Helicobacter pylori* NCTC 11637. The columns represent the diameter of inhibition zones on Brucella-agar in mm. Arithmetic average values were calculated from seven independent inhibition test experiments.

Table 1 *In vitro* inhibitory action of *Lactobacillus plantarum* MLBPL1 crude and precipitated culture supernatant on the growth of *Helicobacter pylori* NCTC 11637

L. plantarum MLBPL1 culture supernatant (treatments)	Inhibition of *Helicobacter*
Crude culture supernatant	+
Crude culture supernatant, fraction <3000 Da	–
Crude culture supernatant, fraction >3000 Da	+
Crude culture supernatant, fraction <10 000 Da	+
Crude culture supernatant, fraction >10 000 Da	–
Crude culture supernatant, treated at 100°C for 10 min	+
Crude culture supernatant, pH raised to 7	–
Crude culture supernatant, pH raised to 7, treated at 100°C for 10 min	–
Culture supernatant, precipitated with ammonium sulfate	+
Culture supernatant, precipitated with ammonium sulfate, treated at 100°C for 10 min	+
Culture supernatant, precipitated with ammonium sulfate, pH raised to 7	+
Culture supernatant, precipitated with ammonium sulfate, pH raised to 7, treated at 100°C for 10 min	+

supernatants of the strains *L. plantarum* MLBPL1, isolated from sauerkraut, and *L. pentosus* from a commercial starter Vege-Start 60 showed the clearest inhibition.

Even though lactic acid inhibits the urease activity of *H. pylori*, several studies have shown other substances secreted by LAB to be behind the mechanism of antibacterial effects (Hamilton-Miller 2003). Coconnier *et al.* (1998) showed that a heat-stable antimicrobial substance secreted by *L. acidophilus* LB is active against *Helicobacter* infection. Also, Michetti *et al.* (1999) who studied *L. acidophilus* La1 concluded that another secreted product in addition to lactic acid contributed to the inhibitory effect observed. In the present study, the substance with anti-*Helicobacter* activity in the culture supernatant of *L. plantarum* MLBPL1 was found to be between 3000 and 10 000 Da in size. Thus, the effect of organic acids and other low molecular mass compounds was excluded. Interestingly, further characterization of *L. plantarum* strain MLBPL1 revealed that the highest anti-*Helicobacter* activity was associated with cells rather than culture supernatant or intracellular fraction. According to the results, the main activity seemed to be associated with the cell wall, from where it is probably extracted into culture supernatant.

It was possible to concentrate the active substance of the *L. plantarum* MLBPL1 culture supernatant by ammonium sulfate precipitation. The anti-*Helicobacter* activity of both the ammonium sulfate precipitate and crude culture supernatant was heat resistant, but unlike crude culture supernatant, the precipitate retained its anti-*Helicobacter* activity at neutral pH. The reason for the loss of anti-*Helicobacter* activity from crude culture supernatant at pH 7 might be a proteolytic enzyme, which is active at neutral pH and is either inactivated or does not precipitate during ammonium sulfate treatment. Similarly, the inhibitory activity of the culture supernatant of *L. acidophilus* La1 was heat resistant, dialysable, and independent of the presence of urea, but was lost when pH of the supernatant was raised to 6 (Michetti *et al.* 1999).

Inactivation after treatment with proteolytic enzymes is a key criterion for bacteriocin characterization. The ammonium sulfate precipitate of the *L. plantarum* MLBPL1 culture supernatant was treated with different enzymes, i.e. pepsin, amylase, proteinase K, N-glycosidase F and neuraminidase. It was not possible to remove the anti-*Helicobacter* activity of MLBPL1 by incubation with any of these enzymes, even though proteinase K is widely used to break down proteins. Therefore, it seems that the anti-*Helicobacter* effect observed is not explained by an already known bacteriocin. On the contrary, incubation with N-glycosidase F, cleaving asparagine-bound N-glycans, increased the anti-*Helicobacter* activity of MLBPL1, suggesting that a component of a larger molecule could be the active compound, which gains the full activity after cleavage from a carrier substance. Wendakoon and Ozimek (2002) reported that the supernatant of *Lactobacillus casei* skimmed milk culture treated with proteinase K or trypsin produced a stronger *Helicobacter* inhibitory action *in vitro* than untreated supernatant. Unlike *L. plantarum* MLBPL1, washed *L. casei* cells showed no inhibition on the growth of *H. pylori*.

