S-layer protein 2 of Lactobacillus crispatus 2029, its structural and immunomodulatory characteristics and roles in protective potential of the whole bacteria against foodborne pathogens

Vyacheslav M. Abramov, Igor V. Kosarev, Tatiana V. Priputnevich, Andrey V. Machulin, Valentin S. Khlebnikov, Sergey Yu. Pchelintsev, Raisa N. Vasilenko, Vadim K. Sakulin, Natalia E. Suzina, Irina O. Chikileva, Evgenia I. Derysheva, Vyacheslav G. Melnikov, Ilya N. Nikonov, Vladimir A. Samoilenko, Eduard E. Svetoch, Gennady T. Sukhikh, Vladimir N. Uversky, Andrey V. Karlyshev

PII: S0141-8130(20)30712-1

DOI: https://doi.org/10.1016/j.ijbiomac.2020.02.065

Reference: BIOMAC 14682

To appear in: *International Journal of Biological Macromolecules*

Received date: 21 January 2020

Revised date: 7 February 2020

Accepted date: 7 February 2020

Please cite this article as: V.M. Abramov, I.V. Kosarev, T.V. Priputnevich, et al., S-layer protein 2 of Lactobacillus crispatus 2029, its structural and immunomodulatory characteristics and roles in protective potential of the whole bacteria against foodborne pathogens, *International Journal of Biological Macromolecules*(2020), https://doi.org/10.1016/j.ijbiomac.2020.02.065

S-layer protein 2 of *Lactobacillus crispatus* 2029, its structural and immunomodulatory characteristics and roles in protective potential of the whole bacteria against foodborne pathogens

Vyacheslav M. Abramov,[a] Igor V. Kosarev,[a] Tatiana V. Priputnevich,[b] Andrey V. Machulin,[c] Valentin S. Khlebnikov,[a] Sergey Yu. Pchelintsev,[a] Raisa N. Vasilenko,[a] Vadim K. Sakulin,[a] Natalia E. Suzina,[c] Irina O.Chikileva,[a,d] Evgenia I. Derysheva,[e] Vyacheslav G. Melnikov,[f] Ilya N. Nikonov,[g] Vladimir A. Samoilenko,[c] Eduard E. Svetoch,[h] Gennady T. Sukhikh,[b] Vladimir N. Uversky,[e,i,*] and Andrey V. Karlyshev[j]

[a]*Institute of Immunological Engineering, 142380 Lyubuchany, Moscow Region, Russia;*

[b]*Kulakov National Medical Research Center for Obstetrics, Gynecology and Perinatology of the Ministry of Health, 117997 Moscow, Russia;*

[c]*Scryabin Institute of Biochemistry and Physiology of Microorganisms, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Science", 142290 Pushchino, Moscow Region, Russia;*

[d]*Laboratory of cell immunity, Blokhin National Research, Center of Oncology Ministry of Health RF, 115478, Moscow, Russia;*

[e]*Institute for Biological Instrumentation, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Science, 142290, Pushchino, Moscow Region, Russia;*

[f]*Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology, Federal Service for Supervision of Consumer Rights Protection and Human Welfare, 152212 Moscow. Russia;*

[g]*Federal Research Center "All-Russian Research and Technological Institute of Poultry" of the Russian Academy of Science, 141311, Sergiev Posad, Moscow Region, Russia*

[h]*State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow Region, Russia;*

[i]*Department of Molecular Medicine, Morsani College of Medicine. University of South Florida, Tampa, FL 33612, USA;*

[j]*Department of Science, Engineering and Computing, Kingston University London, Kingston upon Thames, KT1 2EE, UK*

*** Corresponding author**: VNU, E-mail: vuversky@usf.edu;

Abstract

We have previously demonstrated that human vaginal *Lactobacillus crispatus* 2029 (LC2029) strain is highly adhesive to cervicovaginal epithelial cells, exhibits antagonistic activity against genitourinary pathogens and expresses surface-layer protein (Slp). The aims of the present study were elucidation of Slp structural and immunomodulatory characteristics and its roles in protective properties of the whole vaginal LC2029 bacteria against foodborne pathogens. Enteric Caco-2 and colon HT-29 cell lines were used as the *in vitro* models of the human intestinal epithelial layer. LC2029 strain has two homologous surface-layer (S-layer) genes, *slp1* and *slp2*. Whilst we found no evidence for the expression of *slp1* under the growth conditions used, a very high level of expression of the *slp2* gene was detected. C-terminal part of the amino sequence of Slp2 protein was found to be highly similar to that of the conserved C-terminal region of SlpA protein of *L. crispatus* Zj001 isolated from pig intestines and CbsA protein of *L. crispatus* JCM5810 isolated from chicken intestines, and was substantially variable at the N-terminal and middle regions. The amino acid sequence identity between SlpA and CbsA was as high as 84%, whilst the identity levels of these sequences with that of Slp2 were only 49% and 50% (respectively). LC2029 strain was found to be both acid and bile tolerant. Survival in simulated gastric and intestinal juices of LC2029 cells unable to produce Slp2 was reduced by 2-3 logs. Vaginal *L. crispatus* 1385 (LC1385) strain not expressing Slp was also very sensitive to gastric and intestinal stresses. Slp2 was found to be non-covalently bound to the surface of the bacterium, acting as an adhesin and facilitating interaction of LC2029 lactobacilli with the host immature or fully differentiated Caco-2 cells, as well as HT-29 cells. No toxicity to or damage of Caco-2 or HT-29 epithelial cells were detected after 24 h of colonization by LC2029 lactobacilli. Both Slp2 protein and LC2029 cells induced NF-kB activation in Caco-2 and HT-29 cells, but did not induce expression of innate immunity mediators Il-8, Il-1β, and TNF-α. Slp2 and LC2029 inhibited Il-8 production in Caco-2 and HT-29 cells induced by MALP-2 and increased production of anti-inflammatory cytokine Il-6. Slp2 inhibited production of CXCL1 and RANTES by Caco-2 cells during differentiation and maturation process within 15 days. Culturing Caco-2 and HT-29 cells in the presence of Slp2 increased adhesion of bifidobacteria BLI-2780 to these enterocytes. Upon binding to Caco-2 and HT-29 cells, Slp2 protein and LC2029 lactobacilli were recognized by toll-like receptors (TLR) 2/6. It was shown that LC2029 strain is a strong co-aggregator of foodborne pathogens *Campylobacter jejuni*, *Salmonella enteritidis*, and *Escherichia coli* O157:H used in this study. The Slp2 was responsible for the

ability of LC2029 to co-aggregate these enteropathogens. Slp2 and intact LC2029 lactobacilli inhibited foodborne pathogen-induced activation of caspase-9 and caspase-3 as apoptotic biomarkers in Caco-2 and HT-29 cells. In addition, Slp2 and Slp2-positive LC2029 strain reduced adhesion of tested pathogenic bacteria to Caco-2 and HT-29 cells. Slp2-positive LC2029 strain but not Slp2 alone provided bactericidal effect on foodborne pathogens. These results suggest a range of mechanisms involved in inhibition of growth, viability, and cell-adhesion properties of pathogenic Proteobacteria by the Slp2 producing LC2029, which may be useful in treatment of necrotizing enterocolitis (NEC) in newborns and foodborne infectious diseases in children and adults, increasing the colonization resistance and maintaining the intestinal homeostasis.

Key words: S- layer proteins, *Lactobasilus crispatus,* immunomodulation, antagonistic activity, *Salmonella* Enteritidis, *Campylobacter jejuni, Escherichia coli*

Introduction

Surface-layer proteins (Slps) form regular two-dimensional cell envelope structures found on archaea and many bacteria [1]. Bacterial surface layers are composed of single protein subunits and have significant strength, flexibility, and semi-permeability, resembling chain mail [2]. In archaea, they are involved in the maintenance of the cell shape and cell division [3]. In pathogenic bacteria, Slps, being the hot spots of evolution, where changes occur very quickly, provide effective adhesion to the host cells and are required for virulence [4,5]. Several but not all species of the genus *Lactobacillus* possess the S surface-layer (S-layer) [6]. Some lactobacilli are associated with the host mucosal surface, including the gastrointestinal tract (GIT) and urogenital tract [7]. Slps in these bacteria perform protective functions providing resistance of host tissues to colonization by the pathogenic microorganisms [8]. Slp sequence variability and functions in lactobacilli are not only species but also strain specific [9]. This is one of the reasons for significant strain differences in biological properties of lactobacilli. Healthy vaginal microbiota is dominated by *Lactobacillus spp*., which form a critical line of defense against genitourinary pathogens [10–12]. The functions of vaginal lactobacilli are related to the maintenance of an environment that limits the growth of pathogens [13]. Different factors that increase the risk of bacterial vaginosis (BV), yeast vulvovaginal candidiasis (VVC), and urinary tract infections (UTIs) are associated with the decreased levels of vaginal lactobacilli [14]. These observations have

led to research on the strains and properties of vaginal lactobacilli, which may be responsible for the maintenance of a pathogen-free environment in the genitourinary tract. Healthy vaginal microbiota is typically dominated by *Lactobacillus* species such as *L. iners*, *L. crispatus*, *L. jensenii*, and *L. gasseri* [11,15]. *L. crispatus* appears to be substantially prevailing over the other hydrogen peroxide producing *Lactobacillus* species [16]. A type I microbiota with the dominance of the *L. crispatus* species is important for maintaining of healthy birth tract [11]. Vaginal colonization with probiotic human-derived *L. crispatus* Slp-positive strain CTV-05 has been successful [17]. Slp-positive *L. crispatus* LC2029 strain was isolated upon investigation of vaginal lactobacilli from healthy women of reproductive age and selected as probiotic candidate for prophylactics and treatment of genitourinary infections [18]. Our previous research showed that the Slp was involved in the homeostatic interactions with the cervicovaginal epithelial cells and participated in their effective colonization by vaginal LC2029 strain. S-layer was responsible for the high affinity binding of the LC2029 strain to type IV collagen as one of the main components of the extracellular matrix on cervical HeLa and vaginal VK2/E6E7 epithelial cells (K_d = (8.0±0.7)×10^{-10} M) [18]. Slp producing LC2029 strain provided wide spectrum of antagonistic activity increasing colonization resistance to genitourinary tract infections by BV and VVC associated pathogens [18]. In the same line, Verstraelen and colleagues reported that vaginal strains *L. crispatus* promote stability of the normal vaginal microbiota in pregnancy [19].

Early bacterial populations of neonates depend on environmental factors, such as mode of delivery, formula feeding vs. breast feeding, and other [20]. During the birth of a full-term newborn, a small amount of the mother's vaginal microbiota including *L. crispatus* is naturally ingested and enters the GIT of neonates [21]. *L. crispatus* strain M247, showing a cell aggregation phenotype, was isolated from a fecal sample taken from a weaning baby. The strain adhered to ileal mucus and Caco-2 cells *in vitro.* Three human trials with M247 were performed. M247 could be recovered from either fecal samples or biopsies taken from the colon [22]. However, very little is presently known about the role of vaginal *L. crispatus* strains, evolutionarily selected for dwelling in a vaginal microecological niche of healthy women, in the process of human intestinal homeostasis formation and protection of GIT against foodborne pathogens. The Slp producing *L. crispatus* strains isolated from chicken and pig intestines were found to have antagonistic activity against foodborne pathogens [23–25], but the effects of human vaginal *L. crispatus* strains and their Slps on these pathogens remain poorly understand. Foodborne *Campylobacter jejuni*, *Salmonella enteritidis*, and

Escherichia coli belonging to Proteobacteria are commonly involved in human infectious diseases [26]. It is known that in prematurely born children, the intestinal microbiota contains a high level of Proteobacteria responsible for necrotizing enterocolitis (NEC) [27]. *S. enteritidis* and *C. jejuni* carried by chickens, poultry and swine production, are the major sources of human intestinal infections. *E. coli* O157:H7 is also associated with human intestinal diseases [28,29]. In this work, we investigated the structural and immunomodulatory characteristics of Slp2 and its role in protective properties of whole LC2029 bacteria against foodborne pathogens.

