Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid

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Abstract

Spent culture supernatant (SCS) of the probiotic *Lactobacillus rhamnosus* GG had been reported to exert antibacterial activity against *Salmonella typhimurium*. However, the chemical identity of the antimicrobial compound(s) responsible remained unknown. A survey of the antimicrobial compounds produced by *L. rhamnosus* GG was performed. *Lactobacillus rhamnosus* GG produced a low-molecular weight, heat-stable, non-proteinaceous bactericidal substance, active at acidic pH against a wide range of bacterial species. SCS of *L. rhamnosus* GG grown in MRS medium contained five compounds that could meet the above description, if present at the appropriate concentration. Based on different experimental approaches, it could be concluded that under the growth conditions tested, the strong antimicrobial activity of *L. rhamnosus* GG against *Salmonella* was mediated by lactic acid.

Introduction

In different ecological niches such as the gastrointestinal tract, lactic acid bacteria (LAB) produce a variety of antimicrobial compounds (Ouwehand, 1998; Bongaerts & Severijnen, 2001; Servin, 2004). LAB which, when administered in adequate amounts, confer a health benefit on the host, are referred to as probiotic strains (FAO/WHO, 2001). One such strain is *Lactobacillus rhamnosus* GG (Sherwood & Gorbach, 1996) for which many health benefits have been postulated, including the defeat of intestinal pathogens. *Lactobacillus rhamnosus* GG spent culture supernatant (LGG-SCS) was reported to be antimicrobial against *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and other intestinal pathogens (Silva *et al.*, 1987; Hudault *et al.*, 1997; Lehto & Salminen, 1997). Silva *et al.* (1987) reported that *L. rhamnosus* GG secretes an antimicrobial substance, distinct from lactic acid, with inhibitory activity against other bacteria in the pH range from 3 to 5. Despite many speculations, the purification and/or structural identification of the antimicrobial compound(s) has not been described. Therefore, this study aimed at unequivocally identifying these antimicrobial compound(s) produced by *L. rhamnosus* GG *in vitro*.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus rhamnosus GG (ATCC 53103) (Sherwood & Gorbach, 1996) and *Salmonella typhimurium* SL1344 (Hoiseth & Stocker, 1981) were grown at 37 °C. *Lactobacillus rhamnosus* GG was inoculated from glycerol stocks (–80 °C), propagated twice in De Man–Rogosa–Sharpe medium (MRS; Difco) (De Man *et al.*, 1960) and grown in nonshaking conditions. *Salmonella typhimurium* was grown in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989). Solid media contained 1.5% (w/v) agar.

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Coculture of *L. rhamnosus* GG and *S. typhimurium*

The coculture was performed as described previously (Drago *et al.*, 1997), with modification of the coculture medium used. One liter of coculture-medium was composed of a mixture of the ingredients for 1 L of LB (Sambrook *et al.*, 1989) and for 1 L of MRS medium (De Man *et al.*, 1960) to which 1 L of distilled water was added. For glucose containing medium, filter sterilized glucose solution was added at a final concentration of 20 g L^{-1}.

Preparation of SCS

MRS broth was inoculated with *L. rhamnosus* GG with a starting concentration of 2.35×10^7 CFU per mL. SCS was obtained from a 24 h culture by centrifugation for 30 min at 10 000 ***g*** at 4 °C. To monitor the production of antimicrobial compounds during the growth of *L. rhamnosus* GG, samples were taken and used to prepare SCS at distinct time points. Growth of *L. rhamnosus* GG was monitored by measuring the optical density at 600 nm with an UV-visible light spectrophotometer (UV/VIS Lambda 2, Perkin Elmer). A pH ranging from 4.5 to 3.7 was observed for LGG-SCS over time. For standardization, the pH of tested LGG-SCS was adjusted to 4.5 with HCl or NaOH for all experiments, followed by filter sterilization (0.22 μm; Millipore).

