Compound(s) secreted by Lactobacillus casei strain Shirota YIT9029 irreversibly and reversibly impair the swimming motility of Helicobacter pylori and Salmonella enterica serovar Typhimurium, respectively

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We conducted experiments in order to examine whether the probiotic Lactobacillus casei strain Shirota YIT9029 (LcS) in vitro and in vivo antagonism of Helicobacter pylori and Salmonella, involves inhibition of the swimming motility of these pathogens. We report the irreversible inhibition of the swimming motility of H. pylori strain 1101 and reversible inhibition of Salmonella enterica serovar Typhimurium (S. Typhimurium) strain SL1344 by compound(s) secreted by LcS. In H. pylori 1101, irreversible inhibition results in the helical cells being progressively replaced by cells with 'c'-shaped and coccoid morphologies, accompanied by a loss of FlaA and FlaB flagellin expression. In S. Typhimurium SL1344, transient inhibition develops after membrane depolarization and without modification of expression of FlIC flagellin. The inhibitory activity of strain LcS against both S. Typhimurium and H. pylori swimming motilities is linked with a small sized, heat-sensitive, and partially trypsin-sensitive, secreted compound(s), and needed the cooperation of the secreted membrane permeabilizing lactic acid metabolite. The inhibition of S. Typhimurium SL1344 swimming motility leads to delayed cell entry into human enterocyte-like Caco-2/TC7 cells and a strong decrease of cell entry into human mucus-secreting HT29-MTX cells.

INTRODUCTION

Many bacterial species move by rotating their flagella (Josenhans & Suerbaum, 2002). In an aqueous environment, flagellar swimming allows individual bacteria to swim in three dimensions. Additionally, flagellar swimming in a subset of bacteria coordinates the movement of bacteria across the surface of solid media and host cells (Patrick & Kearns, 2012).

Salmonella enterica serovar Typhimurium (S. Typhimurium), the aetiological agent of gastroenteritis in human, has the ability to interact with, invade and then live intracellularly in host cells (Grassl & Finlay, 2008). Bacterial motility is provided by five to eight flagella that emerge randomly from the surface of the organism. The molecular mechanism by which the Salmonella flagellum is assembled involves the regulation of flagellar gene transcription, and translational and post-translational regulation events. The biogenesis of flagella in S. Typhimurium develops by the coordinated structural assembly of flagellar proteins (Aldridge & Hughes, 2002; Chevance & Hughes, 2008). Two flagellin structural proteins, FlIC and FlJB, and the capping FlID protein comprise the S. Typhimurium flagellar propeller. Moreover, MotA and MotB are cytoplasmic membrane proteins that form the force-generating unit of the flagellar motor in S. Typhimurium (Minamino et al., 2008). S. Typhimurium flagella have been shown to be necessary for the colonization of the intestine (Stecher et al., 2004), the crossing of the intestinal mucus layer followed by attachment to epithelial cells (McCormick et al., 1988), and the promotion of early innate host responses (Winter et al., 2009).

The human pathogen Helicobacter pylori is a member of the Epsilonproteobacteria, a class of bacteria composed almost exclusively of helical and curved organisms (Cover & Blaser, 2009; Salama et al., 2013). H. pylori colonizes the gastric tissue, and although most infected individuals are asymptomatic, this pathogen can induce serious disorders,
including mild gastritis, and the development of chronic gastric inflammation that can lead to ulcers and gastric cancer. The complex hierarchical regulation of flagellar genes in *H. pylori* indicates that flagellar components are assembled in a highly ordered fashion (Lertsathakarn et al., 2011; Salama et al., 2013). A tuft of three to seven sheathed flagella is located at one pole of the bacterium. The *H. pylori* complex filament is composed of two flagellin subpecies, FlmA and FlaB proteins. Moreover, *H. pylori* has acquired particular flagellar properties as a result of adaptation to the harsh gastric environment, including low pH and high proteolytic activity. Moreover, for stomach colonization, in addition to the flagella, the helical cell shape plays a role in the viscosity-dependent enhancement of *H. pylori* swimming velocity within the viscous epithelial mucus layer in which it resides (Cover & Blaser, 2009; Salama et al., 2013).

Probiotic strains are defined as live micro-organisms which, when consumed in appropriate amounts in food, confer a health benefit on the host (FAO/WHO, 2001). Recently, reports have shown inhibition of the swimming motility of *S. Typhimurium* SL1344 and *H. pylori* by probiotic *Lactobacillus* strains (Isobe et al., 2012; Liévin-Le Moal et al., 2011). The probiotic *Lactobacillus casei* strain Shirotta YTT9029 (LcS) has been shown to antagonize both *in vitro* and in vivo gastrointestinal pathogens including *Salmonella* sp. (Asahara et al., 2011; Fayol-Messaoudi et al., 2005, 2007; Lee et al., 2003; Makras et al., 2006; Malago et al., 2010) and *H. pylori* (Avonts & De Vuyst, 2001; Cats et al., 2003; Sgouras et al., 2004). Clinical trials have demonstrated the therapeutic efficacy of LcS against *H. pylori* infection (Cats et al., 2003; Sahagún-Flores et al., 2007). We have conducted experiments in order to examine whether the LcS inhibits the swimming motility of *H. pylori* and *S. Typhimurium*. Results identified the impairment of swimming motility as a mechanism by which probiotic strain LcS affects the virulence mechanism(s) of motile gastrointestinal pathogens.