Materials and methods

Bacterial strains and growth conditions

L. crispatus 2029 (LC2029) Slp-positive strain was originally isolated from a vaginal smear of a healthy woman of reproductive age. The strain was deposited at the All-Russian Collection of Microorganisms at the G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms under the registration number VKM B-2727D. *L. crispatus* (LC1385) S-layer negative strain was originally isolated from a vaginal smear of a woman of reproductive age with clinical diagnosis: recurrent VVC, had history of BV. These strains were grown in Man-Rogosa-Sharpe (MRS) broth or agar containing MRS plates (Himedia, India), 37°C in 5% CO_2 or anaerobically 48h. *Bifidobacterium longum subsp. infantis* (BLI-2780) strain was originally isolated from fecal sample of a healthy, full-term, breast-fed infant at 14 day of age. The strain was grown in MRS, 37°C anaerobically 48h. A complete list of microorganisms used in this study and their growth conditions is provided in **Table 1**.

Transmission electron microscopy of thin sections

Transmission electron microscopy (TEM) was performed as described in [18] with some modifications. Briefly, the LC2029 cells were prefixed with 1.5 % (v/v) glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h. After three washings with the same buffer, the cells were fixed with 1% OsO_4 in 0.05 M cacodylate buffer at 20°C for 3h. After dehydration, the cells were embedded into epoxy resin Epon 812. Ultrathin sections were made on an 8800 ULTROTOME III (LKB, Sweden). The sections were mounted on copper grids covered with a Formvar film, contrasted with uranyl acetate for 30 min and stained with lead citrate [30] at 20°C for 5 min. The sections were investigated using a JEM-100B (JEOL, Japan) electron microscope at an 80 kV accelerating voltage.

Preparation of Slp, SDS-PAGE and Western blotting analysis

Slp was extracted and purified according to [31]. Briefly, Slp was extracted from LC2029 strain by 5 M LiCl. The extract was purified by chromatography on cation-exchange column. The purified Slp was examined by SDS-PAGE containing 12% polyacrylamide gel. Western blotting analysis was performed essentially as described in [32]. A synthetic peptide (YQVSNGKAVSQMPDQKAVVADVNA) corresponding to a unique amino acid sequence 214-237 of Slp2 was synthesized. Additionally, C-terminal cysteine was included into the peptide for conjugation with keyhole limpet hemocyanin (KLH) through this additional residue. The peptide-carrier conjugate was purified by gel-filtration and used for the generation of mouse monoclonal anti-Slp2 antibodies that were used as "primary antibody" in Western blotting analysis. The Goat-Anti-Mouse-Poly-HRP (Pierce), 250 ng/ml in 5% milk were used as a secondary antibody. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) and blue X-ray film (Phenix Research Products, USA) were used for visualization. The LC2029 cells with a depleted Slp2 were tested for tolerance to gastric and intestinal stresses, the ability to adhere to immature Caco-2 and HT-29 cells, co-aggregative and antagonistic activity to foodborne pathogens.

Assays of acid and bile salt tolerance

Determination of *Lactobacillus* strains tolerance to acid and bile salt was performed as described in [33] with a modification. Briefly, bacterial cultures (stationary phase) were grown in MRS medium, pH 6.5 for 48 h at 37°C, 10% CO_2, harvested by centrifugation and washed twice with phosphate buffered saline (PBS), pH 7.4. The cell pellet were either resuspended in fresh MRS broth at pH 2.5 and maintained at 37°C for 10 min, 30 min and 60 min for acid tolerance assay or, alternatively, resuspended in MRS broth followed by inoculation into heat-sterilized fresh swine bile and incubation at 37°C for 5 h. Acid and bile were absent in control samples. The viable cells from the cultures were enumerated on MRS agar incubated at 37°C for 48h. Each experiment was carried out in triplicate.

Intestinal epithelial in vitro models

Immortalized epithelial Caco-2 cells showing marked characteristics of human small intestine enterocytes and HT-29 cells showing marked characteristics of human large intestine colonocytes, including the ability to differentiate, were maintained in a culture

medium composed of Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acid, 1% glutamine. 25mM HEPES buffer. Cells were cultured at 37°C in a 5% CO_2 in humidified atmosphere. Caco-2 cells and HT-29 cells reached confluency by 15 day, a time when morphological and functional differentiation is complete [34]. All cell lines used tested negative for mycoplasma.

Measurement of epithelial cell viability

Caco-2 and HT-29 cell viability was elucidated as described in [35]. Briefly, the CellTiter 96 MTT [3-(4.5-dimethyl-2-thiazolyl)-2.5-diphenyl-2H- tetrazolium bromide] assay (Promega, Madison, WI) was used to assess epithelial cell viability of Caco-2 and HT-29 cells. In addition, the trypan blue assay was used for the enumeration of viable Caco-2 and HT-29 cells after bacterial colonization.

NF-kB luciferase assay

Caco-2 and HT-29 cells were transfected with pHTS-NF-kB firefly luciferase reporter vector (Biomyx Technology, San Diego, CA) and were cultured with LC2029 strain (Manifoldness of Infection (MOI) was 200 bacteria/cell), or in the presence of Slp2 (100 µg/ml), or MaLP-2 (50 nM) as a positive control. Supernatants were removed at the end of the treatment period, and the cells were lysed with GloLysis buffer. The luciferase activity was determined using the Bright-Glo Luciferase Assay System according to the manufacturer's protocol (Promega, Madison, WI). Luminescence was measured using a Victor 2 1420 multilabel microplate counter with Wallac 2.01 software (Perkin Elmer Life Sciences, Boston, MA).

In vitro adhesion assay for lactobacilli

The lactobacilli adhesion to immature Caco-2 cells (*in vitro* biomodelling of small intestinal enterocyte immaturity in preterm infants) and adhesion to immature HT-29 cells (*in vitro* biomodelling of large intestinal colonocytes immaturity in preterm infants) were performed as described in [18] in modification. Briefly, suspensions of lactobacilli in antibiotic-free medium at $2x10^8$ CFU/ml were added to monolayers of immature Caco-2 cells with a layer of 80-100% at MOI 200:1 bacterial cell/epithelial cell ratio. Suspensions of lactobacilli were added to monolayers of immature HT-29 cells at the same conditions. Co-cultures were incubated for 2 h at 37°C in 5% CO_2 atmosphere. After 2 h, co-cultures were washed three times with sterile PBS to remove unbound bacteria, fixed with methanol, stained with azure-

eosin (Pan Eco, Russia) and examined under the Leica DM 4500B microscope (Leica, Canada). Adherent bacteria quantified by using the Leica IM modular applications system (Leica, Canada). Adhesion of lactobacilli cells to epithelial cells was expressed as a percentage of 100 randomly selected epithelial cells with adhering bacteria and as average number of adhering bacteria per epithelial cell.

In vitro adhesion assay for bifidobacteria

The studies of bifidobacteria BLI-2780 strain adhesion to fully differentiated Caco-2 cells and HT-29 cells were performed as described in [36] with some modifications. Briefly, Caco-2 and HT-29 cells were seeded in 24-well plates. Adhesion experiment were performed 15d after confluence. The viable cells number, counted in a Neubauer chamber, was about 5×10^5 cells per well. The 48 h-old BLI-2780 culture was collected by centrifugation, washed with sterile PBS, pH 7.3, and re-suspended in DMEM at approximately 1×10^8 cells /ml. As the reference material (100% values), 1 ml of the original bacteria used in the adhesion assay was centrifuged, the cells were re-suspended in 200 µl trypsin/EDTA plus 200 µl PBS, frozen and stored at -20°C until quantification of the bacteria. BLI-2780 suspension 1×10^8 cells were incubated with a monolayer of fully differentiated Caco-2 or HT-29 cells at 37°C, 5% CO_2 for 2 h. After 2 h of incubation, cell monolayer was washed three times with PBS, to remove unbound bacteria, detached from plastic surface by incubation with 200 µl trypsin/EDTA per well (10 min, 37°C). Cell suspension was incubated in lysis buffer (20 mM Tris-HCL, 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme). Quantification of the adherent bacteria was performed by quantitative PCR targeting the 16S rRNA gene. The primer sequences used in adhesion assay: Bif F (5'-TCGCGTCTGGTGTGAAAG-3') and Bif R (5'-CCACATCCAGCGTCCAC-3') [36]. Estimates of the number of genome copies in the standard were based on the genome size 2.6 Mb. PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, CA) using SYBR Green fluorophore. Amplification was carried out in 20 µl containing 1 µl of each primer, 10 µl SYBR Green PCR Master Mix and 2 µl of bacterial DNA. PCR was conducted at 95°C for 10 min, followed by 40 cycles consisting of 20 s at 95°C, 20 s at 56°C, 30 s at 60°C. Adhesion level was expressed as the number of adherent bacteria divided by total number of bacteria added, multiplied by 100.

ELISA

The concentration of cytokines IL-8, IL-6 in supernatants from Caco-2 cells cultured was measured with ELISA kits (Biosource International, Carlsbad, CA), TNFα, IL-1β was measured with ELISA kits (State Research Institute of Highly Pure Biopreparations, St-Petersburg, Russia), RANTES was determined with ELISA kit (Invitrogen) and CXCL1 was determined with Platinum ELISA (Affymetrix, Santa Clara, CA, USA), following protocols provided by the manufacturer.

Co-aggregation assay

The ability of *Lactobacillus* strains to co-aggregate with foodborne pathogen strains (**Table 1**) was tested according to [25] with some modifications. Briefly, bacteria were grown for 24 h at 37°C and cells harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in acetate buffer (pH 4.0) to give viable counts of approximately 2×10^9 CFU/ml. To analyze co-aggregative abilities, equal volumes of each *Lactobacillus* strain 2×10^9 CFU/ml were mixed with 2×10^9 CFU/ml *C. jejuni*, *S. enteritidis* or *E. coli* by vortexing for 10 s. Control test tubes containing individual bacterial samples were set up at the same time. Test tubes left at room temperature with no agitation were observed every thirty minutes for a total of three hours, analyzed visually and ranked based on a degree of co-aggregation: 0 for no visible co-aggregates in the cell suspension; + for small uniform co-aggregates in the suspension, with small clusters or sand-like grains of cells seen with careful observation, generally with minimal pellet; ++ for aggregates that are easily seen but may not settle immediately, clusters form and are distinct from supernatant or remaining suspension, but do not settle and/or do so very slowly; +++ for larger aggregates which settle and leave some turbidity in the supernatant fluid, with aggregates forming pellets at the bottom of the tubes, but some bacteria remaining in suspension; ++++ for larger aggregates, which settle immediately and leave clear supernatant fluid, often strong aggregation leaves clear supernatant easily visible between very large clusters in suspension.