Sensitivity of antimicrobial compounds to heat and proteolytic enzymes

The physical and enzymatic treatment of the SCS was performed as described previously (Bernet-Camard *et al.*, 1997; Coconnier *et al.*, 1997). Briefly, the LGG-SCS (24 h; pH 4.5) was heated at 110 °C for 1 h. To test the sensitivity to proteases (all purchased from Sigma), the LGG-SCS was incubated at 37 °C for 1 h with and without pronase E (200 μg mL^{-1}), trypsin (200 μg mL^{-1}), proteinase K (100 μg mL^{-1}), or pepsin (200 μg mL^{-1}). As an alternative method to eliminate proteinaceous compounds, the flow-through of a Microcon-SCX filter (Millipore, cut-off 3 kDa) was used.

Determination of organic acid concentration

A commercial kit for the determination of D- and L-lactic acid was used (Roche). For acetic and formic acid, an Aminex HPX 87 H HPLC column (Bio-Rad Laboratories) was used. This column was also used for routine qualitative assessment of organic acids present in SCS. Control experiments were carried out using commercial lactic, acetic and formic acid (VWR International). The column was used with 6 mM HCl as carrier liquid at 0.6 mL min^{-1} flow rate, the column temperature was 35 °C, and the elution of the compounds was monitored at 210 nm.

Tests for antimicrobial activity of SCS

Monitoring bacterial growth via Bioscreen assay

An overnight culture of *S. typhimurium* was washed in phosphate-buffered saline (PBS) and $\pm 10^5$ CFU per mL were applied to growth medium to which LGG-SCS was added and with the final pH adjusted with HCl or NaOH as indicated in the text. Different concentrations of LGG-SCS were tested. 1 : 12 dilutions of SCS were routinely used as this is the highest concentration of LGG-SCS which still allows growth of *Salmonella*. Bacteria were grown, and the optical density at 600 nm was measured automatically each 30 min during at least 80 h in a BioscreenC instrument (Labsystems Oy). For each time point, the average optical density was calculated from three independent measurements. The generation time (g) was calculated as follows: $g = [(t_2 - t_1)\ \log 2]/[\log OD_2 - \log OD_1]$ with t, time; OD, optical density at 600 nm; 1 and 2 are successive time points in exponential growth phase.

Radial diffusion test

The radial diffusion test was performed as described previously (Coconnier *et al.*, 1997). Briefly, 5×10^6 CFU per mL of *S. typhimurium* were added to 10 mL of Trypticase soy broth (TSB) agar [1% agarose, 0.02% (v/v) Tween 20] (42 °C) and poured into a square Petri dish. The test material (15 μL) was applied in a well punched in the agar and incubated for 3 h at 37 °C. Subsequently, the plates were overlaid with 10 mL of sterile TSB agar solution (1% agarose). Clear zones were measured after 18–24 h incubation at 37 °C.

Viability assay

Viability assays were essentially performed as described previously (Coconnier *et al.*, 1997). Briefly, colony count assays were performed by incubating $\pm 10^8$ CFU per mL with the different test solutions at 37 °C. Initially and at predetermined intervals, aliquots were removed, serially diluted and plated on LB agar to determine colony counts. The results of the viability assay experiments are represented as mean values of the three independent replicates.

Microtiter plate assay

Fractions of liquid chromatographic runs were subjected to a modified growth inhibition assay. Specific amounts (10–200 μL) of fractions of different sources were applied to the wells of a 96-well plate and lyophilized. The dry material was resuspended in 100 μL of a *Salmonella* inoculated LB culture (final optical density at 600 nm of 0.150, pH

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5.5). The 96-well plate was incubated at 37 °C (non-shaking) and every 2 h the optical density at 600 nm was measured (VERSAmax, Molecular Devices).

Purification of antimicrobial substances from LGG-SCS

Chromatographic purification steps were performed as previously reported (Huttunen *et al.*, 1995; Coconnier *et al.*, 1997), with minor modifications. Consequently, interpretation of all data refers only to those materials that were soluble in the elution buffers used. Both freeze dried powder and methanol-acetone extract (Coconnier *et al.*, 1997) of the LGG-SCS were dissolved in the elution buffer (50 mM NHAc, pH 4.8) resulting in 660 mg mL^{-1} final concentration of dry material. In the first purification step, 0.5 mL of the sample was loaded and fractionated on a gel filtration Biogel P2 column (1.5 × 30 cm; exclusion 100–1800 Da; Bio-Rad Laboratories). The active fractions of gel filtration were pooled and loaded on DEAE-Sephacell anion exchange column for further purification. Elution was carried out using a gradient elution program (Huttunen *et al.*, 1995). Active fractions originating from DEAE separation were pooled and subjected to Rainin C8 RP-HPLC column (250 × 4.6 mm) for further purification (Huttunen *et al.*, 1995). This resulted in the elution of one major peak with antimicrobial activity (elution time 5.6 min). A portion of the pooled active fractions (20 μL) was subjected to organic acid analysis using the Aminex HPX 87 H HPLC column (Bio-Rad Laboratories). To identify organic acids, standard solutions of lactic acid, acetic acid, formic acid, and pyroglutamic acid (PCA) (Sigma) were used.