**METHODS**

**Bacterial strains.** *S. Typhimurium* strain SL1344 (Cocconnier et al., 1997) was a kind gift from B. A. D. Stocker (Stanford University, Stanford, CA, USA). *S. Typhimurium* SL1344 mutant strains with mutations at different points in the morphogenetic pathway of the flagellum were used (Table 1). *S. Typhimurium* SL1344 and mutant strains were grown overnight in Luria–Bertani (LB) broth (Difco Laboratories) at 37 °C as a static culture. *H. pylori* strain 1101 (Hp1101) isolated from a patient suffering from functional dyspepsia and erosive gastritis (Corthésy-Theulaz et al., 1996) was positive for cagA and develops a vacuolating activity. *H. pylori* 1101 was grown on brain-heart infusion (BHI) agar plates containing 0.25 % yeast extract (Difco Laboratories), 10 % horse serum and 0.4 % *Campylobacter* selective complement (Skoow supplement, SR 69; Oxoid) upside down in a gas jar with microaerophilic atmosphere (gas-generating kit CampyGen; Oxoid) at 37 °C. *H. pylori* colonies on BHI agar plates were scraped into BHI broth.

LcS was from Yakult Honsha. The dairy *L. casei* strains from culture collections were: *L. casei* CNRZ 383, ATCC 334, ATCC 3937 T (=type strain) and ATCC 39392; *L. casei* subsp. *casei* NCIMB 4114 and NCIMB 8822; and *L. casei* subsp. *rhamnosus* CNRZ 205 and ATCC 7469 T. The strains were grown in deMan–Rogosa–Sharpe (MRS) broth (Difco Laboratories) for 24 h at 37 °C. Isolated LcS bacteria and LcS cell-free culture supernatant (CFCS) were obtained by centrifuging an 18 h culture of *Lactobacillus acidophilus* LB at 10 000 g, for 30 min at 4 °C. Before use, the LcS CFCS was passed through a sterile 0.22 μm Millex GS filter unit (Millipore), and the absence of bacterial colonies in the filtered CFCS was confirmed by plating on tryptic soy agar (Cocconnier et al., 2000).

**Treatments of LcS CFCS.** To test the sensitivity of LcS CFCS to proteolytic enzymes, neutralized CFCS was incubated at 37 °C for 1 h with trypsin (200 μg ml⁻¹). BSA was used to check the activity of the trypsin. The heat sensitivity of LcS CFCS was assessed by heating for 1 h at 70 or 100 °C. To test the role of pH, the LcS CFCS was adjusted to neutral pH with NaOH. To determine whether the lactic acid in the LcS CFCS participates in the inhibitory activity, LcS CFCS was subjected to lactic dehydrogenase (LDH) treatment (250 μg ml⁻¹, 2 h at 37 °C). Lactic acid concentration was controlled with a commercial D- and L-lactic acid determination kit (Boehringer Mannheim; combination D-lactic acid/L-lactic acid UV method).

**Bacteria viability.** The method used to determine the viability of *S. Typhimurium* and *H. pylori* subjected to LcS CFCS has been previously described (Fayol-Messaoudi et al., 2005). Colony count assays were performed by incubating approximately 500 μl (10⁶ c.f.u. ml⁻¹) *S. Typhimurium* SL1344 in LB broth or *H. pylori* 1101 in BHI broth with 500 μl Dulbecco’s modified Eagle’s minimal essential medium (DMEM) at 37 °C. Initially and at 60 min of contact, aliquots were removed, serially diluted and plated on LB or BHI agar to determine bacterial colony counts.

**Table 1. List of *S. Typhimurium* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Protein and function</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>χ2421</td>
<td>SL1344 mot-8008 : Tn10</td>
<td>FljB, protein of the filament propeller</td>
<td>Lockman &amp; Curtiss (1990)</td>
</tr>
<tr>
<td>χ8601</td>
<td>SL1344 flgB217</td>
<td>FljB and FljC, proteins of the filament propeller</td>
<td>R. Curtiss III</td>
</tr>
<tr>
<td>χ8602</td>
<td>SL1344 ΔfljC825</td>
<td>CheY, chemotaxis response regulator, transmits chemoreceptor signal to flagellar motor proteins</td>
<td>R. Curtiss III</td>
</tr>
<tr>
<td>M935</td>
<td>SL1344 cheY : Tn10</td>
<td>T3SS protein</td>
<td>Stecher et al. (2004)</td>
</tr>
<tr>
<td>SW399</td>
<td>IR715 invA</td>
<td>FljK, first hook-filament junction; FljB, protein of the filament propeller; T3SS protein</td>
<td>Winter et al. (2009)</td>
</tr>
<tr>
<td>SW358</td>
<td>IR715 flgK fljB invA</td>
<td></td>
<td>Winter et al. (2009)</td>
</tr>
</tbody>
</table>
ATP determination. The intracellular ATP concentration of S. Typhimurium and H. pylori was determined using a quantitative reaction based on bioluminescence (ATP-Lite assay kit; Perkin-Elmer Life Sciences) as previously described (Cocconnier-Polter et al., 2005). The luminescence was measured in a Genios luminometer (Tecan). The ATP content (μM) was calculated using a standard curve plotted from a standard ATP solution.

Membrane depolarization. The cytoplasmic membrane depolarization was determined with the membrane potential-sensitive dye 3,3’-dipropylthiacarbocyanine [DiSC3(5)] (Molecular Probes) as previously described (Liévin-Le Moal et al., 2011). Changes in fluorescence due to the disruption of the membrane potential gradient across the cytoplasmic membrane were recorded using a Genios spectrofluorimeter (Tecan) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Membrane permeabilization. We used the adapted technique described by Cocconnier-Polter et al. (2005). Passage of the fluorescent DNA-binding probe Hoechst 33258 across the bacterial membrane was measured using a black microtitre fluoroplate followed by ECL chemiluminescence detection performed with the appropriate horseradish peroxidase-linked secondary antibody (Cruz Biotechnologies) was used. The blots were then incubated with antibody was used (Leying et al., 2010). For detection of H. pylori 1101 were examined by phase contrast microscopy with a CLSM (model LSM 510 META: Zeiss) equipped with a 488 nm air-cooled argon ion laser, and a 543 nm helium neon laser, and configured with an Axiovert 100M microscope using a Plan Apochromat 40 ×1.2 NA Corr water objective lens. For quantification, the CLSM images were analyzed using Imaris software (version 6.21) (Bitplane). For each sample, ~100–150 cells were examined.