Protection of immature Caco-2 and HT-29 cell monolayers against foodborne pathogens by Slp2 and LC2029 strain

Caco-2 and HT-29 cells were seeded into 6-well plates and grown to 100% monolayers. Cell culture medium was replaced with antibiotic-free medium. Plates with monolayers were divided into two groups. Slp2 (100 μg/ml) was added to the wells of first group, LC2029

strain (10^9 CFU/ml) was added to the wells of second group. The plates were co-incubated for 1 h under 5% CO_2 at 37°C. Each group of plates was divided into 3 subgroups. *Campylobacter* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 1, *Salmonella* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 2, *E. coli* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 3. The plates were co-incubated for 1 h under 5% CO_2 at 37° C. Caco-2 and HT-29 cell monolayers were washed three times with PBS to remove unbound bacteria and Slp2. Then gentamicin (50µg/ml) was added to each well and the monolayers were further incubated for 24 h. Caco-2 and HT-29 cell lysates were prepared for the assessment of caspase-9 and caspase-3 activities.

Caspase-3 and caspase-9 activities assessment

Caspase-3 and caspase-9 activities (as markers of epithelial cell apoptosis) were studied using the Caspase Activity Kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. Caco-2 cell lysates were prepared by incubating cells in extraction buffer or 15 min on ice. After centrifugation (20,000g, 15 min, 4°C), the supernatants were harvested and incubated with the substrate Ac-DEVD-ρNA (caspase-3) or AC-LEND-ρNA (caspase-9) in a 96-well micro plate for 2h at 37°C. The absorbance values of the yellow formazan were determined at 405 nm by a micro plate reader (Thermo Scientific Multiscan, MA., USA).The caspase activity was expressed as the relative enzyme activity to the control group.

Adhesion of C. jejuni, S. enteritidis and E. coli to the monolayers of Caco-2 or HT-29 cells protected by Slp2 or LC2029

For the adhesion assay, plates with Caco-2 or HT-29 monolayers with antibiotic-free medium were divided into two groups. Slp2 (100 µg/ml) was added to the wells of first group, LC2029 strain (10^9 CFU/ml) was added to the wells of second group, whilst sterile PBS was added in the control groups. The plates were incubated for 1 h at 37°C in 5% CO_2. Each group of plates was divided into 3 subgroups. *Campylobacter* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 1, *Salmonella* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 2, *E. coli* cell suspension (10^6 CFU/ml) was added to the wells of subgroup 3. The plates were incubated for 1 h at 37°C under 5% CO_2. Caco-2 and HT-29 cell monolayers were washed three times with sterile PBS to remove unbound bacteria and Slp2, fixed with methanol, stained with azure-eosin (Pan Eco, Russia) and examined under the Leica DM 4500B microscope (Leica, Canada). Adherent bacteria were quantified using

Leica IM modular applications system (Leica, Canada). Adhesion of bacterial cells to epithelial cells was expressed as average number of adhered bacteria per epithelial Caco-2 or HT-29 cell.

Antagonistic activity assay

Antagonistic activity of LC2029 strain to foodborne pathogens was studied by agar spot test according to Schillinger and Lucke [37] in modifications. Briefly, suspension of lactobacilli (3 µl 5×10^9 CFU/ml) were spotted on the surface of MRS 1.2% agar and incubated anaerobically for 24 h at 30°C to form colonies. To assess the antibacterial activity of Slp2, wells were made in the gel of MRS 1.2% agar, 100 µg of Slp2 was added to them and plates were incubated for 24 h at 4°C. Then 100 µl of an overnight culture of the indicator bacteria (approximately 10^8 cells) was mixed with 7 ml of soft brain-heart infusion (BHI) or Luria-Bertrani medium (LB) agar and poured over the plate. The plates were incubated at 37°C 24-48 h and were examined for the presence of the inhibition zones. A clear zone of more than 1 mm around a spot was scored as positive.

Quantitative H_2O_2 determination assay

H_2O_2 production by lactobacilli was tested colorimetrically through detection of the color development by *o*-dianizidine in the presence of horseradish peroxidase (Sigma, USA) as described in [38]. Absorbance reading (A_{405} nm) was measured. Content of peroxide was quantified by comparing the values obtained with those of an H_2O_2 standard curve.

Statistical analysis

The results were analyzed using a one-way ANOVA and represented means ± standard errors of the means (SEM). Statistically significant differences were accepted at $p<0.05$.

Results and discussion

L. crispatus LC2029 strain produces Slp

According to the light microscopy (**Fig. 1A**), the cells of vaginal LC2029 strain are straight or curved rods that do not form abundant auto-aggregates. This property distinguishes the LC2029 strain from other Slp-positive lactobacilli strains *L. crispatus* JCM5810, *L. crispatus* Zj001, and *L. acidophilus* M920 with the auto aggregation ability [24,25,39]. TEM of LC2029 strain (**Fig. 1B**) revealed a characteristic three-layered structure: (i) the innermost

layer is the plasma membrane, (ii) the middle layer - cell wall consist of peptidoglycan, and the third outermost layer represents the S-layer, one part of which is located on the cell wall, and the second part can diffuse into the culture medium (**Fig. 1C**). The genome of LC2029 strain contains genes encoding Slp1 and Slp2 [40]. The predicted molecular masses of the mature Slp1 and Slp2 proteins are is 61.16 kDa and 45.98 kDa respectively. The Slp fraction of LC2029 strain analyzed using SDS-PAGE and Western blotting analysis revealed no presence of Slp1 under the growth conditions we used. In contrast, the Slp2 protein was found to be highly expressed, with the expected size of 46.0 kDa (**Fig. 1D**). *L. crispatus* ZJ001 also has two S-layers genes *slpa* and *slpb* with only *slpa* gene being expressed at the conditions tested [41].

Sequence analysis of Slp2 protein

Slps in other bacteria are known have a variety of functions, some of which are essential for bacterial physiology and survival in specific environments [42]. The human vaginal LC2029 strain is highly adhesive to cervicovaginal epithelial cells due to the presence of Slp2 on its surface [18]. Alignment of the primary structures of Slp2 (slp2-2029) of vaginal *L. crispatus* LC2029, SlpA (slpa-ZJ001) of *L. crispatus* ZJ001 isolated from pig intestines [41], and CbsA (cbsa-5810) of *L. crispatus* JCM 5810 isolated from chicken intestines [43] is shown in **Fig. 2**. The amino acid sequence identity between SlpA and CbsA was as high as 84%, whilst the identity levels of these sequences with that of Slp2 were only 49% and 50% (respectively). The C-terminal regions of the three proteins appear to be more conserved than the N-terminal regions (**Fig. 2**), which is similar to Slps *L. helveticus* and *L. acidophilus* [39,44], but different from Slps of *L. brevis* with N-terminal regions being more conserved [45]. N-terminal regions of Slps are responsible for binding of bacteria to epithelial cells or pericellular tissue components in specific niches. Variation in amino acid sequences in the N-terminal regions of the Slps may contribute to unique biological properties of lactobacilli [42]. The Slp of *L. acidophilus* M92 was proposed to mediate binding to porcine ileal epithelial cells since removal of the Slp by LiCl resulted in significant reduction of adhesion of this strain to intestinal epithelium [39]. *L. helveticus* M92 and its Slps were proved to be involved in competitive exclusion with *S. enterica* serovar Typhimurium FP1 in mice intestine [46]. Extracellular matrix of human cervicovaginal, urethral and intestinal epithelial cells contains type IV collagen [47,48]. The CbsA-expressing strain *L. crispatus* JCM5810 was found to be able to efficiently adhere to immobilized type IV and I collagens, laminin,

and, with a lower affinity, to type V collagen and fibronectin. Slp2-expressing strain LC2029 is able to type IV collagen with high affinity [18] and SlpA-expressing strain *L. crispatus* Zj001 adhered efficiently to human cervical HeLa cells containing type IV collagen in extracellular matrix [24]. Thus, a common property of compared Slps produced by *L. crispatus* strains was their ability to interact with extracellular matrix components: type I and type IV collagens, laminin and fibronectin. *L. acidophilus* is one of the major species of this genus found in human and animal intestines. Slp-expressing strain *L. acidophilus* JCM 1132 did not exhibit detectable adhesiveness to collagens, laminin and fibronectin [49].

Effect of Slp2 on survival of LC2029 strain in simulated gastric and intestinal stresses

The viability of lactobacilli strains during transit through the GIT of the host is very important for the manifestation of their probiotic properties. LC2029 strain was found to be resistant to both acidic conditions and bile (**Table 2**). Removal of Slp2 reduced survival of the LC2029 cells in simulated gastric and intestinal juices by 2-3 logs. A similar pattern was observed for *L. crispatus* ZJ001 containing SlpA on its surface [24]. A vaginal *L. crispatus* strain LC1385 not expressing Slps was very sensitive to gastric and intestinal stresses (**Table 2**). These results confirmed the protective role of Slp2 for the survival of LC2029 strain in the gastric and intestinal environments. Slp2 appears to be able to assist in the transition of vaginal LC2029 cells of the mother into a new intestinal microecological niche of the infant for the implementation of new biological functions protecting newborns from colonization with foodborne pathogens.

Slp2 mediates adhesion of LC2029 lactobacilli and BLI-2780 bifidobacteria strains to Caco-2 and HT-29 cells

One of the important properties of probiotic lactobacilli and bifidobacteria is their ability to adhere to the target sites with epithelial cells and mucosal surfaces leading to formation of a barrier preventing colonization by pathogens. Monolayers of immature Caco-2 cells were used for modelling adhesive properties of immature small intestine enterocytes in extremely low-gestational-age newborns. Adhesion of vaginal LC2029 and LC1385 strains to immature intestinal Caco-2 cells was evaluated by a light microscopy (**Fig. 3**). It could be visually observed that in contrast to Slp-negative LC1385 strain with reduced properties to adhesion, bacterial cells of LC2029 strain expressing Slp2 on their surface tightly cover the immature epithelial monolayer of Caco-2 cells. The adhesion ability of LC2029 strain to immature

Caco-2 cells and immature HT-29 cells significantly decreased, when Slp2 was removed from the bacterial cells by LiCl (**Table 3**). Premature birth results in a delayed and reduced level of gut colonization by *Bifidobacterium* species [50]. This abnormal pattern is thought to affect intestinal development and contribute to a higher risk of foodborne infectious diseases especially NEC. It was shown that birth gestational age acts as a major determinant of bifidobacterial colonization in the premature infant, suggesting the role of gut maturation [50]. The ability of bifidobacteria to bind and interact with the intestinal epithelium plays an important role in gut colonization and modulation of host immune system. *B. infantis* ATCC 15697 grown on human milk oligosaccharides (HMO) had a significantly higher rate of adhesion to both fully differentiated Caco-2 and HT-29 cells [36]. We used fully differentiated Caco-2 and HT-29 cells for *in vitro* biomodelling of Slp2 participation in bifidobacteria-intestinal epithelial cells interactions. Soluble Slp2 enhanced the adhesion of bifidobacteria BLI-2780 strain to Caco-2 and HT-29 cells (**Fig. 4**). The main microecological niche for bifidobacteria is the large intestine. Slp2- treated HT-29 cells absorbed BLI-2780 bacteria more intensively on their surface compared to Caco-2 cells ($p<0.05$). The Slp2 was the key factor of LC2029 strain for adhesion to cervicovaginal mucosal surface [18]. To summarize, these results suggest that Slp2 is playing a role in adhesion of LC2029 cells to host cells and can increase the ability of the small intestinal Caco-2 cells and large intestinal HT-29 cells for interaction with bifidobacteria BLI-2780 strain.

