In vitro evaluation of the probiotic potential of Lactobacillus salivarius SMXD51

Soumaya Messaoudi, Amar Madi, Herve Prévost, Marc Feuilloley, Mohamed Manai, Xavier Dousset, Nathalie Connil

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In vitro evaluation of the probiotic potential of *Lactobacillus salivarius* SMXD51

S. Messaoudi [a], A. Madi [b], H. Prévost [a], M. Feuilloley [b], M. Manai [c], X. Dousset [a], N. Connil [b,*]

[a] *LUNAM University, Oniris, UMR1014 Secalim, Nantes, France*
[b] *Laboratory of Microbiology Signals and Microenvironment EA4312, University of Rouen, 55 rue Saint Germain, Evreux, France*
[c] *Laboratory of Biochemistry and Molecular Biology, Faculty of Sciences of Tunis, Tunisia*

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ABSTRACT

Lactobacillus salivarius SMXD51 was previously isolated from the cecum of a Tunisian poultry and found to produce a bacteriocin-like substance highly active against the foodborne pathogen *Campylobacter jejuni*. The aim of this study was to examine some probiotic properties of the strain: acid and bile tolerance, capacity of adhesion, stimulation of immune defences (IL-6, IL-8, IL-10 and β-defensin 2), and modulation of the barrier integrity. The results showed that *L. salivarius* SMXD51 can tolerate gastrointestinal conditions (acid and bile), adhere to intestinal cells and stimulate the immune system. The bacterium strengthened the intestinal barrier functions through the increase of the transepithelial electrical resistance (TEER) and reinforcement of the F-actin cytoskeleton. One hour pretreatment with *L. salivarius* SMXD51 protected against *Pseudomonas aeruginosa* PAO1-induced decrease of TEER and damage of the F-actin cytoskeleton. Our results highlight that *L. salivarius* SMXD51 fulfils the principle requirements of an efficient probiotic and may be seen as a reliable candidate for further validation studies in chicken.

1. Introduction

Antibiotics have been extensively used in animal feed to improve production in poultry and piglet industries [1]. However, the use of these substances as growth promoters can lead to the development of antibiotic resistances. Such resistances can occur not only in pathogenic bacteria [2,3] which can be transferred from poultry products to human population [4], but also in commensal bacteria [5], constituting a reservoir of resistance genes for pathogenic bacteria [6]. Due to their potential to reduce enteric disease in poultry, probiotics are considered to be a good alternative to the use of antibiotics [7].

It is well-known that probiotics have a number of beneficial health effects in humans and animals. They play an important role in the protection of the host against harmful microorganisms and also strengthen the immune system [8]. Some probiotics have also been found to improve feed digestibility and reduce metabolic disorders [9]. They must be safe, acid and bile tolerant, able to adhere and colonize the intestinal tract [8]. Human enterocyte-like Caco-2 cells have often been used for *in vitro* studies on the mechanisms of cellular adhesion of nonpathogenic *Lactobacilli* [10,11]. Some probiotic strains were shown to prevent attachment and invasion of various bacterial pathogens. *Lactobacillus* and *Bifidobacterium* were found to counteract *Listeria* infection of cultured epithelial cells through both secretion of antagonist molecules and modulation of the epithelial cell's immune response [12]. Compounds secreted by *Lactobacillus* were also shown to decrease *in vivo* the intestinal colonization by pathogenic strains of *Escherichia coli* [13]. Another and synergistic mechanism by which probiotics exert their beneficial effects is the enhancement of the intestinal barrier function. It has been found that treatment with probiotic bacteria may prevent or reverse increased permeability of the epithelium induced by pathogens [14]. These results were essentially obtained by measurement of the transepithelial electrical resistance (TEER) which appears as a reliable method to evaluate *in vitro* the epithelial permeability and compare the probiotic activity of bacterial species [15].

The genus *Lactobacillus* is commonly found in abundance in the upper gastrointestinal tract of humans and animals. Among this genus, within the recent years, *Lactobacillus salivarius* has gained attention as a promising probiotic species [16,17] that influences cytokine profiles and modulates cellular responses to pathogenic challenge. In a recent study, we isolated *L. salivarius* SMXD51 from

* Corresponding author. Tel.: +33 2 32 29 16 42; fax: +33 2 32 29 15 50.
E-mail addresses: soumaya.messaoudi@oniris-nantes.fr (S. Messaoudi), amar.madi@univ-rouen.fr (A. Madi), herve.prevost@oniris-nantes.fr (H. Prévost), marc.feuilloley@univ-rouen.fr (M. Feuilloley), mohamed.manai@fst.rnu.tn (M. Manai), xavier.dousset@oniris-nantes.fr (X. Dousset), nathalie.connil@univ-rouen.fr (N. Connil).