Isolation of flagella. Untreated and Lc CFCS-treated S. Typhimurium SL1344 or H. pylori 1101 were pelleted by centrifuging at 5000 g for 5 min, resuspended in 10 ml 500 mM Tris (pH 8.0), and then blended at high speed for 60 s in an Omni Mixer (Sorvall) (Liévin-Le Moal et al., 2011). Bacteria and debris were pelleted by centrifuging at 8000 g for 15 min. Flagella were pelleted by centrifuging at 100,000 g for 1 h, and then resuspended in loading buffer and heated to 95°C for 5 min.

Western blotting. Proteins were resolved by 10% SDS-PAGE. The gels were transferred onto a PVDF membrane (Amersham Pharmacia Biotech), washed with PBS containing 0.1% Tween, blocked in TBS (0.1% Tween, 3% BSA, 0.5% gelatin) and then incubated with appropriate primary antibodies. For detection of S. Typhimurium SL1344 flagellin FliC, mouse mAb 114/2 and mAb 109/5/2 were used (Sojka et al., 2001). For detection of S. Typhimurium SL1344 outer membrane proteins (Omps) OmpF and OmpP porins from total cell proteins, polyclonal F4 antibody directed against Escherichia coli porins and recognizing Salmonella porins was used (O’Regan et al., 2010). For detection of H. pylori 1101 flagellins, polyclonal AK179 antibody was used (Leying et al., 1992). For detection of H. pylori 1101 OmpF from total cell proteins, an anti-H. pylori Omp Mab (Santa Cruz Biotechnologies) was used. The blots were then incubated with the appropriate horseradish peroxidase-linked secondary antibody (Ozyme), followed by ECL chemiluminescence detection performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

Swimming motility assay and quantification. A previously described method was used (Liévin-Le Moal et al., 2011). Swimming motility of wild-type S. Typhimurium SL1344 or H. pylori 1101 (500 μl culture of a pathogen+ 1000 μl DMEM for S. Typhimurium SL1344 or BHI broth for H. pylori 1101), or S. Typhimurium SL1344 and H. pylori 1101 exposed for 1 h at 37°C to probiotic Lactobacillus cultures or LcS bacteria or LeC CFCS (500 μl externally growing bacteria of a pathogen+ 500 μl test material+ 500 μl DMEM or BHI broth) was examined. Determination of the number of bacteria showing swimming motility was conducted by phase contrast light microscopy (using a Leitz Aristoplan microscope).

A time-lapse examination of pathogen swimming motility track types was conducted by a previously described method (Liévin-Le Moal et al., 2011). Cell movements and trajectories were observed by time-lapse imaging with an inverted microscope, an AxiObserver Z1_Colibri (Zeiss) equipped with an Axiocam MRm CCD camera (6.45 μm pixel size) and a Achroplan 10 ×0.25 NO dry objective lens, at 23 °C (maintained by air conditioning). Phase contrast images were recorded at video rates (14 frames s⁻¹). Time-lapsed images were recorded for a total time of 3 h under all the experimental conditions, with a 10 s exposure time at 10 min intervals with the halogen light source shuttered between image acquisitions. Images were exported to ImageJ (http://rsb.info.nih.gov/ij/) for quantifying the number of bacteria showing the different cell-track types (straight forward run, curvilinear run, tumble without run, non motile).

Determination of H. pylori morphologies. Untreated and Lc CFCS-treated H. pylori 1101 were examined by phase contrast microscopy with a CLSM (model LSM 510 META: Zeiss) equipped with a 488 nm air-cooled argon ion laser, and a 543 nm helium neon laser, and configured with an Axiovert 100M microscope using a Plan Apochromat 40 ×1.2 NA Corr water objective lens. For quantification, the CLSM images were analyzed using Imaris software (version 6.21) (Bitplane). For each sample, ~100–150 cells were examined.

Cell lines and culture. The enterocyte-like TC7 clone (Caco-2/TC7) (Chantret et al., 1994) was used. Cells were routinely grown in DMEM (25 mM glucose) (Life Technologies), supplemented with 15% heat-inactivated (30 min, 56°C) FBS (Life Technologies) and 1% non-essential amino acids. For maintenance purposes, cells were passaged weekly using 0.02% trypsin in Ca²⁺-Mg²⁺-free PBS containing 3 mM EDTA. Fully differentiated cells were used at post-confluence after 15 days in culture for Caco-2/TC7 cells (Liévin-Le Moal et al., 2011).

We used the mucin-secreting HT29-MTX cell subpopulation (Lesuffleur et al., 1990). Cells were routinely grown in DMEM (25 mM glucose) (Life Technologies), supplemented with 10% heat-inactivated FBS (30 min, 56°C). As a control, enterocyte-like HT-29 cells were used. The HT-29 gclc⁻/⁻ cell subpopulation showed growth adaptation when cultured in the presence of glucose (Lesuffleur et al., 1990). HT-29 gclc⁻/⁻ cells express the structural and functional characteristics of enterocyte-like cells as a function of the days in culture. Cells were routinely grown in DMEM supplemented with 20% heat-inactivated FBS. Fully differentiated HT-29 gclc⁻/⁻ and HT29-MTX cells were used at post-confluence after 21 days in culture (Liévin-Le Moal et al., 2005).

