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Exopolysaccharide-producing *Streptococcus thermophilus* CRL1190 reduces the inflammatory response caused by *Helicobacter pylori*

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RESEARCH ARTICLE

Abstract

This work evaluated the ability of the probiotic *Streptococcus thermophilus* CRL1190 strain and its exopolysaccharides to adhere to gastric mucosa. Probiotic bacteria attachment to the human stomach epithelium was confirmed in human stomach tissue samples and the gastric epithelial cell line AGS. In addition, it was demonstrated that *S. thermophilus* CRL1190 strain reduced *Helicobacter pylori* adhesion and attenuated inflammatory response in AGS cells. This is the first demonstration of the capacity of *S. thermophilus* CRL1190 to adhere to the stomach gastric mucosa, and improve protection against *H. pylori* through the reduction of its adhesion and the modulation of the inflammatory response. Therefore, *S. thermophilus* CRL1190 fermented milk is a good candidate for further *in vivo* studying of the protective effect of functional food against *H. pylori* infection and gastric inflammatory damage.

Keywords: gastritis, stomach, inflammation, probiotic, pathogenic bacteria, *S. thermophilus* CRL1190

1. Introduction

The stomach is a particularly challenging niche for bacterial habitation mainly due to the low pH values that affects their luminal colonisation (Wu *et al.,* 2014). Cellular and molecular mechanisms of attachment and colonisation of the gastric mucosa have been well characterised for the pathogenic bacteria *Helicobacter pylori*, which is able to resist the acidic lumen through the synthesis of urease and the *in situ* production of ammonium ions (Salama *et al.,* 2013). Besides, this bacterium has several adhesive factors that are important for protection against acidic pH, mucus and exfoliation during early and chronic phases of infection (Kalali *et al.,* 2014). It is well known that *H. pylori* develops a plurality of mechanisms in the host that are involved in signalling pathways resulting in pro-inflammatory responses, severe inflammation, disruption of the epithelial barrier function, and possibly gastric cancer (Posselt *et al.,* 2013).

In contrast, the stomach microbiome and the mechanism of attachment and colonisation of the normal gastric microflora have been less studied (Wu *et al.,* 2014). Analysis of gastric biopsies identified *Enterococcus*, *Streptococcus*, *Staphylococcus* and *Stomatococcus* as members of the normal gastric microbiota but it remains unknown their mode of interaction with the gastric mucosa (Hakalehto *et al.,* 2011).

Streptococcus thermophilus is widely used in the dairy industry as starter culture for yogurt, cheese, and fermented milks (Delorme, 2008). *S. thermophilus* CRL1190 (ST1190) produces exo-polysaccharides (EPS1190) with high MW (>1,500 kDa) consisting of 33% glucose (mainly as 1,4-linked glucose) and 66% galactose with 1,4- and 1,4,6-galactose residues as main building blocks (Marcial *et al.,* 2013). Previous studies evinced *in vivo* beneficial effects of milk (FM1190) fermented with *S. thermophilus* CRL1190 in a model of gastritis in mice (Rodriguez *et al.,* 2009). Mice were fed with FM1190 for 7 days before (preventive treatment) and after (therapeutic treatment) induction of chronic gastritis by daily administration of acethylsalicilic acid for 10 days. No lesions on gastric mucosa were observed in animals fed with FM1190 regardless the kind of treatment

in comparison to untreated mice that developed gastritis (Rodriguez *et al.,* 2010). The protective effect was associated to the immune regulatory capacity of the EPS1190 in FM1190 which produced an increase in interleukin (IL)-10^+ cells, and a decrease in tumour necrosis factor (TNF)-α^+ and interferon-γ^+ cells in the mouse gastric mucosa (Rodriguez *et al.,* 2009, 2010). Despite these promising results, it remains unknown how *S. thermophilus* CRL1190 and its EPS1190 interact with the gastric mucosa for displaying the protective effect.

In the present study, the complex ST1190-EPS1190 and each single component was evaluated *in vitro* and *in situ* by using cell cultures and tissue cultures to gain insight into their interaction mechanisms with the gastric mucosa. In addition, this work evaluated whether ST1190 is able to exert protective effects against *H. pylori* by influencing adherence and/or inflammation in gastric epithelial cells.

2. Materials and methods

General experimental procedures

Sections of human gastric tissue were kindly provided by Prof. Dr. G. Faller (St. Vincentius Hospital, Karlsruhe, Germany), and were obtained from *H. pylori* negative individuals whose mucosa in the gastric antrum region did not show any significant pathological changes. Chemicals were purchased from Sigma (Deisenhofen, Germany) and VWR (Darmstadt, Germany).

Cultivation of bacteria

S. thermophilus CRL1190, strain EPS^+ was obtained from the Centro de Referencia para Lactobacilos (CERELA) culture collection (Tucumán, Argentina). The bacteria were activated in fresh LAPTlac media and cultivation was carried on according to reference (Marcial *et al.,* 2013). Lyophilised EPS1190 was isolated from fermented milk (Marcial *et al.,* 2013).

Cultivation of AGS cells

Adherent human gastric adenocarcinoma epithelial cell line (AGS) was provided by Prof. Dr. W. Beil (Medizinische Hochschule, Hannover, Germany). Adherent AGS cells were grown in AGS cell culture medium (RPMI 1640, FCS 10%, penicillin/streptomycin 1%) in tissue culture flasks (75 cm^2) at 37 °C with 5% CO_2. Passaging was performed every 7 days; the AGS cells were washed with 5 ml phosphate buffered saline (PBS) and trypsinised (3 ml trypsin/EDTA) for 5 min after removal of the media. The trypsin-AGS suspension was added to 5 ml of AGS medium, centrifuged (440×*g*, 5 min), suspended in fresh medium. Then, cells were inoculated into six-well plates (2×10^5 cells/well) and cultured for 48 h until they reach a minimum confluence of 80%.