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the cecum of a Tunisian poultry [18]. This strain was found to produce a bacteriocin-like substance active against *Campylobacter jejuni*, a bacterium known as a foodborn pathogen in humans and a common commensal of poultry [19]. The bacteriocin, salivaricin SMXD51 was purified and characterized [20]. In addition to *Campylobacter*, salivaricin SMXD51 showed inhibition against a number of foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella*. According to these results, *L. salivarius* SMXD51 may be a promising bioprotective strain for the control of pathogenic bacteria in meat products so in order to get more information about this interesting strain, we recently sequenced its genome [21].

In the present work, we aimed to investigate the *in vitro* probiotic potential of *L. salivarius* SMXD51 to evaluate if this strain could be considered as good alternative to antibiotics use in poultry production.

2. Materials and methods

2.1. Bacterial strains

L. salivarius SMXD51, isolated from the cecum of a Tunisian poultry [18] was cultivated at 37 °C in De Man-Rogosa-Sharpe (MRS) medium [22] for 18–24 h. *Pseudomonas aeruginosa* PAO1, a laboratory reference strain, was cultivated at 37 °C in ordinary nutrient broth (Merck, France).

2.2. Acid and bile tolerance

Acid and bile tolerance were studied according to the protocol used by Anderson et al. (2010) [23]. Briefly, *L. salivarius* SMXD51 was grown overnight at 37 °C in MRS broth and suspended to an approximate cell concentration of 10^8 CFU/ml in MRS broth adjusted to pH 3 for 2 h and in 0.5% w/v bile (Sigma–Aldrich, France) for 4 h. These conditions were chosen to represent the time it takes to pass through the gastrointestinal system and the pH value and bile concentration respectively found in the chicken stomach and intestine. Bacterial viability was assessed by enumeration on MRS agar plates.

2.3. Cell line and culture

The Caco-2/TC7 cells were used between passages 35 and 50. Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, France) supplemented with 15% heat-inactivated foetal calf serum, 2 mM of L-glutamine, 100 U/ml each of penicillin and streptomycin and 1% non-essential amino acids. For experimental assays, the cells were seeded at a density of approximately 10^5 cells/cm^2 in 24-well tissue culture plates (for adhesion, cytotoxicity assay, cytokines and β-defensin 2 quantification) or on inserts (6.4 mm diameter, 3 μm pore size, Falcon) which allow epithelial differentiation (for TEER assay and actin visualisation). The cells were cultured at 37 °C in 5% CO_2-95% air atmosphere and the medium was regularly changed. Caco-2/TC7 grown in 24-well tissue culture plates were incubated to early confluence (undifferentiated state) whereas Caco-2/TC7 cells grown on inserts were used at 21 days post-confluence (fully differentiated state).

2.4. Adhesion capacity

L. salivarius SMXD51 was harvested by centrifugation, resuspended in cell culture medium without serum and antibiotics at a concentration of 10^8 CFU/ml, and then applied on confluent Caco-2/TC7 monolayers. After 4 h of incubation at 37 °C, 5% CO_2, monolayers were washed with PBS to remove non adherent bacteria, and lysed by incubation for 15 min with 0.1% Triton X100. The lysates were then diluted and plated onto MRS agar to determine the number of adherent bacteria.

2.5. Cytotoxicity assay

After 24 h of incubation with *L. salivarius* SMXD51 (10^8 CFU/ml), the supernatants from Caco-2/TC7 monolayers grown on 24-well tissue culture plates were collected. The lactate dehydrogenase (LDH) present in the supernatants was measured using the Cytotox 96R enzymatic assay (Promega, France). LDH is a stable cytosolic enzyme of eukaryotic cells, indicator of necrotic cell death when released. Caco-2/TC7 cells exposed to Triton ×100 (0.9%) were used as a control of total release (100% LDH release) as recommended by the manufacturer's instructions. The background level (0% LDH release) was determined with serum free culture medium. To complete the cytotoxicity assay, the integrity of Caco-2/TC7 monolayers was also estimated by observation with a photonic microscope (×400).