Imaging and quantification of S. Typhimurium SL1344-induced F-actin accumulation. Examination of F-actin in Caco-2/TC7 cells was carried out by direct immunofluorescence labelling using Alexa-488-labelled phalloidin (Molecular Probes) (Liévin-Le Moal et al., 2011). Samples were examined under a CLSM. To quantify the S. Typhimurium-induced F-actin accumulations, F-actin spots were counted using Image J software (version 1.42) (National Institutes of Health, USA). For each sample, images of at least 10 randomly-selected fields, each representing ~100 cells, were recorded. Photographic images were resized, organized, and labelled using Adobe Photoshop software.

Quantification of internalized S. Typhimurium SL1344. The cells were infected with an exponentially growing culture of wild-type S. Typhimurium SL1344 or mutants in DMEM (500 μl, 5 x 10⁵ c.f.u. per well) for the time indicated, at 37°C in an atmosphere of 10% CO₂,90% air (Fayol-Messaoudi et al., 2005). The internalization of S. Typhimurium SL1344 was measured by determining the number of bacteria located within the infected monolayers using the aminoglycoside antibiotic assay. Infected monolayers were washed twice with sterile PBS, and then incubated for 1 h in the presence of DMEM containing 100 μg gentamicin ml⁻¹. Bacteria that adhered to the...
Table 2. Effect of LcS culture, LcS bacteria, LcS CFCS, MRS broth and lactic acid on S. Typhimurium SL1344 and H. pylori 1101 swimming motilities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S. Typhimurium SL1344</th>
<th>H. pylori 1101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LcS culture treated</td>
<td>8 ± 2*</td>
<td>7 ± 3*</td>
</tr>
<tr>
<td>MRS broth (pH 4.5) treated</td>
<td>96 ± 5</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>LcS bacteria treated</td>
<td>98 ± 3</td>
<td>97 ± 6</td>
</tr>
<tr>
<td>LcS CFCS treated</td>
<td>4 ± 2*</td>
<td>5 ± 2*</td>
</tr>
<tr>
<td>Lactic acid treated (15 mM)**</td>
<td>95 ± 4</td>
<td>94 ± 6</td>
</tr>
</tbody>
</table>

*P<0.01 versus untreated bacteria.
†Lactic acid concentration present in LcS CFCS (Sgouras et al., 2004).

Table 3. Effect of CFCSs of culture collection L. casei strains on the swimming motility of S. Typhimurium SL1344

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swimming motility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>LcS</td>
<td>9 ± 4†</td>
</tr>
<tr>
<td>L. casei CNRZ 383</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>L. casei ATCC 334</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>L. casei ATCC 393</td>
<td>62 ± 7†</td>
</tr>
<tr>
<td>L. casei ATCC 39392</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>L. casei subsp. casei NCIMB 4114</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>L. casei subsp. casei NCIMB 8822</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>L. casei subsp. rhamnosus CNRZ 205</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>L. casei subsp. rhamnosus ATCC 7469</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>

*Percentage of motile bacteria measured after 1 h of treatment. Each value shown is the mean percentage ± SD of three experiments. †P<0.01 versus control.

None of the CFCSs of the L. casei strains CNRZ 383, ATCC 334 and ATCC 39392, L. casei subsp. casei strains NCIMB 4114 and NCIMB 8822, and L. casei subsp. rhamnosus strains CNRZ 205 and ATCC 7469 tested exerted an inhibiting activity against S. Typhimurium SL1344 swimming motility after 1 h of contact. Only the strain ATCC 39392 showed a low inhibitory activity against the S. Typhimurium SL1344 swimming motility.

As shown in Fig. 1a, the inhibition of the swimming motilities of both H. pylori 1101 and S. Typhimurium SL1344 develops as a function of the time of contact with LcS CFCS. We went on to use video-microscopy to examine the swimming cell-track types of untreated and LcS CFCS-treated pathogens (Fig. 1b, c). Consistent with a published report (Karim et al., 1998), the observed swimming track types for untreated H. pylori 1101 are curvilinear runs (~40 %) and tumbles without a run (~56 %), and a small number of untreated H. pylori strains are non-motile (~4 %) (Fig. 1b, i). After LcS CFCS treatment (Fig. 1b, ii), the number of bacteria developing curvilinear track runs or tumbles without a run rapidly decreased time dependently. However, it should be noted that bacteria with tumbles without a run seemed to persist for longer than bacteria with curvilinear runs. Consistent with the findings of Turner et al. (2000), ~98 % of untreated S. Typhimurium SL1344 moved by engaging straight forward runs (~97 %), and a small number of bacteria displayed tumbles without a run (~3 %) (Fig. 1c, i). After LcS CFCS treatment (Fig. 1c, ii), there was a transient increase in the number of bacteria developing tumbles without a run and the bacteria showed a rapid decrease of straight forward runs.

LcS CFCS irreversibly and reversibly impaired the H. pylori 1101 and S. Typhimurium SL1344 swimming motilities, respectively