Results and discussion

Production of antimicrobial compounds by *L. rhamnosus* GG parallels its growth curve

A relatively constant antimicrobial activity against *Salmonella typhimurium* SL1344 appeared after 7.5 h in culture of *Lactobacillus rhamnosus* GG (clear zone size in radial diffusion test corresponding to 0 mm at 0, 2.5 and 5 h, 6 mm at 7.5 h, 7.5 mm at 12 h, 8.5 mm at 24, 34, and 48 h). Concentrations of lactic acid, mainly the L-enantiomer, increased during time and were approximately 70 mM at 7.5 h, 136 mM at 12 h, and 215 mM at 24, 34, and 48 h. The pH determined during the time course ranged between 6.0 (0 h) and 3.7 (24–48 h). For subsequent experiments, the SCS prepared from 24 h old MRS *L. rhamnosus* GG cultures (LGG-SCS) was used.

The antimicrobial activity of LGG-SCS was examined as a function of contact time (Fig. 1). *Salmonella* viability decreased rapidly after 1 h of contact with LGG-SCS (±2 logs) and dramatically after 3 h (8 logs) (Fig. 1). In contrast, the controls PBS and MRS (pH 4.5) did not show bactericidal effects.

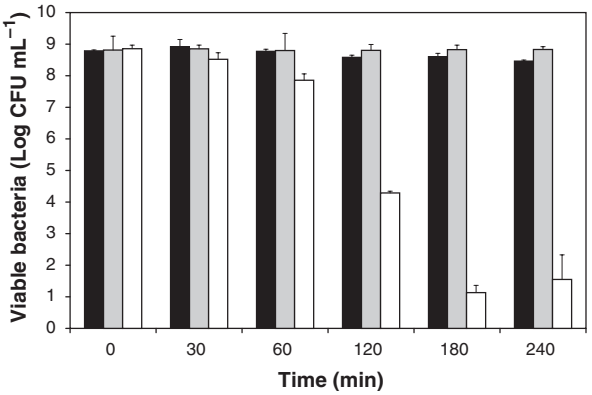


Fig. 1. Evaluation of the bactericidal activity of *Lactobacillus rhamnosus* GG spent culture supernatant (LGG-SCS) on *Salmonella typhimurium* SL1344 as a function of contact time. *Salmonella typhimurium* SL1344 was incubated in sterile De Man–Rogosa–Sharpe medium (MRS) (pH 4.5) (black bars), sterile phosphate-buffered saline (pH 4.5) (grey bars) and LGG-SCS prepared from MRS culture (pH 4.5) (white bars). At the start and at specific intervals, aliquots were removed, serially diluted and plated on LB agar to determine bacterial colony counts. Each value is the mean ± standard deviation (error bar) of three experiments.

Lactobacillus rhamnosus GG produces a pH dependent, heat-stable, nonproteinaceous, low molecular weight antimicrobial compound

When tested at a pH of 6.6, the antimicrobial activity of LGG-SCS was no longer present as is reflected by the faster generation of *Salmonella* compared with the one observed at pH 5 (Table 1). To rule out the possibility that the antimicrobial activity of LGG-SCS was only due to the low pH, the growth in sterile MRS at pH 5 was tested. In this case, a shorter generation time and lag phase of *Salmonella* growth was observed as compared with LGG-SCS at pH 5.0. This can most probably be related to the presence of undissociated organic acids in the LGG-SCS; the pH influences the ratio of dissociated to undissociated acid, as explained by the Henderson–Hasselbach equation. Although both forms can inhibit bacterial growth, the undissociated form of organic acids was reported to be more inhibitory, per mole, than its corresponding dissociated form (Eklund, 1983; Presser *et al.*, 1997).