Binding of *Streptococcus thermophilus* CRL1190 to human gastric epithelium

In situ experiments with histological sections of human gastric mucosa and FITC-labelled ST1190 (FITC-ST1190) were performed as described previously (Falk *et al.,* 1993; Lengsfeld *et al.,* 2004a,b) and evaluated semi-quantitatively by fluorescent microscopy. ST1190 was centrifuged, washed three-times with PBS containing 0.05% of Tween 20, and suspended in PBS. Bacteria concentration was estimated by curves of OD (optical density, λ 550 nm) vs cfu. The FITC labelling was carried out in accordance to Lengsfeld *et al.* (2004a). Briefly, 4.0×10^8 cfu were resuspended in 2 ml of sterile saline solution (pH 8.0). A solution (20 µl) of fluoresceinisothiocyanate (FITC, 10 mg/ml in dimethyl sulfoxide) was added to the bacteria suspension and incubated for 30 min, then bacteria was washed twice with washing buffer (PBS, Tween 20 0.05%) to remove excess of FITC and was gently suspended for further use.

FITC-ST1190 was incubated with different concentrations of EPS1190 (0, 50, 100 µg/ml) at 37 °C during 2 h. Both FITC-bacteria and EPS1190 were suspended in blocking buffer (PBS, BSA 0.2%, Tween 20 0.05%). In the meantime, slides with paraffin-embedded tissue sections were deparaffinated with xylol, isopropanol, water and PBS followed by incubation for 20 min with blocking buffer. After removal of the blocking buffer from the tissue sections, 200 µl of the pretreated bacterial suspension (1×10^7 cfu) were gently transferred onto the respective slides (Lengsfeld *et al.,* 2004b). The gastric tissue sections were then incubated for 1 h in the dark and finally gently washed five times in PBS buffer (Niehues *et al.,* 2010). The slides were then evaluated by fluorescence microscopy. The quantity of adhering fluorescent bacteria to the epithelial surface was evaluated under double blinded conditions.

Laser scanning microscopy

AGS cells were cultured on glass slides (Menzel GmbH, Braunschweig, Germany) inside removable silicone chambers (Greiner bio-one, Frickenhausen, Germany) at 1×10^5 cells per chamber in 300 µl growth medium (RPMI 1640 supplemented with FCS 10% and pen/strep 1%). When cells reached a confluence of 80%, FITC-ST1190 at a multiplicity of infection (MOI) of 1:100 was added with or without the addition of EPS1190 (50 or 100 µg/ml). After 2 h incubation, cells were washed 3 times with PBS to eliminate non-adherent bacteria and fixed with 500 µl of *p*-formaldehyde (3.7% *v/v*) in PBS during 15 min at room temperature. After washing the cells with PBS, a solution of TritonX100 (0.5%) was added and incubated for 10 min. Then 100 µl of bovine serum albumine (BSA)

solution (3%) was added to block unspecific bindings. Subsequently, for staining of cell actin filaments, 50 µl/100 ml of TexasRed-X-phalloidin (Invitrogen, Eugene, OR, USA) in growth medium were added for 30 min. Nucleus staining was done with 1 mg/ml of 40,6-diamino-2-phenyl-indol-dihydrochloride (DAPI, Sigma-Aldrich, Buchs, Schweiz) and incubated for 30 to 60 min. After incubation, the samples were washed three times with PBS, the silicone chambers were removed and cells were fixed. All procedures were performed by exclusion of direct light. Laser scanning microscopy was performed with a Leica TCS-SP2 fluorescence microscope (Leica, Mannheim, Germany). Excitation at 405 nm for DAPI, 488 nm for FITC and 594 nm for Texas-Red. Emission wavelength 410-50 nm for DAPI, 500-570 nm for FITC and 620-700 for Texas-Red (Deters *et al.,* 2010; Marcial *et al.,* 2013; Zippel *et al.,* 2009).

Quantification of adhesion of *Streptococcus thermophilus* CRL1190 to AGS cells

The quantitative flow cytometry adhesion assay was used for quantification of ST1190 adhesion to AGS cells. FITC-ST1190 was co-cultivated with AGS cells at different MOIs (1:20, 1:50 and 1:100) in presence or absence of EPS1190 (100 µg/ml). In the pre-incubation assay, FITC-ST1190 was incubated with EPS1190 during 1 h at 37 °C and then transferred to AGS cells. In the co-incubation assay, FITC-ST1190 and EPS1190 were inoculated at the same time together with AGS cells.

After incubation at 37 °C and 5% CO_2 for 1 h, cells were gently washed three times with 2 ml PBS/well and detached with 600 µl/well trypsin-EDTA (0.05% in D-PBS, 3 min). The trypsinised cells were suspended in 2 ml Pen/Strep-free AGS medium, centrifuged for 5 min at 440× *g* and after discarding the supernatant, the pellet was suspended in a final volume of 750 µl medium.

The flow cytometric analysis was performed by FACSCalibur® (BD, Heidelberg, Germany) as described previously (Niehues *et al.,* 2011). For each flow cytometric measurement a number of 20,000 counts was determined to obtain significance for data evaluation. For each assay, a negative control of AGS cells (cells without bacteria) and positive control of AGS cells incubated with FITC-ST1190 were used.

Anti-adhesive effect of *Streptococcus thermophilus* CRL1190 against *Helicobacter pylori* on AGS cells

The anti-adhesion test was performed according to Messing *et al.* (2014) with some modifications. Pre- and co-incubation experiments were performed as follow: (a) in pre-incubation studies, AGS cells were incubated with ST1190 during 2 h at different multiplicities of infection (MOIs), then cells were washed 3 times with PBS and incubated again with the FITC labelled *H. pylori* (MOI: 1:20) for 1 h; (b) in co-incubation experiments, both ST1190 (at different MOIs) and *H. pylori* were incubated together with AGS cells during 1 h. After incubation, samples were processed and analysed by flow cytometry (FACS-Calibur, BD, Heidelberg, Germany). Polysaccharides with known anti-adhesive *H. pylori* such as Milk Human Oligosaccharide (MO) or *Abelmoschus esculentus* raw polysaccharide (OKRA) were used as positive anti-adhesion controls (Messing *et al.,* 2014).