We next investigated whether the above observed LcS CFCS-induced inhibitory effect against the swimming...
motility of \textit{S. Typhimurium} SL1344 and \textit{H. pylori} 1101 was reversible or not. To do this, the untreated and LcS CFCS-
treated pathogens were centrifuged; the bacteria were then
washed with PBS and replaced in fresh culture medium. The results in Fig. 2a show that the swimming motility of \textit{H. pylori}
1101 is irreversibly inhibited after LcS CFCS treatment, since when the LcS CFCS-treated bacteria were
replaced in a fresh culture medium there was no
reappearance of swimming motility; in contrast, the
reappearance of swimming motility was observed for LcS
CFCS-treated \textit{S. Typhimurium} SL1344. Examination by
CLMS phase contrast microscopy showed that 98% of
untreated \textit{H. pylori} 1101 stably displayed the typical helical
cell morphology (Fig. 2b). In contrast, LcS CFCS-treated
\textit{H. pylori} 1101 showed time-dependent changes in cell
morphology (Fig. 2b). There was a disappearance of cells
with the helical form and appearance of cells with curved-
rod or ‘c’-shaped morphologies. Moreover, results showed
the appearance of a coccoid form at late times of treatment.
Two hours after being replaced in fresh culture medium,
there was a time-dependent decrease of the number of ‘c’-
shaped cells and an increase of cells with the coccoid form
(Fig. 2c). The presence or absence of flagella in non-motile
LcS CFCS-treated \textit{H. pylori} 1101 was examined by Western
blot. Data in Fig. 2d show the decreased presence of \textit{H.
pylori} FlaA and FlaB proteins when the ‘c’-shaped forms
are predominant after a 1 h treatment with LcS CFCS
compared with untreated bacteria. Consistent with the
predominant presence of the coccoid form when LcS
CFCS-treated \textit{H. pylori} 1101 were replaced in fresh culture
medium, there was a progressive disappearance of FlaA and
FlaB proteins compared with untreated bacteria (Fig. 2d).
These results prompted us to examine viability, ATP content and cell morphology in untreated and LcS CFCS-treated \textit{H. pylori} 1101. The viability of \textit{H. pylori} 1101 was decreased by ~1 log after 1 h of contact with LcS CFCS, but without a modification of the ATP content in the bacteria (Table 4). Two hours after being replaced in fresh culture medium, the viability of LcS CFCS-treated \textit{H. pylori} 1101 was decreased by 7 logs and the ATP content level was dramatically decreased (Table 4).

Data in Fig. 3a show that the LcS CFCS-induced inhibition of \textit{S. Typhimurium} SL1344 swimming motility is transient, since a time-dependent recovery of swimming motility was observed. The results of video-microscopy examination showed that the non-motile LcS CFCS-treated \textit{S. Typhimurium} SL1344 successively recovered two cell-track types of swimming motility (Fig. 3b). The first type of swimming motility to be recovered was transient tumbling without a run, a cell-track type not observed in untreated bacteria, and 3 h after being returned to fresh DMEM, all the LcS CFCS-treated bacteria had recovered a normal straight forward run type of swimming motility. A colony count assay and the determination of ATP content show that both the viability and ATP levels were not modified in \textit{S. Typhimurium} SL1344 after 1 h of contact with LcS CFCS compared with untreated bacteria (Table 4). We checked for the presence of flagella in motile untreated and non-motile LcS CFCS-treated \textit{S. Typhimurium} SL1344 by Western blot. Data in Fig. 3c show the presence of flagellar FlIC protein in non-motile LcS CFCS-treated \textit{S. Typhimurium} SL1344 compared with motile untreated bacteria. Consistent with the reversible inhibition of swimming motility, the flagellar FlIC protein was present in LcS CFCS-treated \textit{S. Typhimurium} SL1344 recovering swimming motility when replaced in fresh culture medium (Fig. 3c). The membrane potential-sensitive dye DiSC3(5), which quenches its own fluorescence when it is inside the untreated \textit{S. Typhimurium} SL1344, was released into the medium when the pathogen was treated with the LcS CFCS, indicating a membrane depolarization activity (untreated \textit{S. Typhimurium} SL1344, 0.5 ± 0.02 relative fluorescence intensity; LcS CFCS-treated \textit{S. Typhimurium} SL1344, 7.9 ± 0.3 relative fluorescence intensity). The paralyzed flagella of non-motile LcS CFCS-treated \textit{S. Typhimurium} SL1344 retain the ability to induce the production of IL-8 in basolaterally infected Caco-2/TC7 cells (data not shown).
**Table 4. Viability and ATP content in untreated and LcS CFCS-treated S. Typhimurium SL1344 and H. pylori 1101**

Each value shown is the mean ± SD of three experiments.

<table>
<thead>
<tr>
<th></th>
<th>S. Typhimurium SL1344</th>
<th>H. pylori 1101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability of control untreated bacteria*</td>
<td>7.7 ± 0.4</td>
<td>7.5 ± 0.2</td>
</tr>
<tr>
<td>Viability of LcS CFCS-treated bacteria (1 h)*</td>
<td>7.7 ± 0.6</td>
<td>6.6 ± 0.4†</td>
</tr>
<tr>
<td>Viability of control untreated bacteria replaced for 2 h in fresh culture medium*</td>
<td>8.7 ± 0.5</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>Viability of LcS CFCS-treated bacteria (1 h) replaced for 2 h in fresh culture medium*</td>
<td>8.4 ± 0.7</td>
<td>0.5 ± 0.2†</td>
</tr>
<tr>
<td>ATP content of control untreated bacteria‡</td>
<td>335 ± 23</td>
<td>218 ± 12</td>
</tr>
<tr>
<td>ATP content of LcS CFCS-treated bacteria (1 h)‡</td>
<td>298 ± 19</td>
<td>188 ± 15</td>
</tr>
<tr>
<td>ATP content of control untreated bacteria replaced for 2 h in fresh culture medium‡</td>
<td>312 ± 25</td>
<td>255 ± 19</td>
</tr>
<tr>
<td>ATP content of LcS CFCS-treated bacteria (1 h) replaced for 2 h in fresh culture medium‡</td>
<td>301 ± 14</td>
<td>28 ± 0.9†</td>
</tr>
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</table>

*cfu. ml⁻¹.
†P<0.01 versus untreated bacteria.
‡nM.