2.6. Quantification of IL-6, IL-8, IL-10 and β-defensin 2

After 24 h of treatment with 10^8 CFU/ml of *L. salivarius* SMXD51, the levels of cytokines (IL-6, IL-8 and IL-10) produced by Caco-2/TC7 cells were measured in the culture supernatant using ELISA Quantikine kits (R&D Systems, France). β-defensin 2 was quantified using the Defensin 2, beta (Human) – ELISA Kit (Phoenix, France).

2.7. Transepithelial electrical resistance measurements

The effect of *L. salivarius* SMXD51 on the transepithelial electrical resistance (TEER) of differentiated intestinal culture was studied during 24 h, using the Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). *L. salivarius* SMXD51 was incubated alone at 10^8 CFU/ml on Caco-2/TC7 cell monolayers to determine the effect of this bacterium on the epithelial barrier integrity. The differentiated cells were also exposed to *P. aeruginosa* PAO1, a well-known pathogen able to damage the barrier integrity, or pre-incubated with *L. salivarius* SMXD51 for 1 h before infection with the pathogen. For each conditions tested, TEER values were expressed as percentages of the initial level measured in the insert.

2.8. Actin visualisation

Fully differentiated Caco-2/TC7 monolayers were exposed either to *L. salivarius* SMXD51 or *P. aeruginosa* PAO1 for 24 h or pre-incubated for 1 h with *L. salivarius* SMXD51 before infection with the pathogen. At the end of the experiment, the cells were washed with phosphate-buffered saline (PBS), fixed for 10 min in 3.7% paraformaldehyde and permeabilized for 5 min with 0.1% Triton ×100 at room temperature. The cells were then incubated with 1% bovine serum albumin in PBS for 10 min and the apical F-actin cytoskeleton was stained by incubation with Alexa-488 phalloïdin (Invitrogen, France) for 45 min at room temperature (1U/insert). Following three washes in PBS, cell monolayers were excised from the filter supports, mounted on slides using Fluoromount Plus medium (Clinisciences, France) and examined using a confocal laser scanning microscope (Zeiss, LSM710).

2.9. Statistical analysis

Data are expressed as a mean ± standard error (SE) calculated over three independent experiments performed in triplicate.

Table 1
Evaluation of the probiotic properties of *Lactobacillus salivarius* SMXD51.

Criteria tested	Results
Resistance to acid	Yes. 99% survival after 2 h at pH 3
Resistance to bile	Yes. 99% survival after 4 h at 0.5% w/v bile
Adherence to intestinal cells	Yes. 10^5 UFC/ml (1% of initial bacteria)
Safety/cytotoxicity	No cytotoxicity towards Caco-2/TC7 (see Fig. 1A) No visible damage on the monolayer (see Fig. 1B)
Modulation of immunity	No-induction of IL-6 (see Fig. 2A) Induction of IL-8, 1.8-fold increase (see Fig. 2B) No-induction of IL-10 (see Fig. 2C) Induction of β-defensin 2, 2.6-fold increase (see Fig. 2D)
Barrier integrity	Enhancement in TEER, 1.2 fold increase (see Fig. 3) Protection of F-actin (see Fig. 4)
Antimicrobial activity against pathogenic bacteria	Yes, previous study (Messaoudi et al., 2012) [16]

Analysis of statistical significance was performed by ANOVA with Student's *t*-test.

3. Results and discussion

L. salivarius SMXD51 was evaluated in regard of the typical probiotic properties proposed by Gupta and Garg (2009) [24] and Neville and O'Toole (2010) [17]. Results are summarized in Table 1.

3.1. Acid and bile tolerance

Resistance to acid and bile is generally considered an essential assessment criterion for probiotic evaluation since the strains have to survive the conditions in the stomach and the small intestine [17]. Thus, the survival of *L. salivarius* SMXD51 was tested for 2 h at pH 3 and 4 h in 0.5% w/v bile. The results show that this strain was able to tolerate acidic conditions and the presence of 0.5% w/v bile without any significant loss of viability (99% survival). In these physiological conditions, the behaviour of *L. salivarius* SMXD51 was similar to the results previously obtained by Kirtzalidou et al. [25] for *L. salivarius* C3 isolated from the infant gut microbiota, and by Yun et al. (2009) [26] for *L. salivarius* LB64 from pig feces.