Influence of *Streptococcus thermophilus* CRL1190 on human gastric epithelial cells viability

To determine the influence of probiotic strain on AGS cell viability the assay was performed according to the method described by Messing *et al.* (2014). Bacterial suspensions were added on each well (100 µl/well) in different bacteria:cells ratios (1:10 to 1:100). In addition, non-viable ST1190 was used in the assays. For that, bacterial suspension was autoclaved at 121 °C during 20 min, centrifuged, and the pellet suspended in fresh AGS cell culture medium. Non-viable and viable bacteria were added to AGS cells at a similar concentration, and AGS cells viability was determined by MTT assay after 1 or 2 h of co-incubation.

Cytokine production in AGS cells after *Helicobacter pylori* infection

The cytokine secretion assay was assessed according to Marcial *et al.* (2013) with minor modifications. For investigation on the influence of ST1190, AGS cells were incubated with *H. pylori* in presence of the probiotic strain: (1) 24 h of co-incubation of AGS, ST1190 (variable MOIs) and *H. pylori* (MOI: 1:150); (2) 18 h of co-incubation of AGS with *H. pylori* (MOI: 1:150) following of 6 h co-incubation with ST1190 at different MOIs. 24-h co-incubation of AGS cells with *H. pylori* served as inflammation control. Interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-α were measured in the supernatant by ELISA kit (PeproTech, Hamburg Germany) according the instructions of the manufacturer.

Agar diffusion test

Different dilution or culture supernatant of ST1190 were tested against *H. pylori* to exclude unspecific cell toxicity by agar diffusion test (Marcial *et al.,* 2013). The incubation time was between 3 to 4 days under microaerophilic condition (CampyGen ContainerSystem – Gaspak, Oxoid, Ltd., Basingstoke, UK). Amoxicillin served as positive control (0.5 µg per disk, MPBiomedicals, Irivine, CA, USA).

Statistical analysis

The analysis was performed by using SPSS®. The experimental results are expressed as the mean ± standard deviation. Data were assessed by analysis of variance. In case the analysis indicated significant differences between groups, each group was compared by Dunnett's t-test (two-sided) and $P<0.05$ was considered to be statistically significant.

3. Results

Interaction of *Streptococcus thermophilus* CRL1190 with stomach cells

The adhesion ability of ST1190 was evaluated in an *in situ* model system (Lengsfeld *et al.,* 2004b). Tissue sections from healthy human stomach were incubated with fluorescent-labelled bacteria and attachment of the ST1190 strain was monitored by fluorescent imaging. A clear adhesion of the FITC-ST1190 (without pre-incubation with EPS1190) was observed, with bacterial attachment to the mucus layer and to the epithelial cells (Figure 1A). No influence of EPS1190 (50 or 100 µg/ml) on the bacterial binding was observed (Figure 1B).

The interaction of FITC-ST1190 with AGS cells was analysed by laser scanning microscopy. The cell nuclei was stained with DAPI and actin filaments with phalloidin-TexasRed® (Figure 2). Co-incubation of FITC-labelled bacteria with AGS cells showed adhesion of ST1190 to the gastric cells (Figure 2) which could not be removed by persistent washing with PBS. These results indicated the ability of the bacterium to attach to stomach cells, which has not been described in literature until now. The addition of EPS1190 did not modify the bacterial adhesion, which is in correlation with the *in situ* assays on human gastric tissue showed previously (Figure 1).

The adhesion of ST1190 to AGS cells as well as the influence of EPS1190 was quantified by flow cytometry. For this purpose, pre- and co-incubation assays of AGS cells with ST1190 at different MOIs, in absence or presence of EPS1190 were performed (Figure 3). For evaluation, two gates were defined for quantitative analysis, reflecting bacterial binding (gate M2, high fluorescence activity of individualised cells with adhesive bacteria) and cells with no bacteria attached (gate M1). As shown in Figure 3, a MOI-dependent adhesion of the bacteria to AGS cells was obvious. Addition of EPS1190 at different concentrations to the pre- and co-incubation assay with the AGS cells did not change the level of adhesion, indicating again that bacterial adhesion was not mediated by EPS1190.

Effect of *Streptococcus thermophilus* CRL1190 on *Helicobacter pylori* adhesion to AGS cells

The potential anti-adhesive effects of ST1190 were investigated using FITC-labelled *H. pylori* and AGS cells. At the end of incubation cells with adherent bacteria were quantified by flow cytometry after trypsinisation of the monolayer. Acidic human milk oligosaccharides (MO) structures bearing a 2,3-linked sialic acids capable of inhibiting the adhesins SabA and HpaA, and OKRA a polysaccharide with non-specific inhibitory adhesion activity served as positive controls (Messing *et al.,* 2014). AGS cells co-incubated with labelled *H. pylori* served as untreated control and this was considered as 100%. Pre-treatment of AGS cells with different doses of ST1190 significantly reduced *H. pylori* adhesion to AGS cells in a dose dependent manner (Figure 4). The anti-adhesive activity of the ST1190 strain in a dose of 1:50 was 80.4%, a value that was similar to the MO positive control with 79.2%. The higher dose of ST1190 used (1:100) decreased *H. pylori* adhesion to 65.9%, being similar to the OKRA positive control with 66.1% of adhesion (Figure 4). Co-treatment of AGS cells with ST1190 and *H. pylori* showed similar results to pre-treatment assay (Figure 4).

We also evaluated the direct antibacterial effect of ST1190 against *H. pylori*. The probiotic bacteria or its culture supernatant did not exert any inhibitory effect on *H. pylori* through agar diffusion test in comparison to the positive control, amoxiciline (0.5 µg/disc), which exerted an inhibition zone of 30 mm diameter (data not shown).

Immunomodulatory activity of *Streptococcus thermophilus* CRL1190 in AGS cells

We first evaluated the effect of ST1190 on cellular activity of AGS cells by the MTT assay. ST1190 increased in dose manner the AGS cellular activity at longer incubation time than 1 h. It was observed that viable ST1190 increased the dehydrogenase activity of AGS cells on 20% in comparison to the untreated control at a MOI of 1:100 after 2 h co-incubation, being similar to positive control (FCS 10%). Similar results were found when non-viable bacteria were used (Figure 5).