The characteristics of the antimicrobial activity of LGG-SCS (pH 4.5) were examined, using *S. typhimurium* as indicator strain (Fig. 2). As a control, sterile MRS at the same pH 4.5 was used, showing ± 20% inhibitory activity. This inhibitory activity can be partially attributed to acetic acid as sterile MRS contains 60 mM sodium acetate as a basic ingredient. The antimicrobial activity of LGG-SCS (set at 100%) was compared with that of sterile MRS

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Table 1. Effect of MRS (control) and LGG-SCS in 1 : 12 dilution at different pHs on *Salmonella* growth in LB

Condition	Final pH	Lag (h)	Generation time (g) (h)
LB	6.6	4	0.58
LB + SCS	5	34	2.62
LB + SCS	6.6	4	0.79
LB + MRS	5	22	1.35
LB + MRS	6.6	4	0.79

LB, Luria–Bertani; MRS, De Man–Rogosa–Sharpe; LGG, *Lactobacillus rhamnosus* GG; SCS, spent culture supernatant.

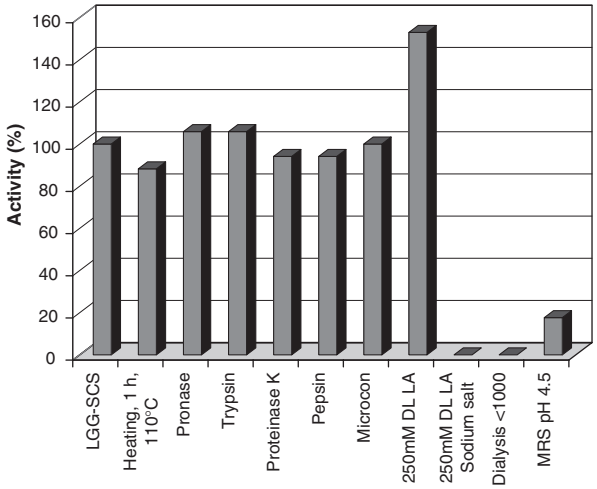


Fig. 2. Effects of physical or chemical treatments on the antimicrobial activity of *Lactobacillus rhamnosus* GG spent culture supernatant (LGG-SCS) against SL1344. LGG-SCS of 24 h old MRS cultures was prepared and treated with different proteases. The SCS was also heated, passed over a Microcon-SCX filter (cut-off 3000 Da) or dialyzed (dialysis < 1000, dialysis with a molecular cut-off of 1000 Da against a HEPES-citrate-Tris buffer at pH 4.5). For comparison, 250 mM lactic acid (LA), 250 mM sodium lactate (LA sodium salt) and sterile De Man–Rogosa–Sharpe medium (MRS) at pH 4.5 (HCl) were included. Antimicrobial activity was determined in a radial diffusion test. The activities are shown in comparison with that of untreated LGG-SCS (100%). Each value shown is the mean of three experiments. The variation in reproducibility was less than 5%.

supplemented with lactic acid. While DL-lactic acid at a concentration of 250 mM and at pH 4.5, had 1.5-fold more antimicrobial activity as compared with LGG-SCS, DL-lactic acid sodium salt (250 mM and at pH 4.5) showed no effect on *Salmonella* growth. The growth inhibitory effect of LGG-SCS could not significantly be alleviated by the addition of proteases, by passing over a Microcon-SCX filter, or by heat treatment. Therefore, the antimicrobial activity of *L. rhamnosus* GG against *S. typhimurium* is not due the production of a bacteriocin (Stevens *et al.*, 1991). This is in line with the broad spectrum of antimicrobial activity of LGG-SCS, including other lactobacilli, *Pediococcus pentosaceus* and even *L. rhamnosus* GG itself (data not shown). The latter would be very unusual in view of bacteriocin production, as generally, the producer strain is immune against its own bacteriocin (Baba & Schneewind, 1998). Consequently, as expected, ammonium sulphate precipitation and subsequent chloroform/methanol extractions could not indicate the presence of bacteriocins in LGG-SCS (data not shown). Note that, although rarely, some bacteriocins are inactivated after chloroform/methanol extraction.