Sequencing of *L. salivarius* SMXD51 by Kergourlay et al. (2012) [21] revealed that several genes known to be important for acid tolerance and bile salt resistance were found in the genome of the bacteria, among them an ornithine decarboxylation-antiporter system and a bile salt hydrolase family protein choloylglycine hydrolase. The presence of these genes may partially explain the ability of *L. salivarius* SMXD51 to resist to the gastrointestinal conditions tested.

3.2. Adhesion capacity

Adherence to intestinal epithelial cells is another reliable criterion for the selection of probiotic. *In vitro* test results using Caco-2 cell for the estimation of adhesion capacity to intestinal epithelium cells have been reported to have a good correlation to *in vivo* results [27] and this characteristic is often strain-specific. Adhesion capacity of *L. salivarius* SMXD51 (10^8 CFU/ml) was examined by incubating the bacteria with confluent Caco-2/TC7 monolayers for 4 h. At the end of incubation period, non adherent bacteria were removed by rinsing and the number of adherent germs was determined by plating. In these conditions 10^5 CFU/ml adherent *L. salivarius* SMXD51, corresponding to 1% of the initial population, were recovered in the Caco-2/TC7 monolayers lysates. This strain demonstrates a capacity of adhesion at least identical or higher than *L. salivarius* C3 previously tested by Kirtzalidou et al. (2011) [25]. We found that *L. salivarius* SMXD51 have high hydrophobic affinity towards *n*-hexadecane (data not shown). This characteristic may promote hydrophobic interactions which are generally considered to play an important role in the adherence of microorganisms to eukaryotic cells [28]. Moreover, analysis of the sequence of *L. salivarius* SMXD51 by Kergourlay et al. (2012) [21] showed recently the presence of two genes encoding cell wall-anchored adhesin that may be involved in adherence to eukaryotic cells, a fibronectin binding protein (FbpA) and a potential mucus adhesion-promoting protein (MapA). The *lsp*A gene from *L. salivarius* UCC118 reported to mediate adhesion was also found with 92.6% identity.

3.3. Cytotoxicity assay

The safety of lactobacilli and bifidobacteria is well documented. In general, the pathogenic potential of these normal colonizers of the human body is considered quite low [29]. Only very rare cases of human infection (commonly endocarditis) have been reported [30,31]. However, further investigation is warranted for probiotic use in at-risk human populations such as severely immunocompromised subjects, neonates or hospitalized patients. For such sensitive populations, the use of appropriate *in vitro* assays to assess the safety of probiotics should not be precluded. *L. salivarius*

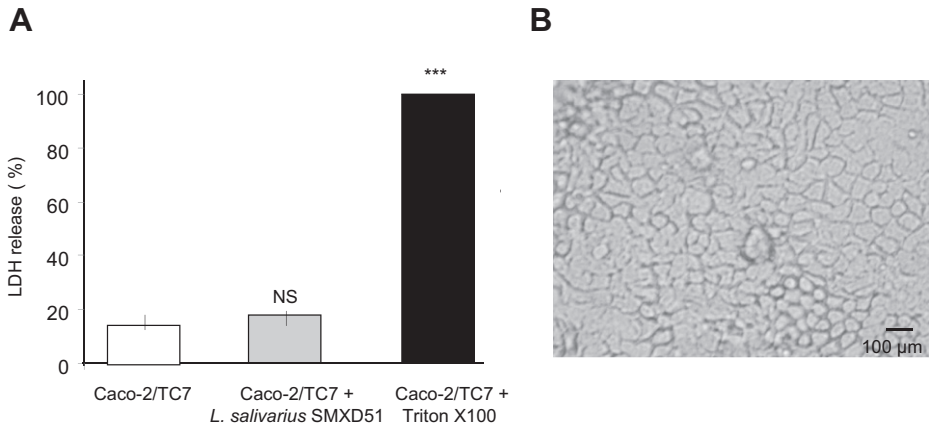

Fig. 1. Cytotoxicity assay. (A) LDH release. Mean ± SE. NS (not significant) and $^{***}P < 0.001$ compared to Caco-2/TC7 control. (B) Observation of Caco-2/TC7 cells treated with 10^8 CFU/ml of *L. salivarius* SMXD51 with a photonic microscope.