Slp2 and LC2029 strain do not have cytotoxic and pro-inflammatory effects on Caco-2 and HT-29 cells

Slp2 and LC2029 strain do not affect Caco-2 cell and HT-29 cell viability (**Fig. 5A**, **C**). At the same time they promoted NF-kB driven luciferase activity in Caco-2 and HT-29 cells similar to that induced by the toll-like receptors (TLR) 2/6 ligand MALP-2 (**Fig. 5 B**, **D**) at significantly higher levels than the medium control ($p<0.01$). However, only MALP-2 in contrast to Slp2 and LC2029 strain, induced a significant IL-8 up-regulation in Caco-2 cells (**Fig. 6A**). Slp2 and LC2029 strain reduced IL-8 production induced by the TLR2/6 ligand MALP-2 in Caco-2 cells (**Fig. 6A**). These data suggest that Slp2 and CL2029 strain are recognized by epithelial cells as TLR2/6 ligands. Recognition of LC2029 strain and its isolated surface structure Slp2 by TLRs is required for intestinal homeostasis. It is known that IL-8 is a powerful chemo attractant and activator of neutrophils [51]. IL-8 has also been shown to play an important role in the pathogenesis of inflammatory bowel disease (IBD)

and mucosal destruction [52]. Our data suggest that LC2029 strain and Slp2 tune in the host innate immune response to avoid production of pro-inflammatory cytokines in the presence of a potent NF-kB activation. MALP-2 induced significant increase in the level of IL-1β ($p<0.01$) and of TNFα ($p<0.001$) in Caco-2 cells, while LC2029 strain and Slp2 had no effect (**Fig. 6 C**, **D**). At the same time, level of IL-6 in LC2029 strain colonized Caco-2 cells or in the presence of Slp2 was increased (**Fig. 6B**). IL-6 is an anti-inflammatory cytokine, a repair and cytoprotective factor [53]. It plays direct role in the protection of various cell types, such as hepatocytes [54], renal [55] and lung epithelia [56] from injury. IL-6 reduces the level of apoptosis among Ag-stimulated cells [57]. It is crucial for the protection of endothelial cells against H_2O_2 – induced cell death [58]. LC2029 strain produces high level H_2O_2 [18], and IL-6 is needed to support homeostatic balance in intestinal tract of newborns. Thus, LC2029 strain together with its Slp2 induce NF-kb activation and at the same time maintain low levels of inflammation-associated cytokines, which is important for the microorganism's potential use as a vaginal probiotic transmitted by mother to child during birth to form a healthy intestinal microbiota that control the pathogenic bacteria. NF-kB is a major transcription factor that plays a key role in inflammatory diseases. The net effect of NF-kB activation depends on the cell and tissue properties, the interaction of intra- and extra-cellular factors, and the nature of the activating signal [59]. Vaginal *Lactobacillus* species (*L. crispatus* , *L. jensenii* and *L. acidophilus*) can cause NF-kB activation and yet maintain low levels of IL-8 and RANTES [35,60]. Vaginal *L. jensenii* can suppress IL-8 induced by TLR ligands [61].

Slp2 inhibits levels of NEC pro-inflammatory mediators CXCL1 and RANTES produced by immature Caco-2 cells during their functional differentiation and maturation

Epithelium of the small intestine of preterm infants is immature unlike full-term infants [50]. Recently, Hayashi and colleagues reported that immature Caco-2 cells secrete CXCL1 and RANTES during their maturation and differentiation [62]. Relevant studies using a mouse model suggested a link between higher incidence of NEC and elevated intestinal expression of CXCL1 mRNA [63]. RANTES is also an effective activator of leukocytes, which play a key role in a wide range of inflammatory disorders, including NEC [64]. We used immature Caco-2 cells as a relevant *in vitro* bio-model to assess the ability of Slp2 to inhibit chemokine CXCL1 and RANTES production in immature small intestine enterocytes during their differentiation and maturation in prematurely infants. In our experimental conditions, the

process of Caco-2 cell differentiation within 15 days is accompanied by an increase in the production of CXCL1 and RANTES (**Fig. 7**). Slp2 inhibited production of CXCL1 (**Fig. 7A**) and RANTES (**Fig. 7 B**). The results indicate feasibility for using Slp2 for the NEC prevention in prematurely born infants at a time when enteral nutrition is contraindicated.

Slp2 and LC2029 strain inhibited foodborne pathogen-induced activation of caspase 9 and caspase 3 in Caco-2 and HT-29 cells

Foodborne pathogens *C. jejuni, S. enteritidis,* and *E. coli* can adhere to enterocytes, activate caspase 9 and caspase 3 and induce apoptosis through a mitochondria-mediated pathway in HT-29 cells [31] and Caco-2 cells [65]. Slps isolated from *L. acidophilus* NCFM [31] and *L. acidophilus* ATCC 4356 [65] strains decreased pathogen-induced Caspase-3 and caspase-9 activation leading to inhibition of apoptosis of HT-29 cells [31] and Caco-2 cells [65]. In our experiments the model foodborne pathogens *C. jejuni, S. enteritidis* and *E. coli* at concentration 10^6 CFU/ml activated caspase 9 and caspase 3 in Caco-2 cells (**Fig. 8A**, **B**) and in HT-29 cells (**Fig. 9A**, **B**). Pre-treatment of Caco-2 and HT-29 cells with Slp2 at concentration 100 μg/ml or LC2029 strain at concentration 10^9 CFU/ml prior to introducing *C. jejuni, S. enteritidis* or *E. coli* into the culture medium, inhibited pathogen-induced activation of caspase 9 and caspase 3 (**Fig. 8A**, **B** and **Fig. 9A**, **B**). This effect was due to the ability of the Slp2 and LC2029 strain to inhibit the adhesion of foodborne pathogens to Caco-2 and HT-29 cells (**Table 4**). The excess of Slp2 or bacterial LC2029 cells added to the culture medium before introducing pathogens was partially spent on interaction with Caco-2 cell and HT-29 cell monolayers, which is necessary to protect them from foodborne pathogens (*C. jejuni, S. enteritidis* or *E. coli*). The remainder of the Slp2 or LC2029 cells took part the formation of aggregates and co-aggregates with foodborne pathogens. In the process of washing of the Caco-2 and HT-29 monolayers, the aggregates and co-aggregates of the foodborne pathogens were removed from the cultural wells. The results of our studies indicate that the Slp2 and LC2029 strain protect epithelial cell monolayers from direct interactions with foodborne pathogens, which induce caspase 9 and caspase 3 activation in the intestinal epithelial cells and apoptosis development.

Bacterial cells of LC2029 strain surrounded by Slp2 co-aggregate with gut pathogens

Vaginal lactobacilli co-aggregation abilities may form a barrier that prevents colonization of women's reproductive system by genitourinary pathogens [66,67]. We evaluated the co-

aggregation capacities of vaginal Slp-positive LC2029 strain and Slp-negative LC1385 strain with model Proteobacteria related to foodborne pathogens (*C. jejuni*, *S. enteritidis* and *E. coli*). Two different vaginal *L. crispatus* strains Slp-positive LC2029 strain and Slp-negative LC1385 strain were found to have differing co-aggregative abilities (**Table 5**). LC2029 is strong co-aggregator of *C. jejuni*, *S. enteritidis* and *E. coli.* The Slp2 was responsible for the ability of LC2029 strain to co-aggregate foodborne pathogens. After treating of LC2029 cells with LiCl, their ability to co-aggregate with foodborne pathogens was lost. It was shown *in vitro* by Wooten that CbsA producing *L. crispatus* JCM5810 of chicken origin is a strong co-aggregator of *C. jejuni* [25]. Strain JCM5810 also effectively reduced *C. jejuni* colonization in chicken intestine. *C. jejuni* growth in co-culture with JCM5810 cells was inhibited after 6 h and was eliminated after 16 h [68]. Thus, the co-aggregation ability of LC2029 strain may contribute to prevention of colonization of human intestinal mucosal surface by foodborne pathogens.

Antagonistic activity of LC2029 strain and Slp2 against foodborne pathogens

As shown in **Table 6**. Slp2 alone did not inhibit the growth of intestinal pathogens *C.jejuni*, *S. enteritidis* and *E. coli.* Slp2-positive LS2029 strain and Slp2-negative LS2029 strain (after shedding of Slp2 from the surface of the lactobacilli cells in the presence of LiCl) were able to inhibit growth of these pathogens more effectively than Slp-negative LC1385 strain ($p< 0.05$). It has been shown earlier [18] that Slp-positive LC2029 strain, in contrast to Slp-negative LC1385 strain, is an effective producer of H_2O_2. Shedding of Slp from the surface of the lactobacilli cells in the presence LiCl do not decrease their viability [69] and effective production of H_2O_2 by Slp2-negative LC2029 strain (**Table 7**). The formation of co-aggregates by Slp-positive LC2029 strain with *C. jejuni*, *S. enteritidis* and *E. coli* (**Table 5**) creates favorable conditions under which the LC2029 strain uses H_2O_2 as a short-term bactericidal weapon against intestinal pathogens. In 90% of cases premature births are the results or urogenital infections due to decreased level of vaginal lactobacilli or their complete disappearance leading to the development of systemic inflammation [70]. In the process of childbirth, such a mother cannot naturally transmit beneficial lactobacilli bacteria similar to LC2029 to the baby. As a result, such a child may develop disbiosis, characterized by increased amounts of Proteobacteria in intestinal microbiota leading to intestinal inflammation and NEC development [27]. NEC is normally induced by non-pathogenic *E. coli* strains, capable of growing under anaerobic conditions in the absence of inhibitory effect

of lactobacilli and bifidobacteria [27]. The results of our studies indicate a possibility of using LC2029 strain as a probiotic for prevention of NEC in preterm infants.

Conclusions

In this study, we revealed new functions of Slp2 and Slp2-positive vaginal LC2029 strain. Slp2 provides tolerance of the LC2029 strain to gastric and intestinal stresses that makes it possible to relocate the strain from the vaginal microecological niche of mother to the child intestinal microecological niche. Slp2 allows the LC2029 strain to colonize the human intestine, assisting in antagonistic activity against foodborne pathogens and preventing apoptotic markers caspase 9 and caspase 3 activation induced by these pathogens in Caco-2 and HT-29 cells. Slp2 was found to possess anti-adhesive activity reducing binding of pathogenic bacteria to the host cells. This is a first study providing the evidences that Slp2 inhibits CXCL-1 and RANTES production by immature Caco-2 cells during their maturation and differentiation, inhibits IL-8 production in Caco-2 and HT-29 cells induced by MALP-2 agonist of TLR2/6 receptors. Treated by Slp2 Caco-2 and HT-29 cells increase their ability to interact with bifidobacteria. These data indicate feasibility of using Slp2 and LC2029 strain for prevention of NEC development in very low birth weight premature infants. The results also indicate the need to continue research using relevant *in vivo* bio-models for elucidation of the roles of Slp2 and LC2029 strain in the formation of intestinal microbiota, leading to prevention of growth of intestinal Proteobacteria and NEC development in preterm infants and to increase in the resistance to foodborne pathogens in children and adults.