Finally, the ability of ST1190 to modulate pro-inflammatory cytokines secretion in AGS cell was evaluated (Figure 6). Treatment of gastric cells with the probiotic bacteria did not induce significant changes in the production of TNF-α or IL-6 (Figure 6A). In addition, no modifications in the levels of IL-8 were observed with exception of the highest concentration of ST1190 used (1:150) which slightly increased IL-8 production by AGS cells (Figure 6A). Challenge of AGS cells with *H. pylori* J99 significantly increased the levels of IL-8, TNF-α, IL-6 in agreement with the capacity of the pathogen to mount a robust

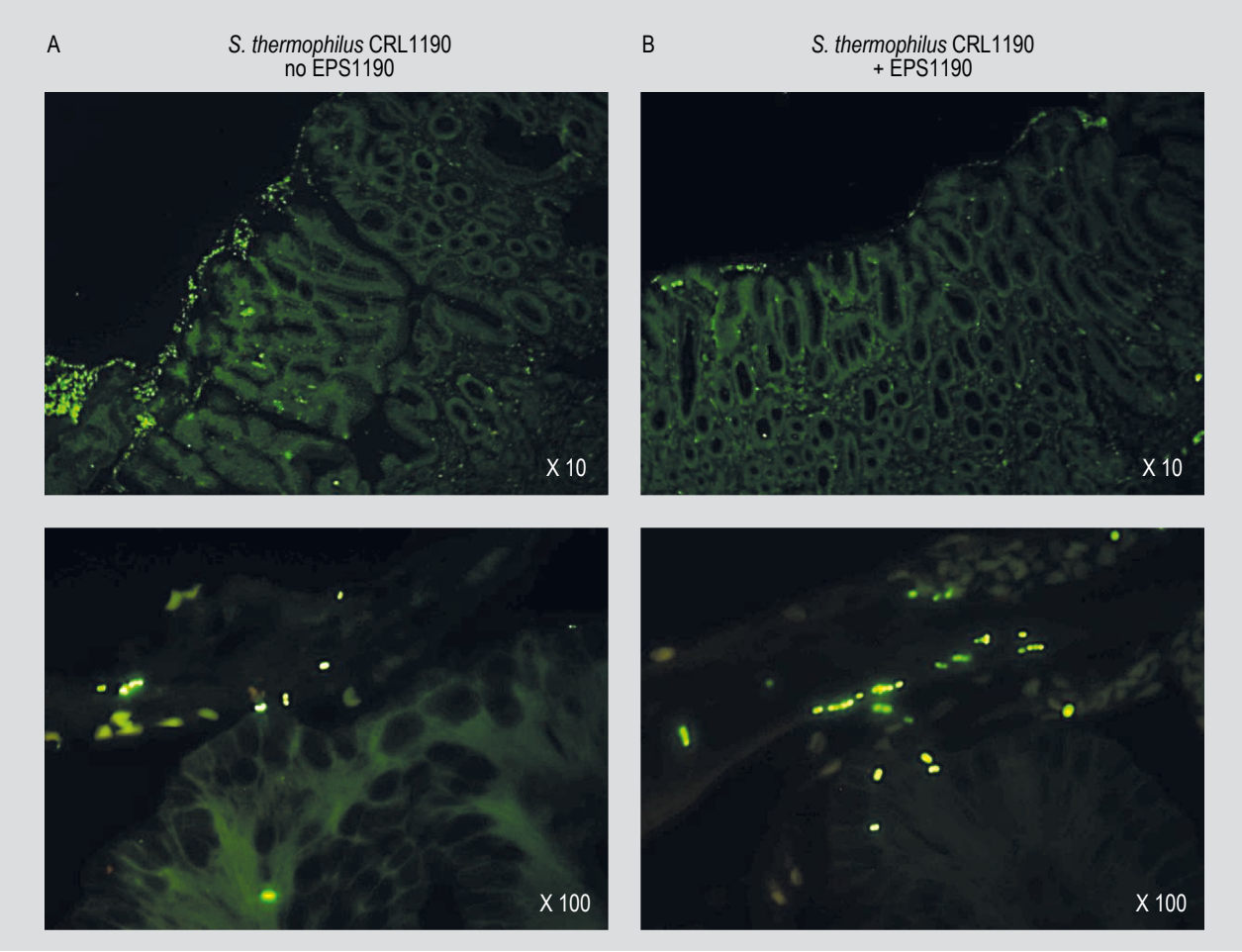

Figure 1. Immunofluorescence microscopy of human stomach tissue sections after different treatment protocols. (A) Adhesion of FITC-labelled *Streptococcus thermophilus* CRL1190 to human gastric mucosa in absence or presence of EPS1190 (magnification 10× and 100×). (B) *S. thermophilus* CRL1190 was pre-incubated with 100 µg/ml of EPS1190.

inflammatory response in gastric cells (Figure 6B, C). When AGS cells were co-incubated with ST1190 and *H. pylori* a significant decrease in IL-8 production was observed while no effect was evident in the levels of TNF-α and IL-6 (Figure 6B). To evaluate whether ST1190 beneficially modulated inflammation after infection with *H. pylori,* AGS cells were challenged with pathogenic gastric bacteria during 18 h and then ST1190 was inoculated until the end of incubation time. Although the anti-inflammatory effect was modest when compared with the reported in co-incubation experiments, a reduction of IL-8 was observed (Figure 6C).

4. Discussion

Fermented dairy products are frequently consumed on a regular basis diet as an excellent source of good quality proteins (Lyer *et al.,* 2010) and are also considered the main vehicles for probiotic lactic acid bacteria (LAB) with specific health functions (Borchers *et al.,* 2009; Collado *et al.,* 2009; Villena *et al.,* 2014). Probiotic LAB are represented mainly by lactobacilli, e.g. *Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus reuteri* and *Lactobacillus acidophilus* among others, which have probiotic effects in the gastrointestinal tract mainly due to interaction with intestinal epithelial cells and the gut associated lymphoid tissue (Kitazawa and Villena, 2014; Lam *et al.,* 2005; Villena and Kitazawa, 2014).