The anti-*Helicobacter* activity of several probiotic LAB strains used in dairy products has been reported in a number of *in vitro* studies and clinical trials, and the results have been encouraging (Hamilton-Miller 2003). The majority of the LAB tested for anti-*Helicobacter* activity have been exemplars of species with minor importance as starters or strains used for probiotic purposes. Technological properties of commercial probiotic culture preparations are often problematic. They are sensitive, and may affect the sensory properties of the product. So, it is common to add them to a completed product. On the contrary, *L. plantarum* has been traditionally used for acidification of foodstuffs and can be used as starter in the production of a wide variety of fermented foods. In this study, the anti-*Helicobacter* activity was present also in cultivations performed in different food matrices. Consequently, the production of a fermented food with anti-*Helicobacter* activity would be easy and cost-efficient, because no additional components besides the food matrix and the starter culture will be needed to complete the

process. Considering the long history of the use of *L. plantarum* in a variety of food applications all over the world and in view of the rapid spread of resistant *H. pylori* strains caused by antibiotic therapy, the addition of a fermented food containing *L. plantarum* to the conventional antibiotic treatment of *Helicobacter* infection should be considered.

Acknowledgements

The authors thank the Finnish Graduate School of Applied Bioscience: Bioengineering, Food & Nutrition, Environment, for financial support.

References

Bringel, F., Curk, M.C. and Hubert, J.C. (1996) Characterization of lactobacilli by southern-type hybridization with a *Lactobacillus plantarum pyrDFE* probe. *Int J Syst Bacteriol* **46**, 588–594.

Coconnier, M.H., Lievin, V., Hemery, E. and Servin, A.L. (1998) Antagonistic activity against *Helicobacter* infection in vitro and in vivo by the human *Lactobacillus acidophilus* strain LB. *Appl Environ Microbiol* **64**, 4573–4580.

Curk, M.C., Hubert, J.C. and Bringel, F. (1996) *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus plantarum*. *Int J Syst Bacteriol* **46**, 595–598.

Felley, C.P., Corthesy-Theulaz, I., Rivero, J.L., Sipponen, P., Kaufmann, M., Bauerfeind, P., Wiesel, P.H., Brassart, D. *et al.* (2001) Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. *Eur J Gastroenterol Hepatol* **13**, 25–29.

Hamilton-Miller, J.M.T. (2003) The role of probiotics in the treatment and prevention of *Helicobacter pylori* infection. *Int J Antimicrob Agric* **22**, 360–366.

Herias, M.V., Hessle, C., Telemo, E., Midtvedt, T., Hanson, L.A. and Wold, A.E. (1999) Immunomodulatory effects of *Lactobacillus plantarum* colonizing the intestine of gnotobiotic rats. *Clin Exp Immunol* **116**, 283–290.

Leal, M.V., Baras, M., Ruiz-Barba, J.L., Floriano, B. and Jiménez-Díaz, R. (1998) Bacteriocin production and competitiveness of *Lactobacillus plantarum* LPCO10 in olive juice broth, a culture medium obtained from olives. *Int J Food Microbiol* **43**, 129–134.

Malaty, H.M. and Nyrén, O. (2003) Epidemiology of *Helicobacter pylori* infection. *Helicobacter* **8**, 8–12.

Malfertheiner, P., Megraud, F., O'Morain, C., Hungin, A.P., Jones, R., Axon, A., Graham, D.Y. and Tytgat, G.; European Helicobacter Pylori Study Group (EHPSG) (2002) Current concepts in the management of *Helicobacter pylori* infection – The Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* **16**, 167–180.

Michetti, P., Dorta, G., Wiesel, P.H., Brassart, D., Verdu, E., Herranz, M., Felley, C., Porta, N. *et al.* (1999) Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonii)* La1 on *Helicobacter pylori* infection in humans. *Digestion* **60**, 203–209.

Perez-Perez, G.I., Rothenbacher, D. and Brenner, H. (2004) Epidemiology of *Helicobacter pylori* Infection. *Helicobacter* **9** (s1), 1–6.

Rose, A.H. (1982) *Economic Microbiology, Fermented Foods*, Vol. 7. London: Academic Press.

Tamminen, M., Mäki, M., Joutsjoki, T., Ryhänen, E.-L. and Joutsjoki, V. (2003) Differentiation of lactobacilli related to *Lactobacillus plantarum* from naturally fermented cucumbers and white cabbage. *Appl Biotech Food Sci Policy* **1**, 125–128.

Tamminen, M., Joutsjoki, T., Sjöblom, M., Joutsen, M., Palva, A., Ryhänen, E.-L. and Joutsjoki, V. (2004) Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR-ELISA. *Lett Appl Microbiol* **39**, 439–444.

Wendakoon, C.N. and Ozimek, L. (2002) Inhibition of *Helicobacter pylori* growth by *Lactobacillus casei* in fermented milk. *Milchwissenschaft* **57**, 506–509.

Wendakoon, C.N., Fedio, W., Macleod A. and Ozimek L. (1998) *In vitro* inhibition of *Helicobacter pylori* by dairy starter cultures. *Milchwissenschaft* **53**, 499–502.