Abbreviations

BHI – brain-heart infusion

BV – bacterial vaginosis

Camp – *Campylobacter jejuni*

DMEM – Dulbecco's modified Eagle's medium

GIT – gastrointestinal tract

HMO – human milk oligosaccharides

IBD – inflammatory bowel disease

KLH – keyhole limpet hemocyanin

LB – Luria-Bertrani medium

MOI – manifoldness of infection

MRS – Man-Rogosa-Sharpe

NEC – necrotizing enterocolitis

NF-kB – nuclear factor kB

PBS – phosphate buffered saline

Sal – *Salmonella* Enteritidis

SEM – standard errors of the means

S-layer – surface-layer

Slp – surface layer protein

TEM – transmission electron microscopy

TLR – toll-like receptor

UTIs – urinary tract infections

VVC – vulvovaginal candidiasis

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

References

[1] M. Sára, U.B. Sleytr, S-layer proteins, J. Bacteriol. 182 (2000) 859–868. doi:10.1128/JB.182.4.859-868.2000.

[2] E. Baranova, R. Fronzes, A. Garcia-Pino, N. Van Gerven, D. Papapostolou, G. Péhau-Arnaudet, E. Pardon, J. Steyaert, S. Howorka, H. Remaut, SbsB structure and lattice reconstruction unveil Ca2+ triggered S-layer assembly, Nature. 487 (2012) 119–22. doi:10.1038/nature11155.

[3] U.B. Sleytr, P. Messner, D. Pum, M. Sára, eds., Crystalline bacterial cell surface layers, Springer Berlin Heidelberg, Berlin, Heidelberg, 1988. doi:10.1007/978-3-642-73537-0.

[4] E. Calabi, F. Calabi, A.D. Phillips, N.F. Fairweather, Binding of Clostridium difficile surface layer proteins to gastrointestinal tissues, Infect. Immun. 70 (2002) 5770–5778. doi:10.1128/IAI.70.10.5770-5778.2002.

[5] J. Kern, O. Schneewind, BslA, the S-layer adhesin of B. anthracis, is a virulence factor for anthrax pathogenesis, Mol. Microbiol. 75 (2010) 324–32. doi:10.1111/j.1365-2958.2009.06958.x.

[6] K. Masuda, T. Kawata, Distribution and chemical characterization of regular arrays in the cell walls of strains of the genus Lactobacillus, FEMS Microbiol. Lett. 20 (1983) 145–150. doi:10.1111/j.1574-6968.1983.tb00105.x.

[7] B.R. Johnson, T.R. Klaenhammer, Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms, Antonie Van Leeuwenhoek. 106 (2014) 141–56. doi:10.1007/s10482-014-0171-y.

[8] U. Hynönen, A. Palva, Lactobacillus surface layer proteins: Structure, function and applications, Appl. Microbiol. Biotechnol. 97 (2013) 5225–5243. doi:10.1007/s00253-013-4962-2.

[9] H.J. Boot, C.P. Kolen, B. Pot, K. Kersters, P.H. Pouwels, The presence of two S-layer-protein-encoding genes is conserved among species related to Lactobacillus acidophilus, Microbiology. 142 (Pt 9 (1996) 2375–84. doi:10.1099/00221287-142-9-2375.

[10] F.E. Keane, C.A. Ison, D. Taylor-Robinson, A longitudinal study of the vaginal flora over a menstrual cycle., Int. J. STD AIDS. 8 (1997) 489–94. doi:10.1258/0956462971920631.

[11] J. Ravel, P. Gajer, Z. Abdo, G.M. Schneider, S.S.K. Koenig, S.L. McCulle, S. Karlebach, R. Gorle, J. Russell, C.O. Tacket, R.M. Brotman, C.C. Davis, K. Ault, L. Peralta,

L.J. Forney, Vaginal microbiome of reproductive-age women, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 4680–4687. doi:10.1073/pnas.1002611107.

[12] M.C. Verdenelli, M.M. Coman, C. Cecchini, S. Silvi, C. Orpianesi, A. Cresci, Evaluation of antipathogenic activity and adherence properties of human Lactobacillus strains for vaginal formulations, J. Appl. Microbiol. 116 (2014) 1297–1307. doi:10.1111/jam.12459.

[13] P. Mastromarino, P. Brigidi, S. Macchia, L. Maggi, F. Pirovano, V. Trinchieri, U. Conte, D. Matteuzzi, Characterization and selection of vaginal Lactobacillus strains for the preparation of vaginal tablets, J. Appl. Microbiol. 93 (2002) 884–893. doi:10.1046/j.1365-2672.2002.01759.x.

[14] G. Reid, Probiotic agents to protect the urogenital tract against infection, in: Am. J. Clin. Nutr., 2001. doi:10.1093/ajcn/73.2.437s.

[15] S.I. Pavlova, A.O. Kilic, S.S. Kilic, J.-S. So, M.E. Nader-Macias, J.A. Simoes, L. Tao, Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences., J. Appl. Microbiol. 92 (2002) 451–9. doi:10.1046/j.1365-2672.2002.01547.x.

[16] M.A. Antonio, S.E. Hawes, S.L. Hillier, The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species., J. Infect. Dis. 180 (1999) 1950–6. doi:10.1086/315109.

[17] M.A.D. Antonio, S.L. Hillier, DNA fingerprinting of Lactobacillus crispatus strain CTV-05 by repetitive element sequence-based PCR analysis in a pilot study of vaginal colonization., J. Clin. Microbiol. 41 (2003) 1881–7. doi:10.1128/jcm.41.5.1881-1887.2003.

[18] V. Abramov, V. Khlebnikov, I. Kosarev, G. Bairamova, R. Vasilenko, N. Suzina, A. Machulin, V. Sakulin, N. Kulikova, N. Vasilenko, A. Karlyshev, V. Uversky, M.L. Chikindas, V. Melnikov, Probiotic Properties of Lactobacillus crispatus 2,029: Homeostatic Interaction with Cervicovaginal Epithelial Cells and Antagonistic Activity to Genitourinary Pathogens., Probiotics Antimicrob. Proteins. 6 (2014) 165–76. doi:10.1007/s12602-014-9164-4.

[19] H. Verstraelen, R. Verhelst, G. Claeys, E. De Backer, M. Temmerman, M. Vaneechoutte, Longitudinal analysis of the vaginal microflora in pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora, BMC Microbiol. 9 (2009). doi:10.1186/1471-2180-9-116.

[20] B.L. Frost, B.P. Modi, T. Jaksic, M.S. Caplan, New Medical and surgical insights into neonatal necrotizing enterocolitis a review, JAMA Pediatr. 171 (2017) 83–88. doi:10.1001/jamapediatrics.2016.2708.

[21] Y.L. Song, N. Kato, Y. Matsumiya, C.X. Liu, H. Kato, K. Watanabe, Identification of and hydrogen peroxide production by fecal and vaginal lactobacilli isolated from Japanese women and newborn infants., J. Clin. Microbiol. 37 (1999) 3062–4. http://www.ncbi.nlm.nih.gov/pubmed/10449509 (accessed December 10, 2019).

[22] C. Cesena, L. Morelli, M. Alander, T. Siljander, E. Tuomola, S. Salminen, T. Mattila-Sandholm, T. Vilpponen-Salmela, A. Von Wright, Lactobacillus crispatus and its nonaggregating mutant in human colonization trials, J. Dairy Sci. 84 (2001) 1001–1010. doi:10.3168/jds.S0022-0302(01)74559-6.

[23] M. Horie, A. Ishiyama, Y. Fujihira-Ueki, J. Sillanpää, T.K. Korhonen, T. Toba, Inhibition of the adherence of Escherichia coli strains to basement membrane by Lactobacillus crispatus expressing an S-layer, J. Appl. Microbiol. 92 (2002) 396–403. doi:10.1046/j.1365-2672.2002.01539.x.

[24] X. Chen, J. Xu, J. Shuai, J. Chen, Z. Zhang, W. Fang, The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157:H7 and Salmonella typhimurium, Int. J. Food Microbiol. 115 (2007) 307–312. doi:10.1016/j.ijfoodmicro.2006.11.007.

[25] J.M. Wooten, The coaggregative abilities of lactobacilli and Campylobacter jejuni, University of Illinois, Urbana-Champaign, IL, 2015. http://hdl.handle.net/2142/89236.

[26] G. Rizzatti, L.R. Lopetuso, G. Gibiino, C. Binda, A. Gasbarrini, Proteobacteria: A common factor in human diseases, Biomed Res. Int. 2017:93515 (2017). doi:10.1155/2017/9351507.

[27] M. Pammi, J. Cope, P.I. Tarr, B.B. Warner, A.L. Morrow, V. Mai, K.E. Gregory, J. Simon Kroll, V. McMurtry, M.J. Ferris, L. Engstrand, H.E. Lilja, E.B. Hollister, J. Versalovic, J. Neu, Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: A systematic review and meta-analysis, Microbiome. 5 (2017). doi:10.1186/s40168-017-0248-8.

[28] A. Farzan, R.M. Friendship, A. Cook, F. Pollari, Occurrence of Salmonella, Campylobacter, Yersinia enterocolitica, Escherichia coli O157 and Listeria monocytogenes in swine., Zoonoses Public Health. 57 (2010) 388–96. doi:10.1111/j.1863-2378.2009.01248.x.

[29] W.A. Ferens, C.J. Hovde, Escherichia coli O157:H7: animal reservoir and sources of human infection., Foodborne Pathog. Dis. 8 (2011) 465–87. doi:10.1089/fpd.2010.0673.

[30] E.S. Reynolds, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy., J. Cell Biol. 17 (1963) 208–212. doi:10.1083/jcb.17.1.208.

[31] J. Meng, Q.X. Zhang, R.R. Lu, Surface layer protein from Lactobacillus acidophilus NCFM inhibit intestinal pathogen-induced apoptosis in HT-29 cells, Int. J. Biol. Macromol. 96 (2017) 766–774. doi:10.1016/j.ijbiomac.2016.12.085.

[32] H. Luo, G.O. Rankin, S. Straley, Y.C. Chen, Prolonged incubation and stacked film exposure improve sensitivity in western blotting, J. Pharmacol. Toxicol. Methods. 64 (2011) 233–237. doi:10.1016/j.vascn.2011.06.001.

[33] W.-H. Lin, B. Yu, S.-H. Jang, H.-Y. Tsen, Different probiotic properties for Lactobacillus fermentum strains isolated from swine and poultry., Anaerobe. 13 (n.d.) 107–13. doi:10.1016/j.anaerobe.2007.04.006.

[34] G.J. Mahler, M.L. Shuler, R.P. Glahn, Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability., J. Nutr. Biochem. 20 (2009) 494–502. doi:10.1016/j.jnutbio.2008.05.006.

[35] R.N. Fichorova, H.S. Yamamoto, M.L. Delaney, A.B. Onderdonk, G.F. Doncel, Novel vaginal microflora colonization model providing new insight into microbicide mechanism of action., MBio. 2 (2011) e00168-11. doi:10.1128/mBio.00168-11.

[36] M. Chichlowski, G. De Lartigue, J.B. German, H.E. Raybould, D.A. Mills, Bifidobacteria isolated from infants and cultured on human milk oligosaccharides affect intestinal epithelial function., J. Pediatr. Gastroenterol. Nutr. 55 (2012) 321–7. doi:10.1097/MPG.0b013e31824fb899.

[37] U. Schillinger, F.K. Lücke, Antibacterial activity of Lactobacillus sake isolated from meat., Appl. Environ. Microbiol. 55 (1989) 1901–1906.

[38] R. Martín, J.E. Suárez, Biosynthesis and degradation of H2O2 by vaginal lactobacilli, Appl. Environ. Microbiol. 76 (2010) 400–405. doi:10.1128/AEM.01631-09.