SMXD51 (10^8 CFU/ml) were applied on Caco-2/TC7 monolayers for 24 h and the cytotoxicity was evaluated by measurement of LDH release and microscopic observation. The results show that the amount of LDH release in the supernatant of Caco-2/TC7 cultured in the absence or presence of the bacteria was unchanged, indicating that *L. salivarius* SMXD51 didn't cause cell lysis (Fig. 1A). The absence of cytotoxicity of *L. salivarius* SMXD51 against Caco-2/TC7 cells was confirmed by microscopic studies. Even after 24 h of incubation with the bacteria, the integrity of the monolayer was fully preserved (Fig. 1B).

3.4. Quantification of IL-6, IL-8, IL-10 and β-defensin 2

Another criterion often screened for the selection of probiotics is their capacity to modulate the immune system [32,33]. The quantification of IL-6, IL-8, IL-10 and β-defensin 2 using ELISA assays showed that *L. salivarius* SMXD51 induces 1.8-fold increase in IL-8 secretion (Fig. 2B) and 2.6-fold increase in β-defensin 2 secretion (Fig. 2D) compared to untreated Caco-2/TC7 cells. On the contrary, for IL-6 (Fig. 2A) and IL-10 (Fig. 2C), no variation of the basal secretion of Caco-2/TC7 cells was observed after exposure to the bacteria. These results are consistent with those of Perez-Cano et al. [34] who showed that *Lactobacillus fermentum* CECT5716 and *L. salivarius* CECT5713 display *in vitro* immunomodulatory activity by induction of IL-8. However, in contrast with our results, *L. fermentum* CECT5716 and *L. salivarius* CECT5713 also promoted the production of the pro-inflammatory IL-6 and anti-inflammatory IL-10. These differences are not surprising since the immunomodulatory effects are often strain-specific [35,36]. We also observed that *L. salivarius* SMXD51 induced the secretion of β-defensin 2. It is known that inducible β-defensins play an important role in the intestinal barrier function and i*n vitro* studies have shown that clinically effective probiotics induce the production of antimicrobial β-defensin 2 [37–39]. Induction of β-defensin 2 by probiotics, including *L. salivarius* SMXD51, might be an alternative and a complementary new strategy to strengthen innate defence mechanisms.

3.5. Effect of L. salivarius SMXD51 on TEER and the F-actin cytoskeleton

Modulation of the barrier integrity was studied to provide further support for the probiotic properties of *L. salivarius* SMXD51.

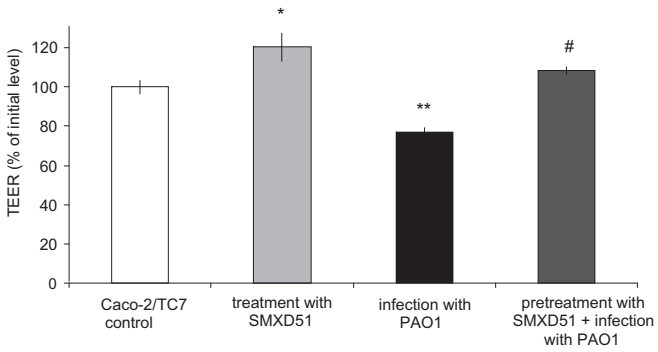

Fig. 3. TEER of Caco-2/TC7 cells exposed to *L. salivarius* SMXD51, *P. aeruginosa* PAO1 or a combination of the two strains. Data are expressed as percentages of the initial level measured in the insert and as a mean ± SE. $^*P < 0.05$ and $^{**}P < 0.01$ compared to Caco-2/TC7 control. $\#P < 0.01$ compared to infection with PAO1.

Caco-2/TC7 cells were treated with *L. salivarius* SMXD51 during 24 h. The results showed that the bacteria induce a 20 ± 7% increase of the TEER of the monolayer (Table 1, and Fig. 3). Confocal microscopy observations confirmed that the network of the F-actin cytoskeleton was more dense after 24 h incubation with *L. salivarius* SMXD51 (Fig. 4B) compared to an untreated control (Fig. 4A). The use of transepithelial electrical resistance of intestinal epithelial cell monolayers as a mean to evaluate probiotic activity was first proposed by Klingberg et al. [15]. They investigated the probiotic potential of five lactobacilli strains and found that *Lactobacillus plantarum* MF1298 and *L. salivarius* DC5 showed the highest increase in TEER in a dose dependent manner. The maximal increase of TEER obtained was about 40% after 24 h of incubation with the probiotics.