On the other hand, the antimicrobial activity of LGG-SCS was lost upon dialysis (Fig. 2). Conclusively, and in line with previous results with *Escherichia coli* as indicator strain (Silva *et al.*, 1987), under the conditions tested, the antimicrobial activity of LGG-SCS is due to (a) heat-stable, nonproteinaceous low molecular weight compound(s).

Purification of antimicrobial substance(s) of *L. rhamnosus* GG

Throughout purification, the organic acid composition of the antimicrobial fractions was monitored, as preliminary characterization suggested that undissociated organic acids might mediate the antimicrobial activity of LGG-SCS. As a control, the organic acid profile of sterile MRS medium was analysed, revealing besides several non-identified minor peaks, peaks of two unidentified acids (5.7 and 7.6 min), of acetic acid (AA) (14.6 min), and of pyroglutamic acid (PCA) (17.6 min) (Fig. 3a). Compared with this control chromatogram (Fig. 3a), the one of LGG-SCS (Fig. 3b) contained two additional peaks, one corresponding to lactic acid (LA) (11.9 min) and one to formic acid (FA) (13.3 min) (with lactic acid being the major compound).

The methanol-acetone extract of LGG-SCS contained lactic, formic and acetic acid, PCA and traces of several unidentified acids (Fig. 3c). The same major acid compounds were found in the active fractions separated by the Biogel-2 column. The major peak with antimicrobial activity, eluted from a RP-HPLC column (data not shown), showed to contain after organic acid analysis an unidentified compound also present in sterile MRS (5.7 min), lactic acid (11.9 min), acetic acid (14.6 min), PCA (17.9 min) and a compound having the same retention time as formic acid (13.3 min) (Fig. 3d). Note that the enrichment during purification obviates estimating the actual concentrations of organic acids present in LGG-SCS.

PCA seemed a good potential candidate, as already previously suggested to be responsible for the antimicrobial activity in LGG-SCS (Yang *et al.*, 1997; Lehto & Salminen, 1997). However, this study showed that PCA is already present in sterile MRS medium, and that its amount does not significantly change during growth of *L. rhamnosus* GG (Fig. 3a and b). The fact that glutamic acid is converted to

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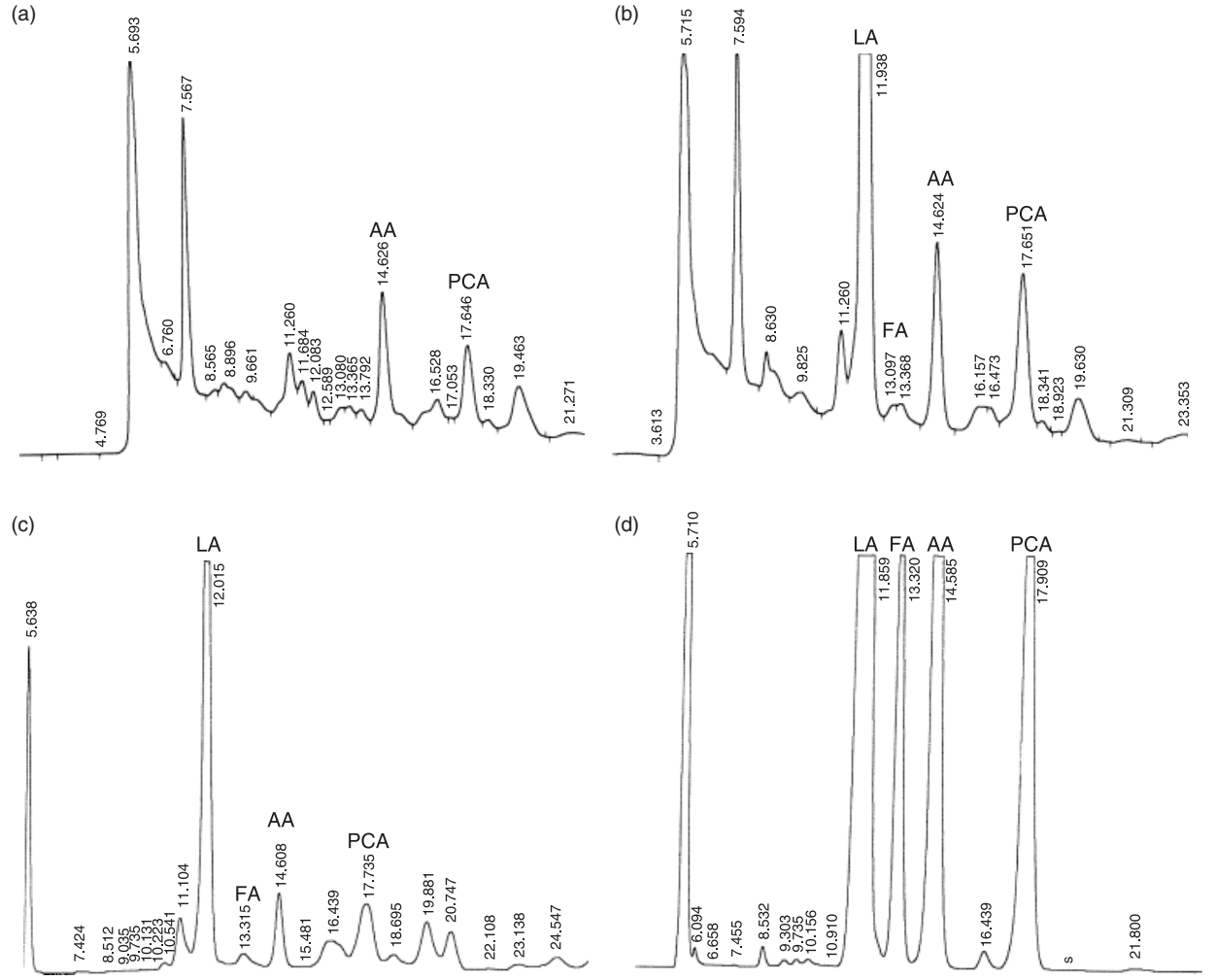