**Characteristics of LcS secreted compound(s) triggering the inhibition of bacteria swimming motility**

Results in Fig. 4a show that the inhibitory effect of LcS CFCS against S. Typhimurium SL1344 swimming motility develops as a function of the concentration. Results in Fig. 4b show that when neutralized, the LcS CFCS loses its inhibitory activity against both S. Typhimurium SL1344 and H. pylori 1101 swimming motilities. After 1 h at 100°C, the inhibitory activity of LcS CFCS was entirely abolished. Trypsin treatment decreased but did not entirely abolish the inhibitory activity of LcS CFCS. After dialysis (cut-off 1000 Da), the inhibitory activity of LcS CFCS entirely disappeared. LcS CFCS was subjected to LDH treatment in order to determine whether or not the produced lactic acid participates in the inhibitory activity. Despite lactic acid being devoid of activity against S. Typhimurium SL1344 and H. pylori 1101 swimming motilities (Table 2), we found that the LDH treatment of LcS CFCS produces a strong decrease of its inhibitory activity against S. Typhimurium SL1344 and H. pylori 1101 swimming motilities (Table 5). The LDH-treated LcS CFCS recovered its inhibitory activity against S. Typhimurium SL1344 swimming motility when lactic acid (50 mM) was added. Consistent with previous observations (Alakomi et al., 2000; Liévin-Le Moal et al., 2011), lactic acid exerted a membrane permeabilization activity (untreated S. Typhimurium SL1344, 3.5 ± 1.2 relative fluorescence intensity; S. Typhimurium SL1344 + lactic acid, 15.9 ± 4 relative fluorescence intensity of DNA-binding probe Hoechst 33258).

**LcS CFCS-treated S. Typhimurium SL1344 showed a delayed ability to induce membrane remodelling and to penetrate into cultured human enterocyte-like Caco-2/TC7 cells**

We checked whether the inhibition of swimming motility by LcS CFCS had a consequence for host cell infection. To do this, we examined whether or not the LcS CFCS-induced inhibition of S. Typhimurium SL1344 swimming motility affects the capability of the enteroinvasive pathogen to enter within cultured human host enterocyte-like Caco-2/TC7 cells. In untreated wild-type S. Typhimurium SL1344-infected cells, dense and localized F-actin spots were observed at the cell surface (Fig. 5a, b). In contrast, there were dramatically fewer F-actin spots in cells infected with LcS CFCS-treated S. Typhimurium SL1344 at both 5 min and 1 h post-infection (p.i.) (Fig. 5b); but at 3 h p.i., the numbers of F-actin spots in LcS CFCS-treated S. Typhimurium SL1344-infected cells had increased (Fig. 5b). The control, the S. Typhimurium SL1344 treated with MRS broth at pH 4.5, showed a normal formation of F-actin spots (Fig. 5b). We observed that the number of internalized bacteria was dramatically lower at both 5 min and 1 h p.i. for S. Typhimurium SL1344 treated with LcS CFCS than for untreated bacteria (~4.0 logs lower), and by 3 h p.i. all the LcS CFCS-treated S. Typhimurium SL1344 had been internalized (Fig. 5c). Examined as controls, S. Typhimurium SL1344 flagella mutants γ8601 (fljB) and γ8602 (fljB, flIC), and a non-motile flagella motor mutant γ3421 (motA, motB), showed a delayed cell entry compared with wild-type (Fig. 5d). Consistent with the above results showing that both trypsin and heat treatments dramatically decreased the inhibitory activity of LcS CFCS against S. Typhimurium SL1344 swimming motility, both trypsin and heat treatments reversed the inhibitory activity of LcS CFCS against S. Typhimurium SL1344 cell association and cell entry (data not shown).

We found that when a mild centrifugal force accelerating the contact between bacteria and cultured epithelial cells was applied (Jones et al., 1992), for the LcS CFCS-treated S. Typhimurium SL1344 the bacteria showed the same time-course of cell internalization as untreated S. Typhimurium SL1344 (Fig. 6a). As for LcS CFCS-treated S. Typhimurium SL1344, mutants of S. Typhimurium having defects in the expression of flagellar proteins (γ8601 and γ8602), or paralyzed flagella after mutation in the flagellar motor motA or motB genes (γ3421), showed a decreased cell-entry capacity, which was reversed when a mild centrifugal force
Each value shown is the mean percentage of bacteria showing motility or each cell-track type. Controls for gel loading. In (a, b) the results are expressed as the percentage of bacteria showing motility or each cell-track type. LcS CFCS-treated H. pylori controls for gel loading. In (a, b) the results are expressed as the culture medium. OmpF and OmpD porins were visualized as LcS CFCS-treated H. pylori. (a) Time-course analysis of the recovery of swimming track types in LcS CFCS-treated bacteria were returned to fresh culture medium. (b) Time-course analysis of swimming motility of untreated and LcS CFCS-treated S. Typhimurium SL1344. (c) Western blot showing the presence of FliC flagellin in S. Typhimurium SL1344. (a) Time-course analysis of swimming motility of untreated and LcS CFCS-treated S. Typhimurium SL1344 after bacteria were replaced in fresh DMEM culture medium. The arrow indicates the time when the bacteria were returned to fresh culture medium. (b) Time-course analysis of the recovery of swimming track types in LcS CFCS-treated S. Typhimurium SL1344 replaced in fresh DMEM culture medium. (c) Western blot showing the presence of Flc flagellin in untreated and LcS CFCS-treated H. pylori 1101 (60 min), and in LcS CFCS-treated H. pylori 1101 (60 min) replaced in fresh culture medium. OmpF and OmpD porins were visualized as controls for gel loading. In (a, b) the results are expressed as the percentage of bacteria showing motility or each cell-track type.

was applied (Fig. 6b). In contrast, the flagellated, motile SW399 (invA) and non-motile SW358 (flgK fljB invA) mutants defective for expression of InvA, a component of Salmonella pathogenicity island 1, which is necessary for efficient invasion of epithelial cells (Galán, 2001), displayed an abolished capacity to invade the cells compared with wild-type (~4.0 logs lower at 1 h), which remained abolished even when a mild centrifugal force was applied (Fig. 6c). Collectively, these results show that LcS CFCS-treated S. Typhimurium SL1344 displays a delayed capacity to induce F-actin mobilization and to enter polarized enterocyte-like Caco-2/TC7 cells as the results of a transiently impaired swimming motility, without modifying the invasiveness capacity.