3.6. Protection of the barrier integrity by L. salivarius SMXD51

We evaluated if *L. salivarius* SMXD51 can prevent the loss of barrier integrity provoked by a pathogen, *i.e. P. aeruginosa* PAO1. This strain was chosen as model for alteration of the intestinal epithelium because it has been previously shown to have a strong cytotoxic activity towards Caco-2/TC7 cells and be able to disrupt the F-actin cytoskeleton [40]. Our results revealed that when Caco-2/TC7 cells were infected with *P. aeruginosa* PAO1 (10^6 CFU/ml), the TEER was decreased by a mean of 25 ± 3% (Fig. 3) and the F-actin cytoskeleton

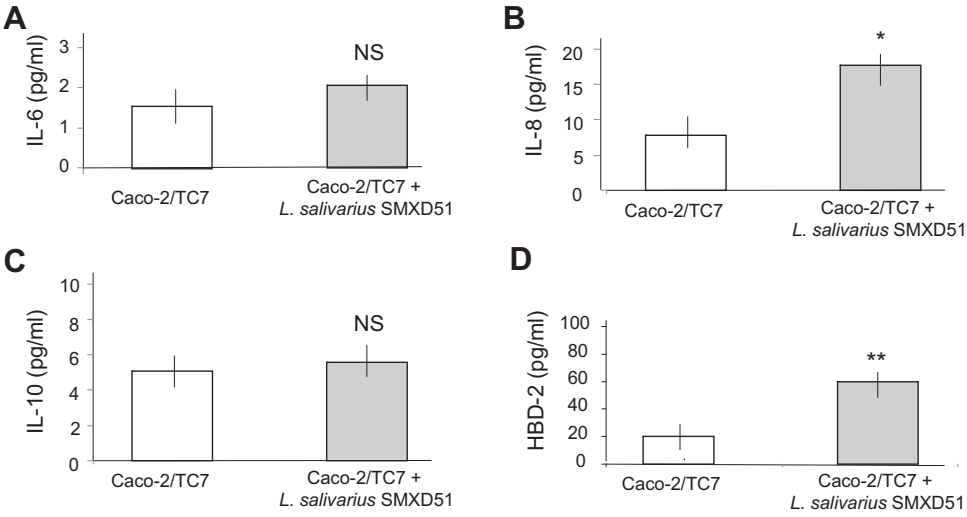

Fig. 2. Quantification of cytokines and β-defensin 2 (HBD-2) in the supernatants of Caco-2/TC7 cells after exposition to *L. salivarius* SMXD51. (A) IL-6, (B) IL-8, (C) IL-10 and (D) HBD-2. Mean ± SE. NS (not significant), $^*P < 0.05$ and $^{**}P < 0.01$ compared to Caco-2/TC7 control.