Fig. 3. Organic acid composition of the sterile De Man–Rogosa–Sharpe (MRS) medium (a), *Lactobacillus rhamnosus* GG spent culture supernatant (LGG-SCS) (b), methanol acetone extract of the freeze-dried LGG-SCS (c), and the fraction exhibiting antimicrobial effect from the last purification step (RP-HPLC, C8) (d). The *x*-axis refers to the retention time of the acids (in minutes): lactic acid (LA), formic acid (FA), acetic acid (AA) and pyroglutamic acid (PCA). Chromatograms were recorded at 210 nm using a Waters 486 tunable UV detector connected to a Hewlett Packard HP3396 series II integrator (attenuation 8, 0.250 A full scale). Note that ammonium acetate was used in the buffers of all the purification steps.

PCA when heated above 180 °C (Hartmann *et al*., 1981) can explain why it is also detected in sterile MRS. As in this study production of PCA by *Lactobacillus* could not be confirmed, it is not the compound conferring the antimicrobial activity in LGG-SCS.

Antimicrobial activity of organic acids identified in the enriched antimicrobial fraction of LGG-SCS

The only compounds present in the active fraction of purified LGG-SCS and absent in sterile MRS are lactic acid and formic acid. *Lactobacillus rhamnosus* GG is classified as a facultative heterofermentative *Lactobacillus* (Kandler & Weiss, 1986): glucose is mainly converted into lactic acid while acetic acid and formic acid are produced in much smaller amounts [i.e. at $\pm$20-fold lower concentration than lactic acid (12 mM), data not shown]. While MRS supplemented with such low concentrations of these acids (72 mM acetate final concentration and 12 mM formic acid, respectively), at pH 4.5, did not effect *S. typhimurium* viability (Fig. 4), 3 h of contact with MRS supplemented with lactic acid (Fig. 4) or with LGG-SCS (Fig. 1), drastically decreased *Salmonella* viability (8 logs).