The adhesion of *Lactobacillus* to the intestinal epithelium was defined as a characteristic of interest for selection of probiotic strains, since it prevents its immediate elimination by intestinal peristalsis and represents the first step in the formation of a barrier to prevent colonisation by undesirable microorganisms due to competition for nutrients and adherence sites (Collado *et al.,* 2005). Various *in vitro*, *ex vivo* and *in vivo* models have been used for the study of lactobacilli adhesion to the intestinal epithelium and significant advances have been performed in the understanding of the cellular and molecular mechanisms involved in probiotic adhesion to intestinal epithelial cells by nonspecific physical interactions, such as steric and hydrophobic interactions, which result in reversible

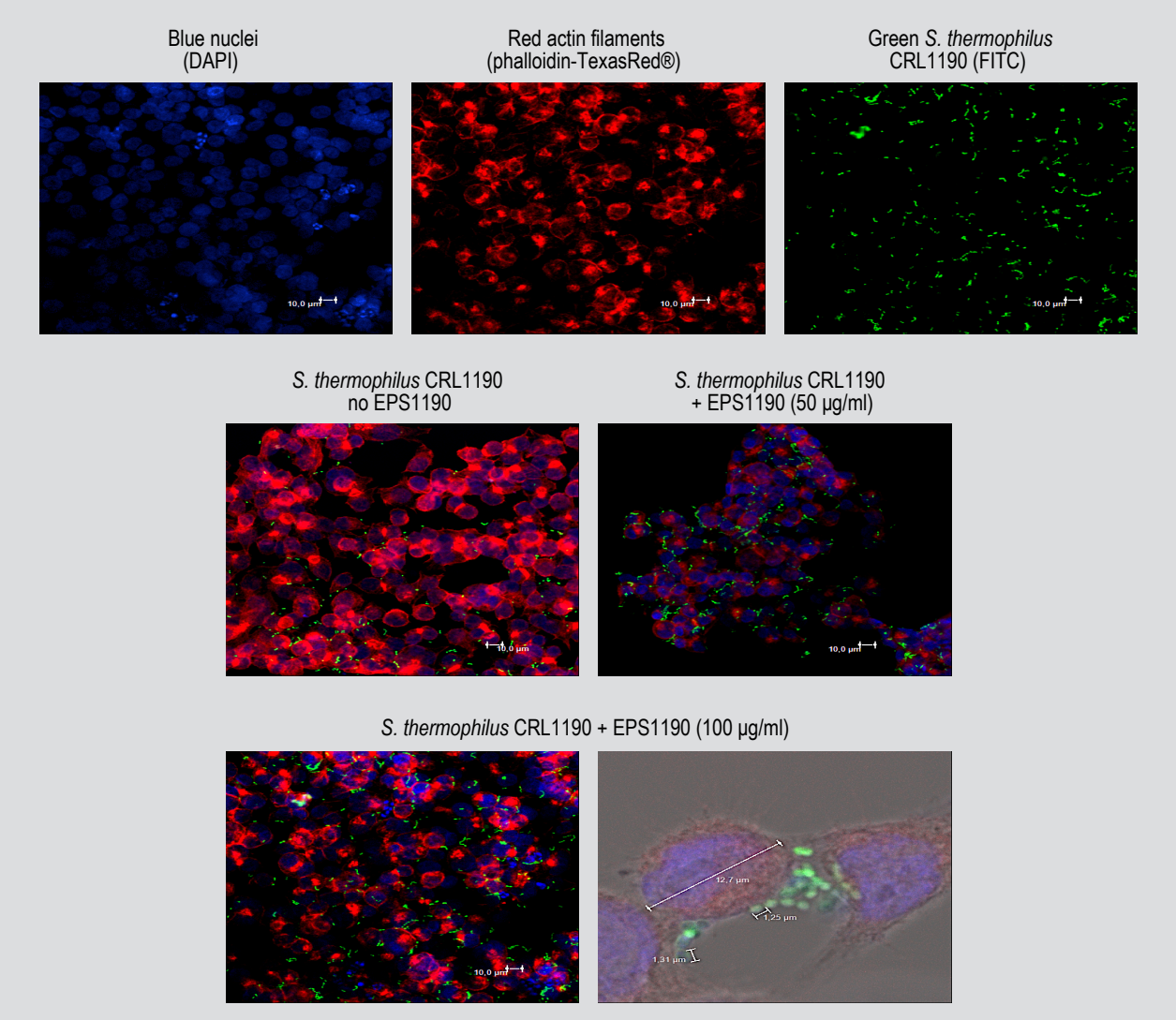

Figure 2. Laser scanning microscopy of AGS cells after different treatment protocols. Adhesion of green-yellow fluorescence FITC-labelled *Streptococcus thermophilus* CRL1190 after 2 h incubation with AGS cells in absence or presence of EPS1190. *S. thermophilus* CRL1190 was pre-incubated with 50 or 100 µg/ml of EPS1190. Cell nuclei was stained with DAPI and actin filaments with phalloidin-TexasRed®.

attachment. This initial bacterial adhesion then allows specific interactions between adhesins and their receptors (Van Tassel and Miller, 2011). Moreover, surface adhesins of probiotic microbes are believed to be crucial in bacterial retention in the gut and in host-bacterial communications (Segers and Leeber, 2014). In addition to surface proteins, lactobacilli EPS have been shown to influence probiotic adhesion. In this regard, the probiotic *L. rhamnosus* GG contains two major types of polysaccharides: long galactose-rich polysaccharides and shorter glucose/mannose-rich polysaccharides that have been associated to its adherent abilities (Francius *et al.,* 2008). Recent studies (Ren *et al.,* 2014) suggested that the quantity of EPS produced by lactobacilli strains could be related to their adhesive properties. The work demonstrated that *L. salivarius* CICC 23174 and *L. plantarum* CGMCC 1557 were the most adhesive strains producing the highest quantity of exo-polysaccharide.