A chemotaxis system operating via methyl-accepting chemotaxis proteins and Che proteins regulates both the anticlockwise and clockwise movement of the bacteria flagellum (Harshey, 2003). We found that the LcS CFCS treatment blocks the swimming motility (untreated 98 ± 0.5 versus LcS CFCS-treated, 5 ± 2% motile bacteria measured after 1 h of treatment) and the cell entry into Caco-2/TC7 cells (Fig. 6d) of the chemotaxis M935 (cheY) mutant similar to that above observed with LCS CFCS-treated S. Typhimurium SL1344. Moreover, the blockade of M935 mutant cell entry was reversed by mild centrifugal force (Fig. 6d). This result indicates that LcS CFCS inhibits the swimming motility and cell entry of S. Typhimurium SL1344 independently of chemotaxis.

**LcS CFCS-treated S. Typhimurium showed a decreased capacity to enter human mucus-secreting HT29-MTX cells**

The mucus layer that covers the surface of the intestinal and gastric epithelial cells provides a physical barrier against gastrointestinal pathogens. Consistent with this, we found a decreased level of internalized S. Typhimurium SL1344 within mucin-secreting HT29-MTX cells compared with those internalized within enterocyte-like HT-29 glc−/− cells (Fig. 7a). The non-motile LcS CFCS-treated S. Typhimurium SL1344 displayed a strongly decreased capacity to enter HT29-MTX cells compared with untreated S. Typhimurium SL1344 (Fig. 7a). The same decrease in bacteria cell entry was observed after infection of HT29-MTX cells with non-motile S. Typhimurium SL1344 flagellar or motor mutants (Fig. 7b).

**DISCUSSION**

Lactobacillus-secreted antibacterial molecules, including hydrogen peroxide and lactic acid, small peptides, and bacteriocins, exert antagonistic effects against Gram-negative gastroenterovirulent bacteria (Servin, 2004). Moreover, by modulating the deleterious effects of these pathogens on the host intestinal cell structure, machinery and functions, other secreted Lactobacillus compound(s) contribute to the protection of the intestinal epithelial barrier functions (Bron et al., 2012; Kleerebezem et al., 2010; Lebeer et al., 2008). The data reported here show that the compound(s) secreted by the probiotic LcS promotes a loss of swimming motility in S. Typhimurium SL1344 and H. pylori 1101 strains. The inhibitory effect is not due to the acidic pH of LcS CFCS since there was a lack of activity when the pathogens are
subjected to MRS broth at pH 4.5 or lactic acid. Altogether, our data indicate that the secreted compound(s) supporting the inhibitory activity against both *S. Typhimurium* SL1344 and *H. pylori* 1101 swimming motilities are of proteinaceous or peptidic nature, heat sensitive and small in size. In addition, we found that lactic acid by its membrane permeabilization activity (Alakomi *et al.*, 2000; Liévin-Le Moal *et al.*, 2011) participates in the LcS CFCS inhibitory activity against both *S. Typhimurium* SL1344 and *H. pylori* 1101 swimming motilities. For the killing activity of hydrogen peroxide against *Gardnerella vaginalis*, *uropathogenic E. coli* and *S. Typhimurium* (Atassi & Servin, 2010;
Pridmore et al., 2008), lactic acid acts cooperatively by increasing the sensitivity of target bacteria (Atassi & Servin, 2010). It is tempting to propose that lactic acid secreted by the strain LcS through an increase of membrane permeability favours the entry within the target pathogens of secreted compound(s) that block swimming motility.

The swimming motility of *H. pylori* has been observed to be modified by compounds and drugs acting by different mechanisms. *Lactobacillus johnsonii* NCC533 secreted small peptide(s) of molecular mass >10 kDa that inhibited the motility of *H. pylori* by an unknown mechanism and without affecting cell viability (Isobe et al., 2012). Proton pump inhibitor, rabeprazole, and its thioether derivative, inhibited the motility of *H. pylori* (Tsutsui et al., 2000). The other proton pump inhibitor, trifluoromethyl ketone derivative 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone, inhibits the motility of clarithromycin-susceptible and -resistant *H. pylori* by altering swimming more than tumbling, suggesting that the molecule works as an uncoupler of the flagellar motor rather than affecting the clockwise rotation (Spengler et al., 2004). *H. pylori* motility was diminished by ranitidine bismuth citrate without any loss of viability or change in cell morphology, and colloidal bismuth citrate induced *H. pylori* cell fragmentation and flagellum detachment, whereas ranitidine hydrochloride did not (Worku et al., 1999a). Moreover, the expression of the flagellar *flaA* and *flaB* genes of *H. pylori* was down-regulated in the presence of celecoxib (Wang et al., 2010).

Diverse compounds have been described as blocking the swimming motility of *Salmonella* by different mechanisms. Fumarate promoted the clockwise rotation of *S. Typhimurium*, but did not promote switching, and malate, maleate and succinate all promoted switching, whereas aspartate and lactate did not (Prasad et al., 1998). Micocin L depolarized the bacterial cytoplasmic membrane of *S. enterica* serovars *Typhimurium* and *Enteritidis* (Morin et al., 2011). Our group has recently reported that the *L. acidophilus* LB CFCS transiently impaired the swimming motility of *S. Typhimurium* SL1344 by depolarizing the bacterial membrane by means of secreted, heat-stable, trypsin-insensitive, low molecular weight molecule(s) (Liévin-Le Moal et al., 2011).