Interestingly, when using PBS instead of MRS, the bactericidal effect of lactic acid on *Salmonella* disappeared (Fig. 4). This clearly demonstrates that the specific composition of the medium or solvent influences the results of comparative viability tests. This medium dependency can be attributed to the equilibrium of dissociated to

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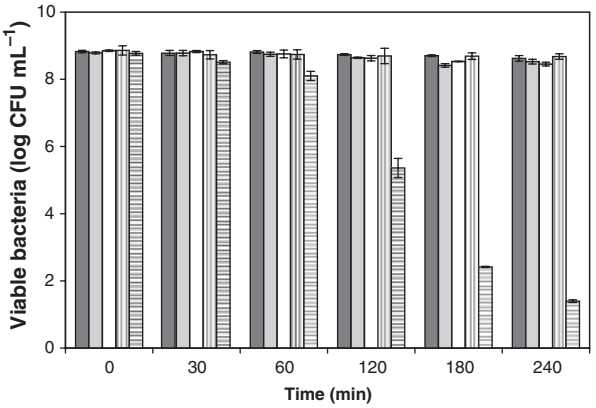


Fig. 4. Evaluation of the bactericidal activity of organic acids in different media on the viability of *Salmonella typhimurium* SL1344 as a function of time. *Salmonella typhimurium* SL1344 was incubated in sterile De Man–Rogosa–Sharpe medium (MRS) (black bars), MRS supplemented with acetic acid (0.72 g L^{-1}) (grey bars), MRS supplemented with formic acid (0.55 g L^{-1}) (white bars), PBS supplemented with lactic acid (20 g L^{-1}) (vertically striped bars) and MRS supplemented with lactic acid (20 g L^{-1}) (horizontally striped bars), all at final pH 4.5. Initially and at specific intervals, aliquots were removed, serially diluted and plated on LB agar to determine bacterial colony counts. Each value is the mean ± standard deviation (error bar) of three experiments.

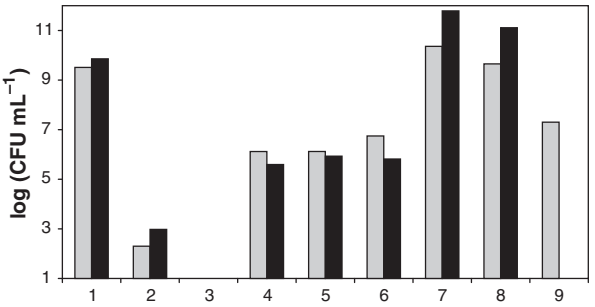


Fig. 5. Growth of *Salmonella* in coculture with *L. rhamnosus* GG. (1) Control condition containing only *Salmonella* bacteria in coculture medium with glucose, at initial pH 7. (2) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^5 CFU mL^{-1}; coculture medium with glucose, at initial pH 7. (3) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^9 CFU mL^{-1}; coculture medium with glucose, at initial pH 7. (4) Control condition containing only *Salmonella* bacteria; coculture medium without glucose, at initial pH 5. (5) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^5 CFU mL^{-1}; coculture medium without glucose, at initial pH 5. (6) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^9 CFU mL^{-1}; coculture medium without glucose, at initial pH 5. (7) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^5 CFU mL^{-1}; coculture medium without glucose, at initial pH 7. (8) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^9 CFU mL^{-1}; coculture medium without glucose, at initial pH 7. (9) *Salmonella* in coculture with *L. rhamnosus* GG added at initial concentration of 10^5 CFU mL^{-1}; coculture medium with sugar, at initial pH 7, in dialysis tube (cut-off 1000 Da, dialysis against coculture medium (with glucose added)). SL1344 was added at a starting concentration of 10^5 CFU mL^{-1}. The CFU per mL of SL1344 and *L. rhamnosus* GG were calculated by plating out serial dilutions on, respectively, LB supplemented with streptomycin and MRS-agar, at 24 h (grey bars) and 48 h (black bars). For the dialysis experiment, the bacterial counts at 48 h were not determined. Data are reported for *Salmonella* only as the log of the obtained CFU mL^{-1}. Values obtained for *L. rhamnosus* GG counts were 10 and 9 (log of CFU mL^{-1}) for growth in coculture medium with and without glucose added, respectively. The results shown are from a single experiment representative of three independent experiments.

nondissociated forms of organic acids, which is influenced by the pH and composition of the medium (Thomas *et al.*, 2002). Therefore, care should be taken when conclusions about the role of lactic acid as antimicrobial compound are drawn based on experiments that compare the antimicrobial activity of SCS with the inhibitory effect of solutions containing externally added lactic acid at concentrations higher than present in the SCS (Silva *et al.*, 1987; Bernet-Camard *et al.*, 1997; Coconnier *et al.*, 1997).