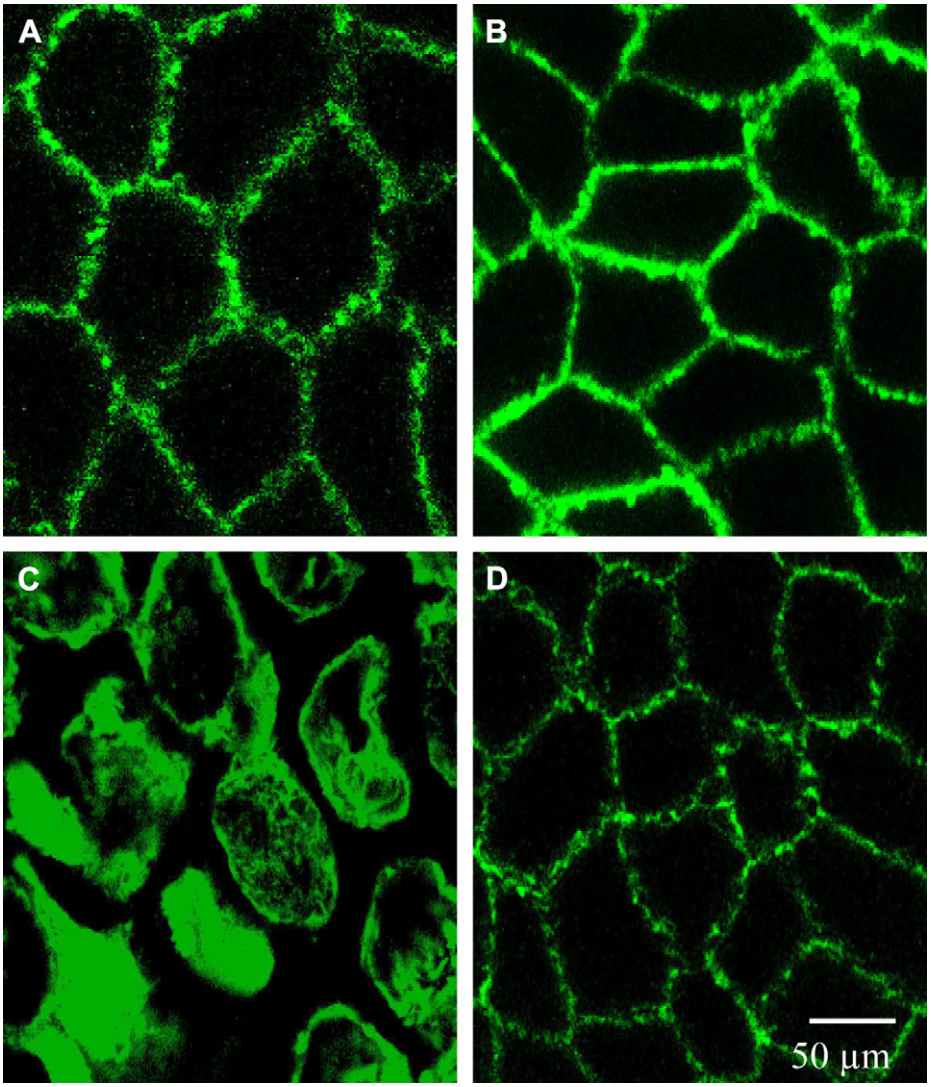

Fig. 4. Staining of F-actin cytoskeleton with Alexa-488 phalloïdin of Caco-2/TC7 cells without treatment (A), treated with 10^8 CFU/ml of *L. salivarius* SMXD51 (B), infected with 10^6 CFU/ml of *P. aeruginosa* PAO1 (C), or pretreated with SMXD51 and then infected with PAO1 (D).

was seriously damaged (Fig. 4C). Preincubation of Caco-2/TC7 cells with *L. salivarius* SMXD51 (10^8 CFU/ml) prevented the deleterious *P. aeruginosa* PAO1-induced TEER changes (Fig. 3) and the cytoskeleton disruption (Fig. 4D). However, this effect was only observed when *L. salivarius* SMXD51 is added 1 h before the infection with the pathogen. When the two bacteria were added simultaneously, *L. salivarius* SMXD51 failed to prevent the loss of barrier integrity (data not shown). It could be suggested that when *L. salivarius* SMXD51 is used in prevention to the infection, the bacteria may form a physical protection barrier on the monolayer that refrains the access of the pathogen to the eukaryotic receptor by occupying the target sites. Moreover, during the pretreatment, *L. salivarius* SMXD51 may have time to secrete some molecules that will have antagonistic activities against *P. aeruginosa* PAO1. Similar mechanisms for other probiotics have been described previously. Liu et al. [41] found that *L. plantarum* prevented the decrease in the expression of tight junction proteins and F-actin in NCM460 cells infected with enteroinvasive *E. coli* (EIEC) or enteropathogenic *E. coli* (EPEC). Another study found that *Lactobacillus rhamnosus* GG protected epithelial monolayers against enterohaemorrhagic *E. coli* (EHEC)-induced redistribution of the claudin-1 and ZO-1 tight junction proteins. *Lactobacillus acidophilus* protected against F-actin rearrangement induced in an epithelial cell line upon exposure to a pathogenic *E. coli* [42]. *Streptococcus thermophilus* and *L. acidophilus* maintained (actin, ZO-1) or enhanced (actinin, occludin) cytoskeletal and tight junctional protein structures in epithelial cell lines [14]. Similarly, *E. coli* Nissle 1917 can counteract the disruptive effects of EPEC on T-84 epithelial cells tight junctions [43].

4. Conclusion

This study shows that *L. salivarius* SMXD51 fulfils to all the principle requirements and properties of an efficient probiotic (tolerance to gastrointestinal conditions, adhesion to intestinal cells, stimulation of immunity, and reinforcement of epithelial barrier). These findings demonstrated that *L. salivarius* SMXD51 could be a reliable candidate for future use as probiotic in chicken feed supplement. Further investigations will be conducted in animal experiments to assess the *in vivo* efficacy of the strain.

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