Most of the studies regarding probiotic adhesive capacities have been performed with intestinal epithelial cells. On the contrary, few works have been done on probiotic LAB using the stomach as ecological niche where neutralisation of local gastric pH and attachment into the stomach mucus layer are typical strategies for bacteria survival. Viable LAB were found in stomach biopsies from healthy gastroscopy patients; however, it remained unknown whether these bacteria were adhered to the gastric mucosa or slightly attached to unspecific surface structures (Hakalehto *et al.,* 2011). Later, the adhesion of members of the genus *Lactobacillus* isolated from the stomach of healthy humans to the gastric mucosa was evaluated *in vitro* using the

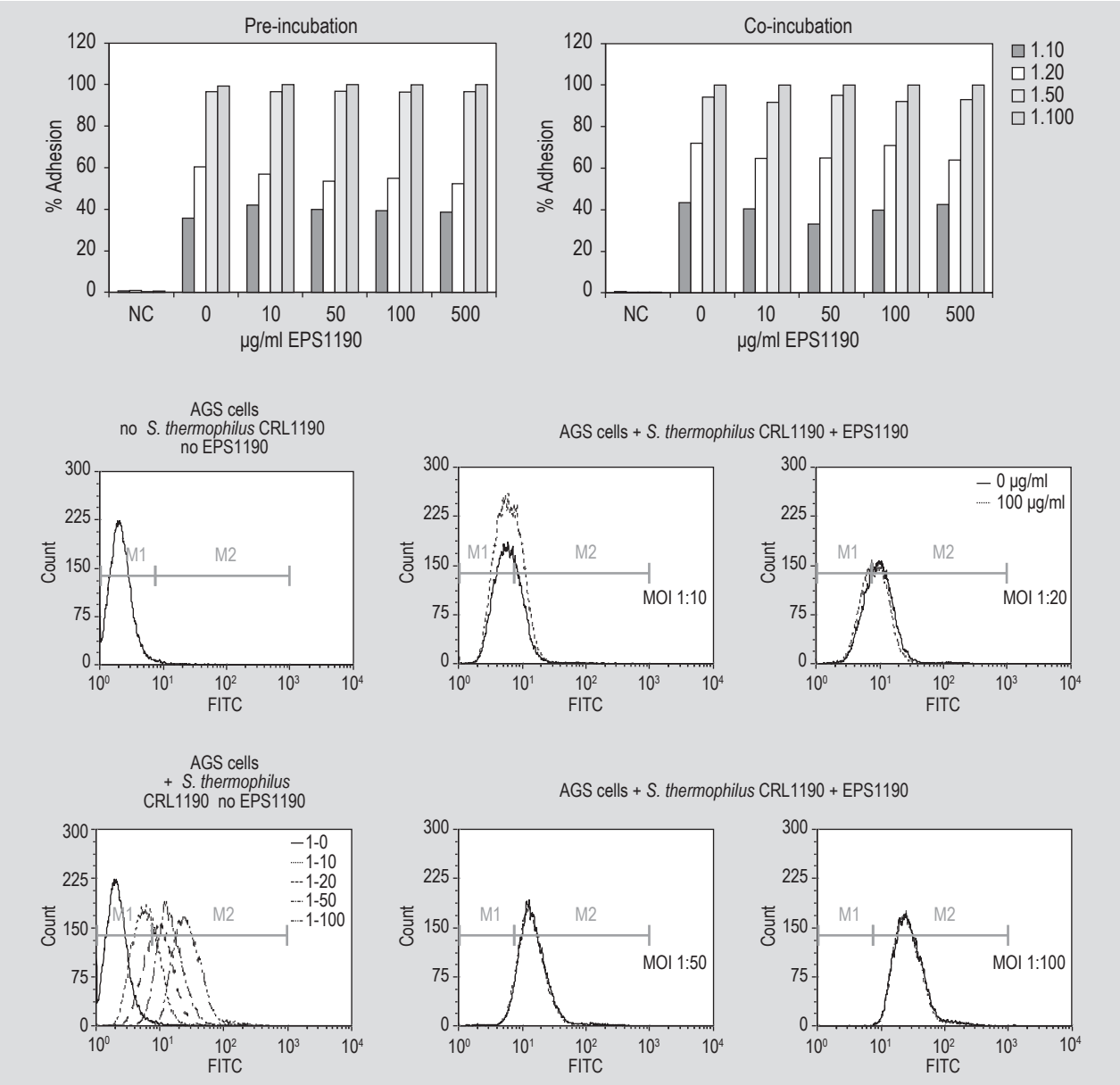

Figure 3. Flow cytometry of AGS cells after different treatment protocols. Cells were pre- or co-incubated with different MOIs of *Streptococcus thermophilus* CRL1190 in presence or absence of EPS1190. *S. thermophilus* CRL1190 was incubated with 100 µg/ml of EPS1190. Results are expressed as percentage of adhesion. NC = negative control, untreated AGS cell control, not treated with EPS, not incubated with bacteria. M1 = low fluorescence gate, indicating AGS cells with low fluorescence label. M2 = high fluorescence gate, indicating AGS cells with high fluorescence.

gastric cell line AGS (Delgado *et al.,* 2015). Similar to other probiotic traits, it was found that the percentage of adhesion to gastric cells was strain-dependent. Moreover, some studies reported that adhesive *Lactobacillus* strains could inhibit *H. pylori* adherence to human gastric epithelial cells and confer protection against the infection (Chen *et al.,* 2012). On the contrary, to our knowledge, no reports of the adherence of probiotic *Streptococcus* strains to gastric mucosa have been published.