[39] B. Kos, J. Šušković, S. Vuković, M. Sĭmpraga, J. Frece, S. Matošić, Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92, J. Appl. Microbiol. 94 (2003) 981–987. doi:10.1046/j.1365-2672.2003.01915.x.

[40] A. V. Karlyshev, V.G. Melnikov, V.C. Khlebnikov, V.M. Abramov, Draft Genome Sequence of Lactobacillus crispatus 2029, Genome Announc. 2 (2014). doi:10.1128/genomea.01221-13.

[41] X. Chen, Y. Chen, X. Li, N. Chen, W. Fang, Characterization of surface layer proteins in Lactobacillus crispatus isolate ZJ001., J. Microbiol. Biotechnol. 19 (2009) 1176–83. doi:10.4014/jmb.0901.010.

[42] R.P. Fagan, N.F. Fairweather, Biogenesis and functions of bacterial S-layers, Nat. Rev. Microbiol. 12 (2014) 211–222. doi:10.1038/nrmicro3213.

[43] J. Wooten, X. Liu, M.J. Miller, Draft Genome Sequence of Lactobacillus crispatus JCM5810, Which Can Reduce Campylobacter jejuni Colonization in Chicken Intestine., Genome Announc. 4 (2016). doi:10.1128/genomeA.00255-16.

[44] A. Waśko, M. Polak-Berecka, A. Kuzdraliński, T. Skrzypek, Variability of S-layer proteins in Lactobacillus helveticus strains, Anaerobe. 25 (2014) 53–60. doi:10.1016/j.anaerobe.2013.11.004.

[45] M. Jakava-Viljanen, S. Avall-Jaaskelainen, P. Messner, U.B. Sleytr, A. Palva, Isolation of three new surface layer protein genes (slp) from Lactobacillus brevis ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions, J. Bacteriol. 184 (2002) 6786–6795. doi:10.1128/JB.184.24.6786-6795.2002.

[46] J. Beganović, J. Frece, B. Kos, A. Leboš Pavunc, K. Habjanič, J. Sušković, Functionality of the S-layer protein from the probiotic strain Lactobacillus helveticus M92., Antonie Van Leeuwenhoek. 100 (2011) 43–53. doi:10.1007/s10482-011-9563-4.

[47] B. Westerlund, T.K. Korhonen, Bacterial proteins binding to the mammalian extracellular matrix, Mol. Microbiol. 9 (1993) 687–694. doi:10.1111/j.1365-2958.1993.tb01729.x.

[48] B. Westerlund, P. Kuusela, J. Risteli, L. Risteli, T. Vartio, H. Rauvala, R. Virkola, T.K. Korhonen, The O75X adhesin of uropathogenic Escherichia coli is a type IV collagen-binding protein, Mol. Microbiol. 3 (1989) 329–337. doi:10.1111/j.1365-2958.1989.tb00178.x.

[49] T. Toba, R. Virkola, B. Westerlund, Y. Bjorkman, J. Sillanpaa, T. Vartio, N. Kalkkinen, T.K. Korhonen, A collagen-binding S-layer protein in Lactobacillus crispatus, Appl. Environ. Microbiol. 61 (1995) 2467–2471.

[50] M.-J. Butel, A. Suau, F. Campeotto, F. Magne, J. Aires, L. Ferraris, N. Kalach, B. Leroux, C. Dupont, Conditions of bifidobacterial colonization in preterm infants: a prospective analysis., J. Pediatr. Gastroenterol. Nutr. 44 (2007) 577–82. doi:10.1097/MPG.0b013e3180406b20.

[51] K. Matsushima, Y. Terashima, E. Toda, F. Shand, S. Ueha, Chemokines in

inflammatory and immune diseases, Inflamm. Regen. 31 (2011) 11–22. doi:10.2492/inflammregen.31.11.

[52] C. Banks, A. Bateman, R. Payne, P. Johnson, N. Sheron, Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease, J. Pathol. 199 (2003) 28–35. doi:10.1002/path.1245.

[53] I.L. Dodge, M.W. Carr, M. Cernadas, M.B. Brenner, IL-6 production by pulmonary dendritic cells impedes Th1 immune responses., J. Immunol. 170 (2003) 4457–64. doi:10.4049/jimmunol.170.9.4457.

[54] A. Bohan, W.-S. Chen, L.A. Denson, M.A. Held, J.L. Boyer, Tumor necrosis factor alpha-dependent up-regulation of Lrh-1 and Mrp3(Abcc3) reduces liver injury in obstructive cholestasis., J. Biol. Chem. 278 (2003) 36688–98. doi:10.1074/jbc.M304011200.

[55] J.M. Ueland, J. Gwira, Z.-X. Liu, L.G. Cantley, The chemokine KC regulates HGF-stimulated epithelial cell morphogenesis., Am. J. Physiol. Renal Physiol. 286 (2004) F581-9. doi:10.1152/ajprenal.00289.2003.

[56] N.S. Ward, A.B. Waxman, R.J. Homer, L.L. Mantell, O. Einarsson, Y. Du, J.A. Elias, Interleukin-6-induced protection in hyperoxic acute lung injury., Am. J. Respir. Cell Mol. Biol. 22 (2000) 535–42. doi:10.1165/ajrcmb.22.5.3808.

[57] I. Rochman, W.E. Paul, S.Z. Ben-Sasson, IL-6 increases primed cell expansion and survival., J. Immunol. 174 (2005) 4761–7. doi:10.4049/jimmunol.174.8.4761.

[58] A.B. Waxman, K. Mahboubi, R.G. Knickelbein, L.L. Mantell, N. Manzo, J.S. Pober, J.A. Elias, Interleukin-11 and interleukin-6 protect cultured human endothelial cells from H2O2-induced cell death., Am. J. Respir. Cell Mol. Biol. 29 (2003) 513–22. doi:10.1165/rcmb.2002-0044OC.

[59] P.P. Tak, G.S. Firestein, NF-κB: A key role in inflammatory diseases, J. Clin. Invest. 107 (2001) 7–11. doi:10.1172/JCI11830.

[60] H.S. Yamamoto, Q. Xu, R.N. Fichorova, Homeostatic properties of Lactobacillus jensenii engineered as a live vaginal anti-HIV microbicide., BMC Microbiol. 13 (2013) 4. doi:10.1186/1471-2180-13-4.

[61] W.A. Rose, C.L. McGowin, R.A. Spagnuolo, T.D. Eaves-Pyles, V.L. Popov, R.B. Pyles, Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures., PLoS One. 7 (2012) e32728. doi:10.1371/journal.pone.0032728.

[62] N. Hayashi, A. Yokotani, M. Yamamoto, M. Kososhi, M. Morita, C. Fukunishi, N. Nishizawa, N. Gotoh, Extracellular Signals of a Human Epithelial Colorectal

Adenocarcinoma (Caco-2) Cell Line Facilitate the Penetration of Pseudomonas aeruginosa PAO1 Strain through the Mucin Layer., Front. Cell. Infect. Microbiol. 7 (2017) 415. doi:10.3389/fcimb.2017.00415.

[63] J. Lu, M. Pierce, A. Franklin, T. Jilling, D.M. Stafforini, M. Caplan, Dual roles of endogenous platelet-activating factor acetylhydrolase in a murine model of necrotizing enterocolitis, Pediatr. Res. 68 (2010) 225–230. doi:10.1203/PDR.0b013e3181eb2efe.

[64] I. Von Luettichau, P.J. Nelson, J.M. Pattison, M. Van De Rijn, P. Huie, R. Warnke, C.J. Wiedermann, R.A.K. Stahl, R.K. Sibley, A.M. Krensky, Rantes chemokine expression in diseased and normal human tissues, Cytokine. 8 (1996) 89–98. doi:10.1006/cyto.1996.0012.

[65] P. Li, Y. Yin, Q. Yu, Q. Yang, Lactobacillus acidophilus S-layer protein-mediated inhibition of Salmonella-induced apoptosis in Caco-2 cells., Biochem. Biophys. Res. Commun. 409 (2011) 142–7. doi:10.1016/j.bbrc.2011.04.131.

[66] S. Boris, J.E. Suárez, C. Barbés, Characterization of the aggregation promoting factor from Lactobacillus gasseri, a vaginal isolate., J. Appl. Microbiol. 83 (1997) 413–20. doi:10.1046/j.1365-2672.1997.00250.x.

[67] G. Reid, J.A. McGroarty, R. Angotti, R.L. Cook, Lactobacillus inhibitor production against Escherichia coli and coaggregation ability with uropathogens, Can. J. Microbiol. 34 (1988) 344–351. doi:10.1139/m88-063.

[68] J.M. Neal-McKinney, X. Lu, T. Duong, C.L. Larson, D.R. Call, D.H. Shah, M.E. Konkel, Production of Organic Acids by Probiotic Lactobacilli Can Be Used to Reduce Pathogen Load in Poultry, PLoS One. 7 (2012). doi:10.1371/journal.pone.0043928.

[69] S. Lortal, J. Van Heijenoort, K. Gruber, U.B. Sleytr, S-layer of Lactobacillus helveticus ATCC 12046: Isolation, chemical characterization and re-formation after extraction with lithium chloride, J. Gen. Microbiol. 138 (1992) 611–618. doi:10.1099/00221287-138-3-611.

[70] G.G. Donders, K. Van Calsteren, G. Bellen, R. Reybrouck, T. Van Den Bosch, I. Riphagen, S. Van Lierde, Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy, BJOG An Int. J. Obstet. Gynaecol. 116 (2009) 1315–1324. doi:10.1111/j.1471-0528.2009.02237.x.

Figure legends

Figure 1. Detection of S-layer on the surface of LC2029 strain.

(A) Light microscopy of *L. crispatus* LC2029 cells after methylene blue staining. (B, C) Transmission electron microscopic image showing: (B) S-layer located on the bacterial surface, (C) S-layer non-covalently bound to a cell wall can desquamate from its surface and enter the culture medium. CM, cytoplasmic membrane; CW, cell wall; S, S-layer. (D) Western blotting of extracted and purified Slp2 from bacterial surface. Lane 1. Slp2. Sizes of the molecular mass marker proteins are indicated on the left.

Figure 2. Alignment of the primary structures of Slps from *L. crispatus* LC2029 (2029-slp2), from *L. crispatus* ZJ001 (ZJ001-slpa), and from *L. crispatus* JCM 5810 (5810-cbsa). Sequences of three Slps of *L. crispatus* strains were aligned with the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/).

"*****" - means that the residues are identical in all sequences in the alignment.

":" - means that conserved substitutions are observed.

"." - means that semi-conserved substitutions have been observed.

Figure 3. Adhesion of the LC2029 and LC1385 lactobacilli strains to monolayers of immature Caco-2 cells.

(A) – intact immature Caco-2 cells (control), (B) - adhesion of bacterial cells *L. crispatus* LC2029 strain containing Slp2 on their surface, (C) - adhesion of bacterial cells *L. crispatus* LC1385 Slp-negative strain. Light microscopy after azure-eosin staining. Monolayers of immature Caco-2 cells were used.

Figure 4. Effect of Slp2 on the adhesion of *B.infantis* BLI-2780 strain to fully differentiated Caco-2 and HT-29 cells.

(A) Adhesion of *B. infantis* BLI-2780 to fully differentiated Cao-2 cells. (B) Adhesion of *B. infantis* BLI-2780 to fully differentiated HT-29 cells.