The similarity in sensitivity of LcS CFCS to treatments was suggestive that the observed inhibitory effect against the swimming motility of both *S. Typhimurium* SL1344 and *H. pylori* 1101 probably results from identical secreted molecules. Interestingly, our results revealed that the two gastrointestinal pathogens examined responded in different ways to these secreted inhibitory compounds. Indeed, LcS CFCS-treated *S. Typhimurium* SL1344 transiently lost its swimming motility following membrane depolarization, and without modification of the flagella expression. It was noted that LcS CFCS-treated *S. Typhimurium* SL1344 showed no alteration of cell viability and ATP intracellular content. In contrast, LcS CFCS-treated *H. pylori* 1101 undergoes a change of morphology starting with the appearance of ‘c’-shaped forms and terminated by the formation of coccoid forms, a phenomenon accompanied by a loss of flagella and a dramatic decrease in cell ATP content. The helical shape of *H. pylori* serves an important function in pathogenesis by enhancing the flagellar motility of *H. pylori* through the viscous epithelial mucus layer in which it resides by a corkscrew mechanism (Cover & Blaser, 2009). Mutant *H. pylori* cells with a pronounced ‘c’-shaped morphology showed a decreased expression of flagella accompanied by a perturbed swimming motility (Sycuro et al., 2012). Moreover, the *H. pylori* coccoid form had few small flagella or coiled flagella (Cocconnier et al., 1998; Sato et al., 2003) consistent with the loss of swimming motility observed for precoccoidal or coccoidal forms (Worku et al., 1999b). The fact that LcS CFCS-treated *H. pylori* undergoes a transformation into the coccoid form is consistent with this form being known to be a stressed cell morphology resulting from a protection mechanism switched on by the bacterium (Azvedo et al., 2007; Saito et al., 2008). The conversion of *H. pylori* from spiral form to coccoid form has been observed during exposure to environmental stresses (Can et al., 2008; Mouery et al., 2006), antibiotics (Berry et al., 1995; DeLoney & Schiller, 1999; She et al., 2001; Sörberg et al., 1998), colloidal bismuth subcitrate (Bland et al., 2004), non-treated and heat-treated *L. acidophilus* LB CFCS (Cocconnier et al., 1998), and live *L. johnsonii* NCC533 (Sgouras et al., 2005). Observation that the *S. Typhimurium* SL1344 seems to be more resistant than *H. pylori* 1101 to LcS CFCS treatment is probably explained by *Salmonella* having robust systems of resistance or adaptation to stress and antibacterial compounds (Shen & Fang, 2012).
Motility is a key factor in the adaptation of many bacterial pathogens to enable them to colonize in vivo host mucosal surfaces. Results reported here showed that compound(s) secreted by LcS by inhibiting swimming motility of S. Typhimurium SL1344 in turn decreased the capacity of the pathogen to invade human enterocyte-like Caco-2/TC7 cells. These results fit well with the role of flagella for Salmonella interaction with cultured intestinal cells (Dibb-Fuller et al., 1999; Lockman & Curtiss, 1990; McCormick et al., 1988; Michetti et al., 1994; Pontier-Bres et al., 2012;
**Fig. 5.** LcS CFCS treatment retarded *S. Typhimurium* SL1344-induced F-actin mobilization and cell entry into enterocyte-like Caco-2/TC7 cells. (a) Observation of direct labelling of F-actin with FITC-phalloidin revealing the absence of F-actin spots at the apical cell domain of control cells and the presence of F-actin spots in cells infected with untreated *S. Typhimurium* SL1344. (b) Counting of F-actin spots during the time-course in cells infected with untreated or MRS broth-treated or LcS CFCS-treated *S. Typhimurium* SL1344. (c) Time-course of cell entry in cells infected with untreated or LcS CFCS-treated *S. Typhimurium* SL1344. (d) Time-course of cell entry of SL1344 mutants χ8601 (fljB), χ8602 (fljB, fliC) and χ3421 (motA, motB). In (a) images are representative of three experiments, each performed in duplicate. In (b–d) each value shown is the mean±SD from three experiments (three successive passages of cells). *P <0.01 versus SL1344.

**Fig. 6.** Effect of a mild centrifugal force on the cell entry within Caco-2/TC7 cells of LcS CFCS-treated *S. Typhimurium* SL1344 and SL1344 flagella mutants. (a, b) Restoration of a normal cell entry of LcS CFCS-treated *S. Typhimurium* SL1344 (a), and SL1344 mutants χ8602 (fljB, fliC) and χ3421 (motA, motB) (b) by exposure to a mild centrifugal force (500 g for 5 min). (c) Absence of a restoration of a normal cell entry of SW399 (DinvA) and SW358 (DflgK fljB invA) after exposure to a mild centrifugal force. (d) LcS CFCS treatment inhibits the cell entry of chemotaxis *S. Typhimurium* SL1344 mutant strain M935 (cheY), an effect reversed by exposure to a mild centrifugal force. Cell entry in (d) was measured at 30 min p.i. Each value shown is the mean from two experiments (two successive passages of cells).
The swimming motility of H. pylori has been shown to play a central role in the ability of the pathogen to swim into the protective mucus layer to escape the harsh environment of the stomach, and to colonize the gastric epithelium (Schreiber et al., 1999). The present results showing that molecule(s) secreted by LcS blocked the swimming motility of S. Typhimurium and in turn reduced their entry into human enterocyte-like and mucus-secreting cells are of interest in explaining previous results showing that LcS antagonized the colonization of the stomach and intestine by several motile gastrointestinal pathogens in rodent models (Asahara et al., 2011; de Waard et al., 2002; Fayol-Messaoudi et al., 2007; Sgouras et al., 2004). It may be possible that the inhibition of the swimming motility of motile gastrointestinal pathogens by products secreted by several well-characterized Lactobacillus strains has the effect in vivo of prolonging the exposure of the pathogens to the luminal, gastric and intestinal defences of the host.

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