S. thermophilus is widely used in the milk industry as starter culture in combination with *Lactobacillus bulgaricus* ssp. *delbrueckii* for yoghurt fermentation. However, there are little evidences concerning its potential beneficial health effects. In this regard, we have recently reported a reduction in gastric inflammation when mice were fed with the FM1190 obtained by milk fermentation with the exo-polysaccharide producing strain ST1190 (Rodriguez *et al.,* 2009, 2010). The healing effect was ascribed to both an improved mucus secretion and the regulation of inflammatory cytokines by the probiotic strain; however,

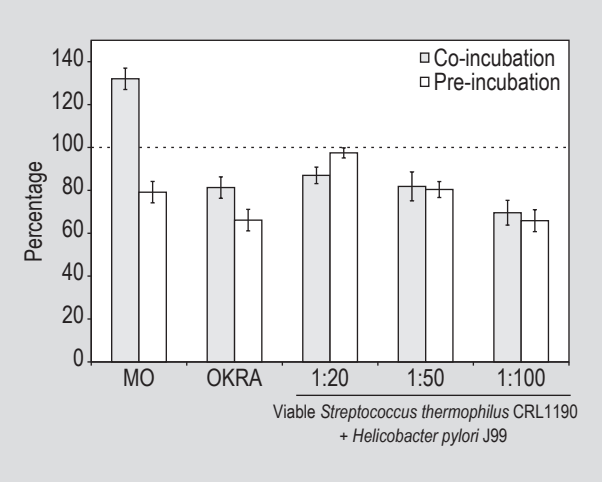

Figure 4. Effects of *Streptococcus thermophilus* CRL1190 on adhesion of *Helicobacter pylori* to AGS cells. Pre- and co-incubation experiments were performed. Different MOIs of *S. thermophilus* CRL1190 were evaluated (1:20, 1:50, 1:100) against *H. pylori* (MOI of 1:20). MO and OKRA served as a positive control. (---) means AGS incubated only with FITC-labelled *H. pylori* (100% adhesion).

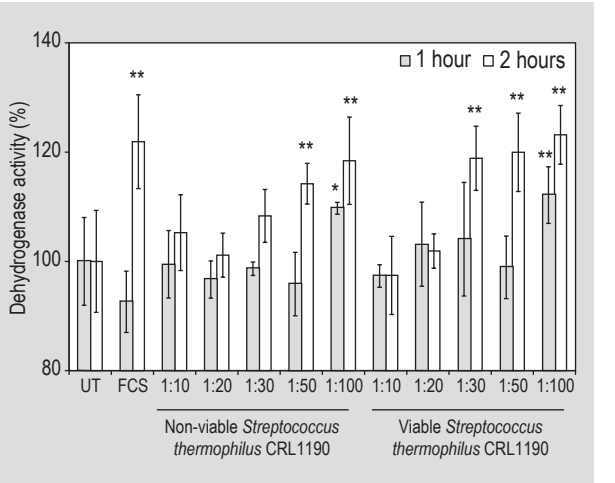

Figure 5. Influence of viable and non-viable *Streptococcus thermophilus* CRL1190 at different MOIs on AGS cellular activity after 1 or 2 h incubation. 100% of dehydrogenase activity correspond to basal activity of AGS cells. FCS (10%) was used as a positive control. Bars represent standard deviation (SD) with *$P<0.05$, **$P<0.01$ compared to the untreated control (UT).

from these results it was not possible to determine whether or not the probiotic ST1190 was able to adhere to the gastric tissue.

The present study showed evidence of the strong adhesion of ST1190 to the AGS cell line and to human tissue sections (*in vitro* and *in situ* adhesion assays, respectively). The adhesion of the bacteria was not dependent on the presence of the EPS; however, it should not be discarded the role the biopolymer may play in bacterial survival under the stomach acid conditions. Our group (Marcial *et al.,* 2013) suggested that the adhesion ability of ST1190 to the gastric mucosa might be enhanced when the bacteria is included in dairy products, e.g. yoghurt or fermented milks, where the peptides and other metabolites formed during milk fermentation would interact with the EPS1190 forming a multidimensional mesh in which the bacteria are included. A similar network of milk proteins-polysaccharide was detected in different dairy food matrix, *e.g.* fermented milk, yogurt and Cheddar cheese (Ayala-Hernandez *et al.,* 2008; Hassan *et al.,* 2002). Bioadhesive polysaccharides containing polygalactan or polygalacturonid structures are the key players for biofilm formation. It is supposed that they initiate the formation of mucus-like polysaccharide layers on the cells and tissue surfaces followed by the inclusion of proteins and bacteria into this polysaccharide layer (Schmidgall and Hensel, 2002). On the other hand, *Streptococcus* spp. has another strategy to survive in acidic environment by synthesising urease. The production of ammonia neutralises acidity and protects the bacteria from the low pH (Arioli *et al.,* 2007; Chen *et al.,* 2000). Previous results of our group found that ST1190 is urease positive, which is another factor able to contribute to resistance against gastric condition.

Whether ST1190, through their stomach epithelium adhesive properties, was able to avoid colonisation of gastric pathogens such as *H. pylori* was also an interesting topic for investigation. The results of this work clearly demonstrated that the ST1190 strain is able to significantly reduced *H. pylori* adhesion to AGS cells in a dose-dependent manner. Moreover, it was demonstrated here that ST1190 is able to modulate the production of the pro-inflammatory cytokine IL-8 in gastric epithelial cells in response to *H. pylori* challenge.

Inflammatory disorders have been well recognised as the key risk factors for many types of cancers. *H. pylori* infection and the resultant chronic inflammation in the gastric mucosa is a major step in the initiation and development of gastric cancer (Wang *et al.,* 2014). *H. pylori* induces an inflammatory response both in the gastric epithelial cells and the circulating immune cells recruited to the site of infection through multiple pathways. It has been reported that *H. pylori* infection can up-regulate several pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and RANTES (Lamb *et al.,* 2013). These cytokines are considered important mediators of gastric pathophysiology and may play critical roles in the development of gastric inflammation and gastric cancer. Moreover, activation of NF-κB and up-regulation of IL-8 in gastric epithelial cells were suggested as the critical mechanisms responsible for *H. pylori*-induced chronic inflammation and gastric carcinogenesis (Brandt *et al.,* 2005). Then, strategies to