Fully differentiated Caco-2 cells or HT-29 cells were treated by Slp2 for 30 min at 37°C in anaerobic condition, then cell monolayers were washed three times with sterile PBS, to remove unbound Slp2. Bifidobacteria 2×10^8 cells/ml were incubated with Caco-2 cells or HT-29 cells for 2 h at 37°C in anaerobic conditions, then were washed three times with sterile PBS, to remove unbound bacteria. Quantification of adhered bifidobacteria was based

on 16S rRNA encoding gene. The results are presented as the percentage of bacteria recovered vs. total applied to monolayers. Values are means and SEM. All data were representative of six independent experiments, tested in triplicate. *p<0.05 - adhesion of *B. infantis* BLI-2780 to epithelial cells at concentration of Slp2 in medium 25-50 µg/ml vs. 0: **p<0.01 at concentration of Slp2 in medium 75-100 µg/ml vs. 0.

Figure 5. Effect of Slp2 and LC2029 strain on the Caco-2 and HT-29 cell viability and NF-kB activity.

(A) Viability of Caco-2 cells and (C) HT-29 cells assessed at 24 h postcolonization with LC2029 strain. (MOI was 200 bacteria/cell) or in the presence of Slp2 (100 µg/ml). (B) Induction of NF-kB expression in Caco-2 cells and (D) in HT-29 cells. 24-h lysates from Caco-2 cells or HT-29 cells cultured with LC2029, Slp2 or MaLP-2 (50 nM) as a positive control. Luciferase activity measured in lysates from cultures. Bars are means and SEM. All data were representative of six independent experiments, tested in triplicate. ***p<0.001 Slp2, LC2029 and MALP-2 vs. medium control.

Figure 6. Level of cytokines IL-8, IL-6, TNFα and IL-1β secreted by immature Caco-2 cells in the presence of Slp2 or LC2029.

IL-8 and Il-6 levels determined in supernatants cultured for 24 h, TNFα and IL-1β levels determined in supernatants cultured for 8 h in the presence of Slp2 (50 µg/ml), LC2029 or MALP-2 as a positive control. Bars are means and SEM. All data were representative of six independent experiments, tested in triplicate. (A) **p<0.01 medium, Slp2 or LC2949 vs. MALP-2; *p<0.05 MALP-2 vs. MALP-2+Slp2 or MALP-2+LC2029. (B) *p<0.05 Slp2 or LC2029 vs. medium; **p<0.01 MALP-2 vs. medium; (C) ***p<0.001 MALP-2 vs. medium, Slp2 and LC2029; (D) **p<0.01 MALP vs. medium, Slp2 or LC2029.

Figure 7. Slp2 from LC2029 strain inhibits CXCL1 and RANTES secretion by Caco-2 cells during their 15-days functional differentiation.

(A) CXCL1 and (B) RANTES in supernatants from Caco-2 cells determined by ELISA.

Grey bars – supernatant from Caco-2 cells in DMEM; white bars – supernatant from Caco-2 cells in DMEM+Slp2 (100 µg/ml). Bars are means and SEM. All data were representative of six independent experiments, tested in triplicate. *p<0.05 supernatant from Caco-2 cells in DMEM (10 days) vs. DMEM+Slp2 (10 days); **p<0.01 supernatant from Caco-2 cells in

DMEM (15 days) vs. DMEM+Slp2 (15 days); #p<0.001 supernatants from Caco-2 cells in DMEM (15 days) vs. Control (fresh DMEM).

Figure 8. Effects of Slp2 and LC2029 strain on the caspase activity in foodborne pathogens-infected Caco-2 cells.

(A) Caspase-9 activity in Caco-2 cells. (B) Caspase-3 activity in Caco-2 cells. Bars are means and SEM. All data were representative of six independent experiments, tested in triplicate. **p<0.01 vs. medium control; ##p < 0.01 Slp2+Camp and LC2029 +Camp vs. Camp alone; Slp2+Sal and LC2029+Sal vs. Sal alone; Slp2+*E. coli* and LC2029+*E. coli* vs. *E. coli* alone.

Figure 9. Effects of Slp2 and LC2029 strain on the caspase activity in foodborne pathogens-infected HT-29 cells.

(A) Caspase-9 activity in HT-29 cells. (B) Caspase-3 activity in HT-29 cells. Bars are means and SEM. All data were representative of six independent experiments, tested in triplicate. **p<0.01 vs. Medium control; ##p<0.01 Slp2+Camp and LC2029+Camp vs. Camp alone; Slp2+Sal and LC2029+Sal vs. Sal alone; Slp2+*E. coli* and LC2029+*E. coli* vs. *E. coli* alone.

Table 1. Microorganisms used in this study

Microorganism	Strain	Growth conditions	Comments
Lactobacillus crispatus	LC 2029	MRS*37°C in 5% CO_2 or anaerobically 48 h	IBPM RAS[a]. Vaginal strain was originally isolated in healthy woman.
Lactobacillus crispatus	LC 1385	The same	IIE[b]. Vaginal clinical isolate (recurrent VVC, had history of BV).
Bifidobacterium longum subsp. infantis	BLI-2780	MRS 37°C anaerobically 48 h	IIE. Intestinal strain was originally isolated in healthy infant at 14 day of age.
Campylobacter jejuni	33291	BHI** 42°C in microaerophilic conditions LB*** 18 h at 37°C	ATCC[c]
Salmonella Enteritidis	25928	The same	ATCC
Escherichia coli 0157:H7	43889	The same	ATCC

[a] Russian Collection of Microorganisms at the Skryabin Institute of Biochemistry and Physiology of Microorganisms, Federal Research Center "Pushchino Scientific Center for Biological Research, RAS" Pushchino, Moscow Region, Russia.

[b] Collection of Microorganisms at the Institute of Immunological Engineering (IIE). Department of Biochemistry of Immunity and Biodefence, Lyubuchany, Moscow Region, Russia.

[c] American Type Culture Collection, Manassas, VA, USA.

* Man-Rogosa-Sharpe (MRS) broth or agar containing plates. (Himedia, India).

** Brain-Heart Infusion (BHI) broth supplemented with 0.5% yeast extract or agar containing BHI plates. (Sigma-Aldrich, St. Louis, MO, USA).

***Luria-Bertrani medium (LB) broth or agar. (Himedia, India).

Table 2. Tolerance of vaginal LC2029 strain containing Slp2 on its surface to gastric and intestinal stresses.

Strain	Gastric stress						Intestinal stress	
	10 min		30 min		60 min		5 hours	
	CFU/ml		CFU/ml		CFU/ml		CFU/ml	
	Experiment	Control	Experiment	Control	Experiment	Control	Experiment	Control
LC2029	1.80×10^7	2.16×10^7	1.75×10^7	2.18×10^7	1.05×10^7	2.20×10^7	1.75×10^6	2.43×10^7
LC2029*	1.56×10^5	1.14×10^7	1.62×10^5	1.17×10^7	1.15×10^4	1.63×10^7	1.20×10^4	2.25×10^7
LC1385**	0.86×10^5	4.26×10^7	0.67×10^5	4.31×10^7	0.53×10^4	4.25×10^7	3.75×10^4	4.16×10^7

Controls – strains cultured in the absence of gastric and intestinal stresses. Values represent means and SEM. All data were representative of six independent experiments, tested in triplicate. * LC2029 strain after removal of the Slp2 from the cell surface by LiCl; **Slp-negative vaginal *L. crispatus* strain.

Table 3. Effect of Slp2 on the adhesion of LC2029 strain to immature Caco-2 cells and immature HT-29 cells.

Epithelial cells	Strain of lactobacilli	% cells with adhering lactobacilli	No. of adhering bacteria/cell
Caco-2	LC 2029	100***	51.7 ± 8.6***
	LC 2029 (LiCl treatment)	7.3 ± 3.4	4.6 ± 1.3
	LC 1385	6.5 ± 2.7	3.5 ± 0.7
HT-29	LC 2029	100***	39.4 ± 6.2***
	LC 2029 (LiCl treatment)	5.8 ± 1.7	3.5 ± 1.3
	LC 1385	4.9 ± 2.6	2.8 ± 0.9

Monolayers of immature Caco-2 and HT-29 cells were used with a layer of 80%. Values represent means and SEM. The adhesion experiment was performed six times, tested in triplicate. ***$p<0.001$ differences between intact Slp-positive LC2029 strain and LC2029 treated by LiCl or Slp-negative LC1385 strain.

Table 4. Slp2 and LC2029 strain reduce of foodborne pathogens adhesion to Caco-2 cells and HT-29 cells.

Intestinal epithelial cells	Adherent bacteria per epithelial cell		
	Campylobacter jejuni ATCC 33291	*Salmonella enteritidis* ATCC 25928	*Escherichia coli 0157:H7* ATCC 43889
Caco-2	38.4 ± 2.35	21.9 ± 3.64	26.5 ± 4.27
Caco-2 + Slp 2	0.9 ± 0.11*	0.8 ± 0.12$^{\#}$	0.6 ± 0.05$^{\chi}$
Caco-2 + LC2029	0.6 ± 0.08*	0.7 ± 0.05$^{\#}$	0.8 ± 0.07$^{\chi}$
HT-29	24.1 ± 2.75	32.6 ± 2.83	27.8 ± 3.52
HT-29 + Slp 2	0.5 ± 0.06*	1.1 ± 0.09$^{\#}$	0.9 ± 0.08$^{\chi}$
HT-29 + LC2029	0.7 ± 0.05*	0.8 ± 0.06$^{\#}$	0.7 ± 0.05$^{\chi}$

$^{*}p<0.001$ adhesion of *C. jejuni* to Caco-2 or HT-29 cells vs. adhesion of *C. jejuni* to Caco-2+ Slp2; Caco-2+LC2029; HT-29+ slp2; HT-29+ LC2029. $^{\#}p< 0.001$ adhesion of *S. enteritidis* to Caco-2 or HT-29 cells vs. adhesion of *S. enteritidis* to Caco-2+ Slp2; Caco- 2+LC2029; HT-29+ slp2; HT-29+ LC2029. $^{\chi}p<0.001$ adhesion of *E. coli* 0157:H7 to Caco-2 or HT-29 cells vs. adhesion of *E. coli* 0157:H7 to Caco-2+ Slp2; Caco- 2+LC2029; HT-29+ slp2; HT-29+ LC2029. All data were representative of six independent experiments, tested in triplicate.

Table 5. Co-aggregative abilities of vaginal LC2029 strain with foodborne pathogens.

Strain of lactobacilli	Co-aggregation with:		
	Campylobacter jejuni ATCC 33291	*Salmonella enteritidis* ATCC 25928	*Escherichia coli* 0157:H7 ATCC 43889
L. crispatus 2029	+ + +	+ + +	+ + + +
L. crispatus 2029*	0	0	0
L. crispatus 1385**	0	0	0

Number of + symbols illustrate degree of co-aggregation. *LC2029 strain after removal of the Slp2 from the cell surface by LiCl; **Slp-negative vaginal LC1385 strain. All data were representative of six independent experiments, tested in triplicate.

Table 6. Antagonistic activity of Spl2 and LC2029, LC2029#, LC1385 strains to foodborne pathogens.

Target bacteria	Inhibition zone values (mm) Mean ± SEM			
	Antagonist			
	Slp2	LC 2029	LC2029#	LC 1385
Campylobacter jejuni ATCC 33291	0	13.5 ± 1.7*	11.8 ± 0.9*	2.2 ± 0.8
Salmonella enteritidis ATCC 25928	0	12.9 ± 1.8*	11.5 ± 1.4*	2.1 ± 0.6
Escherichia coli 0157:H7	0	14.6 ± 1.2*	13.8 ± 1.3*	2.4 ± 0.5

LC2029# strain after removal of Slp2 from the cell surface by LiCl; * $p < 0.05$ LC2029 and LC2029# strain vs. LC1385 strain. All data were representative of six independent experiments, tested in triplicate.