Lactic acid as antimicrobial substance produced by *L. rhamnosus* GG

The residual inhibitory effect of SCS present after removal of lactic acid was evaluated. After elimination of the lactic acid by dialysis of LGG-SCS against a buffer at pH 4.5, its antimicrobial activity disappeared completely (Fig. 2). However, when this dialysis was performed against a lactic acid solution (20 g L^{-1}) at pH 4.5, the antimicrobial activity remained unaltered, pointing towards lactic acid as major antimicrobial compound.

Additional evidence supporting this role of lactic acid came from a second experiment in which the viability of *Salmonella* was monitored in coculture with *L. rhamnosus* GG under different conditions (Fig. 5). As compared with the reference condition at pH 7 (no *L. rhamnosus* GG present, Fig. 5, condition 1), adding *L. rhamnosus* GG severely inhibited *Salmonella* growth (Fig. 5, conditions 2 and 3). Dialysis of the coculture decreased the inhibitory effect of *L. rhamnosus* GG (Fig. 5, condition 9). The residual antimicrobial activity was of the same magnitude as that observed in the condition without *L. rhamnosus* GG, at low pH (Fig. 5, condition 4). Comparing the conditions without *L. rhamnosus* GG at pH 7 (Fig. 5, condition 1) and pH 5 (Fig. 5, condition 4) indeed showed that low pH can only be partially responsible for the observed *Salmonella* growth inhibition in coculture.

Omission of sugar in the medium does not significantly affect growth of *L. rhamnosus* GG, while the production of lactic acid is drastically reduced. This medium was used in conditions 4–8 (Fig. 5). In the absence of sugar, and at pH 7 (Fig. 5, conditions 7 and 8), *Salmonella* viability was restored to a level observed in the reference [pH 7, no *L. rhamnosus* GG present (Fig. 5, condition 1)]. Similarly, no

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significant difference in viability could be observed between the reference condition at pH 5 (Fig. 5, condition 4), and the cocultures at the same pH 5.0 lacking a sugar source (Fig. 5, conditions 5 and 6). Although not very plausible, antimicrobial compounds, if present in these conditions, are clearly independent of the presence of a fermentable sugar and their effect exerted on *Salmonella* just equals that of low pH. The increased antimicrobial effect observed in conditions 2 and 3 is thus clearly dependent on the presence of fermentable sugars and thus must be related to a fermentation product of *L. rhamnosus* GG, able to be dialysed, identified as lactic acid.

So far, in literature the role of lactic acid in the antimicrobial activity of *L. rhamnosus* GG is controversial with hypotheses being formulated that range from no role at all (Silva *et al*., 1987; Fayol-Messaoudi *et al*., 2005) to lactic acid being the major player (Makras *et al*., 2005). In this study, however, clear indications for the latter hypothesis were found. The exact mode of action underlying this observed antimicrobial effect of lactic acid is not yet completely clarified (Ricke, 2003). Besides exerting its activity through lowering the pH and through its undissociated form (Rubin *et al*., 1982; Presser *et al*., 1997), lactic acid is also known to function as a permeabilizer of the Gram-negative bacterial outer membrane (Alakomi *et al*., 2000) allowing other compounds to act synergistically with lactic acid (Niku-Paavola *et al*., 1999). By their chelating properties, organic acids such as lactic acid can capture elements essential for growth, such as iron, being another potential mechanism for inhibition (Presser *et al*., 1997). Lactic acid also specifically influences the expression of the *Salmonella* key virulence gene *hilA* (Durant *et al*., 2000).

Conclusions

Conclusively, HPLC analysis identified five components in the antimicrobial fraction of LGG-SCS: an unidentified compound with retention time of 5.7, acetic acid, PCA, formic acid and lactic acid. As the first three compounds were also present in sterile MRS medium and formic acid, at its actual concentration in LGG-SCS was not bactericidal against *Salmonella*, lactic acid was identified as the main antimicrobial compound produced by *L. rhamnosus* GG. Despite the fact that differences in *L. rhamnosus* GG physiology between *in vivo* and *in vitro* conditions could complicate generalization of these results, lactic acid remains an intriguing compound for further studies.

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