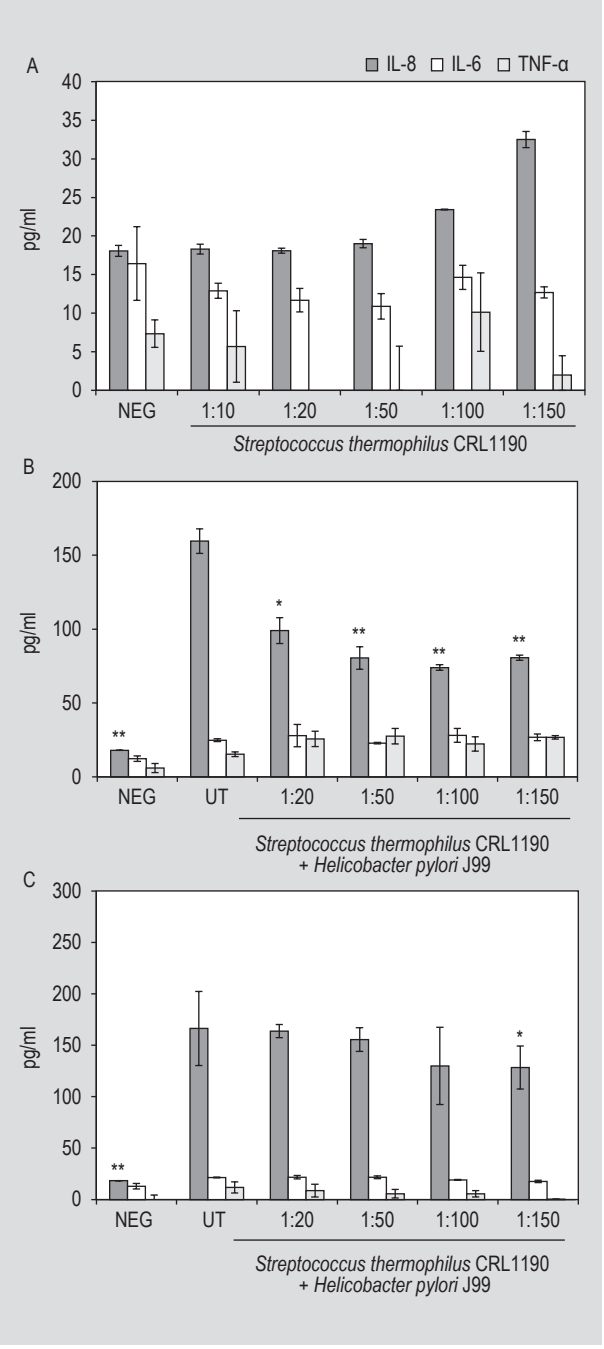

Figure 6. Secretion of cytokines by AGS cells. (A) Secretion of interleukin (IL)-8, IL-6 and tumour necrosis factor (TNF)-α by AGS cells under normal condition. (B) Secretion of IL-8, IL-6 and TNF-α by AGS cells infected with *Helicobacter pylori* in absence or presence of *Streptococcus thermophilus* CRL1190 at different MOIs. (C) Secretion of IL-8, IL-6 and TNF-α by AGS cells previously incubated with *H. pylori* and then treated with *S. thermophilus* CRL1190. Bars represent standard deviation with * *P*<0.05, ** *P*<0.01 compared to the untreated control (UT). NEG = negative control.

reduce *H. pylori*-induced chronic inflammation have been focus in the modulation of IL-8.

Some studies have documented the ability of probiotics to beneficially modulate IL-8 production during *H. pylori* infection. In this regard, it was reported that co-incubation of *L. gasseri* OLL2716 with a MK45 cell line infected with *H. pylori* inhibited expression of IL-8 when compared with the control (Ushiyama *et al.,* 2003). Further investigation of the ability of *L. gasseri* OLL2716 to inhibit IL-8 yielded similar results, but indicated that the probiotic strain did not inhibit adhesion of *H. pylori* to infected cells (Tamura *et al.,* 2006). Similarly, by using the SS1 mouse model of *H. pylori* infection, it was showed (Sgouras *et al.,* 2005) that the probiotic strain *Lactobacillus johnsonii* La1 although did not exert an antimicrobial activity on *H. pylori in vivo*, but distinct attenuation in the neutrophilic polymorphonuclear inflammatory infiltration of the lamina propria of the lactobacillus-administered animals was evident. Consistent with those *in vivo* observations, authors demonstrated that *L. johnsonii* La1 administration reduced gastric mucosal levels of macrophage-inflammatory protein-2 and keratinocyte chemoattractant, and also observed a significant decrease in IL-8 levels secreted by human gastric epithelial cells infected with *H. pylori in vitro*. These studies suggest that probiotic lactobacilli may exert an anti-inflammatory activity without necessarily affecting *H. pylori* colonisation, indicating that immunomodulatory activity is mediated by a mechanism independent of inhibition of the adhesion of the pathogen. Therefore, the results of this work indicate that ST1190 have two independent beneficial properties that could be used to protect the host against *H. pylori*-mediated diseases. To evaluate *in vivo* the anti-*H. pylori* effect of ST1190 and/or its fermented milk is an interesting topic for near future investigations.

In conclusion, this work demonstrated that ST1190 is able to attach directly to the gastric cells and stomach tissue. To our knowledge, this is the first evidence of adhesion of probiotic *S. thermophilus* to the stomach mucosa. By this attachment ST1190 could be partially responsible of the beneficial effects reported for this strain including the immune-regulatory effect in chronic gastritis (Rodriguez *et al.,* 2009, 2010) and the strong stimulatory activity on epithelial cell regeneration and immunological innate defence mechanisms (Marcial *et al.,* 2013). Moreover, we demonstrated here that ST1190 could confer protection against *H. pylori*-mediated diseases through two independent mechanisms: inhibition of adhesion and beneficial modulation of the inflammatory response.

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Conflict of interest

The authors state that there are no conflicts of interest including any financial, personal or other relationships with other people or organisations within three years of beginning of the submitted work that could inappropriately influence, or be perceived to influence, their work.

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