Table 7. Production of H_2O_2 by vaginal LC2029 , LC2029# , LC1385 strains.

Strain	Production of H_2O_2 (mg/l)	Differences between strains	
		LC2029 vs. LC2029#	LC2029 , LC2029# vs. LC1385
LC2029	118 ± 6	$p>0.05$	
LC2029#	115 ± 7		
LC1385	9 ± 1		$p<0.001$

LC2029# strain after removal of Slp2 from the cell surface by LiCl; values represent means and SEM from six independent experiments, tested in triplicate.

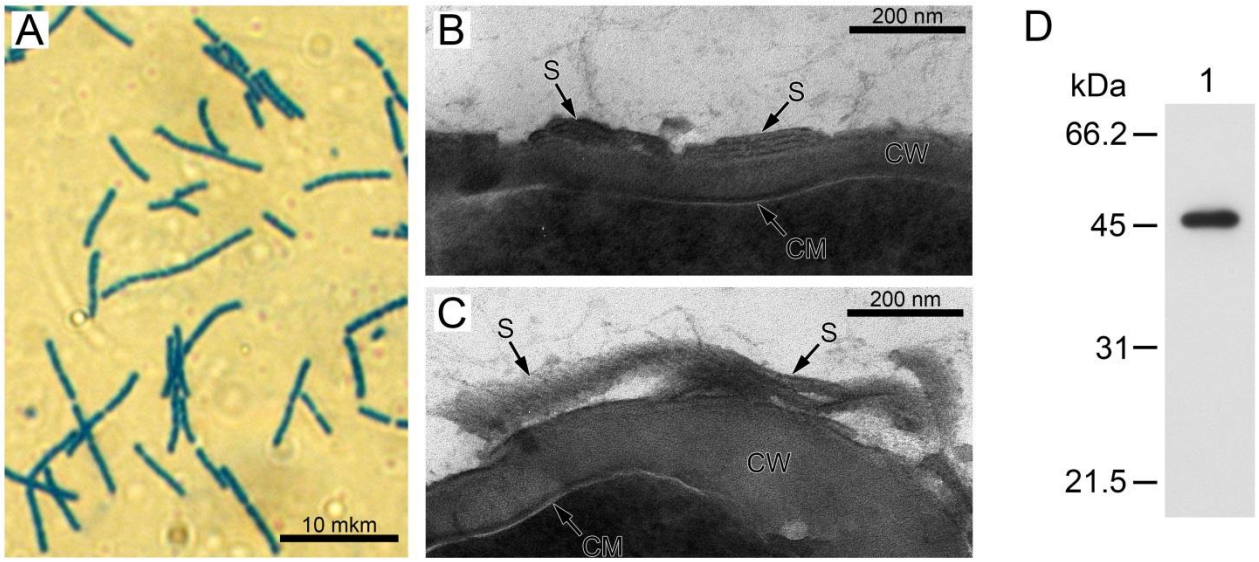

Figure 1

```
2029-slp2    -AATTTNTVYTVINADGTAINTPADAKYDVDVTPNLTAIAASTVNGQTINGSITGNITAS 59
ZJ001-slpA   ------ASSSAVQTATNIGTVLPLTDGSTVNVKPNISLNT-SAYEGV--KANISVSFSAT 51
5810-cbsA    DAVSSANNSNLGNNNNGTFTVLPLNNGATVNVKPNISLNT-SAYEGV--KANISVSFSAT 57
                    .     .  .     *      *:*.**::  : *: :*   :..*: .::*:

2029-slp2    YNGQSYTGTLDTKNGKVSVIDSKDRPVIH-------FGKLANGLYTVIVKGVSFNFGIAN 112
ZJ001-slpA   VDGTTATSNFTPNASTIELWKNEKNKVTQVTYLQQVTSSNAGATYQVKMTQVGLNFGSQN 111
5810-cbsA    VDGTTATSNFTPNASTIELWKNEKDKVTQVTDLQQVTSSNAGATYQVKMTQVGLNFGSQN 117
              :* : *..:  : ..:.: ..:.  * :        .. *.. * * :. *.:***  *

2029-slp2    AHNTITLGSKNSNVEFSTDEGKSFANTKKVELTQDGTLKNPISIKVSNVNALDLSNASGI 172
ZJ001-slpA   ANKKVTLTFPEGDMF--KTADTSLAQSHEVKLDQNGTITLPE--VVMNVTAKDFANPAVV 167
5810-cbsA    ANKKVTLTFPEGDMF--KTADTSLAQSHEVQLDKNGTTTLPE--VVMNVTAKNFANPTVV 173
             *::.:**   :.::   .  ..*:*::::*:* ::** . *    * **.* :::* : :

2029-slp2    NFYNASNGSQVTKGSVNVTAGV-NGRLNVSTVASEILKNFAAYQVSNGKAVSQMPDQKAV 231
ZJ001-slpA   NWYNTATNAVVSTGNIELFAGSDAGKMNVAQVVSATEKKYHASNY--GTKANQESSTISY 225
5810-cbsA    TWLNGTTSAPVTAGNITLYAGSDAGKMNVAQVVAEARKNYVAMGA----------KVADP 223
             .: * :..: *: *.: : **   *::**: *.:   *:: *             .

2029-slp2    VADVNAALKAANIPVDNAGWFAAPTSFSVNVKVTSSINGVDATLPVTVNVANGKDTTVPS 291
ZJ001-slpA   TNNLKDALKAMNVDVDAQGWFVAPKSFTFNMTAKANNNDASSTLAVTVSVPNGKDMTVPS 285
5810-cbsA    TNNIKEALKAMNIDVDARGWFVAPKSFTFNLTAKSDVNDATATLPVTVNVPNGKDTTVPS 283
             . ::: **** *: **  ***.**.**:.*:...:. *.. :** ***.* **** ****

2029-slp2    QSKTIMHNAYFYDKNAKRVGTDKLTRYKSVTVAMNTTTINGKAYYEVIENGKATGKFINA 351
ZJ001-slpA   QSKTVMHNAFFYDKNGKRVGSDKVTRYNSATVAMNTTTINGKAYYEVIENGKATGKFINA 345
5810-cbsA    QSKTVMHNAYFYDKNGKRVGSDKVTRYNSATVAMSTTTIKGKAYYEVIENGKATGKFINA 343
             ****:****:*****.****:**:***:*.****.****:**********************

2029-slp2    DNITGTKRTLKHNAYVYKTSKKRANKVTLKKGTEVITYGGTYTFKNGKQYYKIGDNTEKT 411
ZJ001-slpA   ANIDGTKRTLKHNAYVYKSSKKRANKVVLKKGTEVVTYGGAYTFKNGKQYYKIGNNTDKT 405
5810-cbsA    ANIDGTKRTLKHNAYVYKSSKKRANKVVLKKGTEVTTYGGAYTFKNGKQYYKIGNNTDKT 403
              ** **************:********.******* ****:*************:**:**

2029-slp2    YVKASNF   418
ZJ001-slpA   YVKVSNF   412
5810-cbsA    YVKASNF   410
             ***.***
```

Figure 2

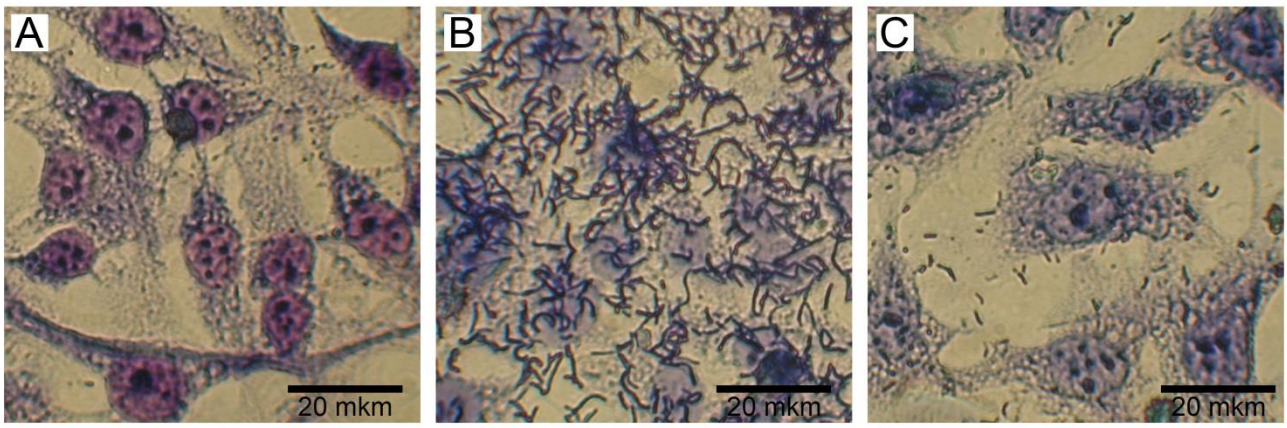

Figure 3

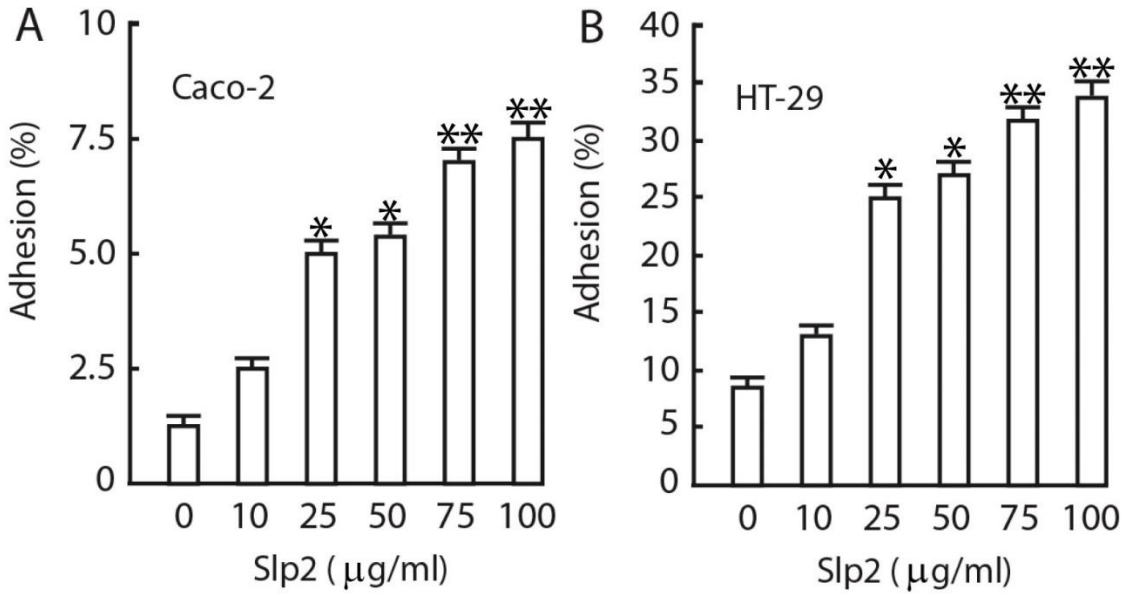

Figure 4

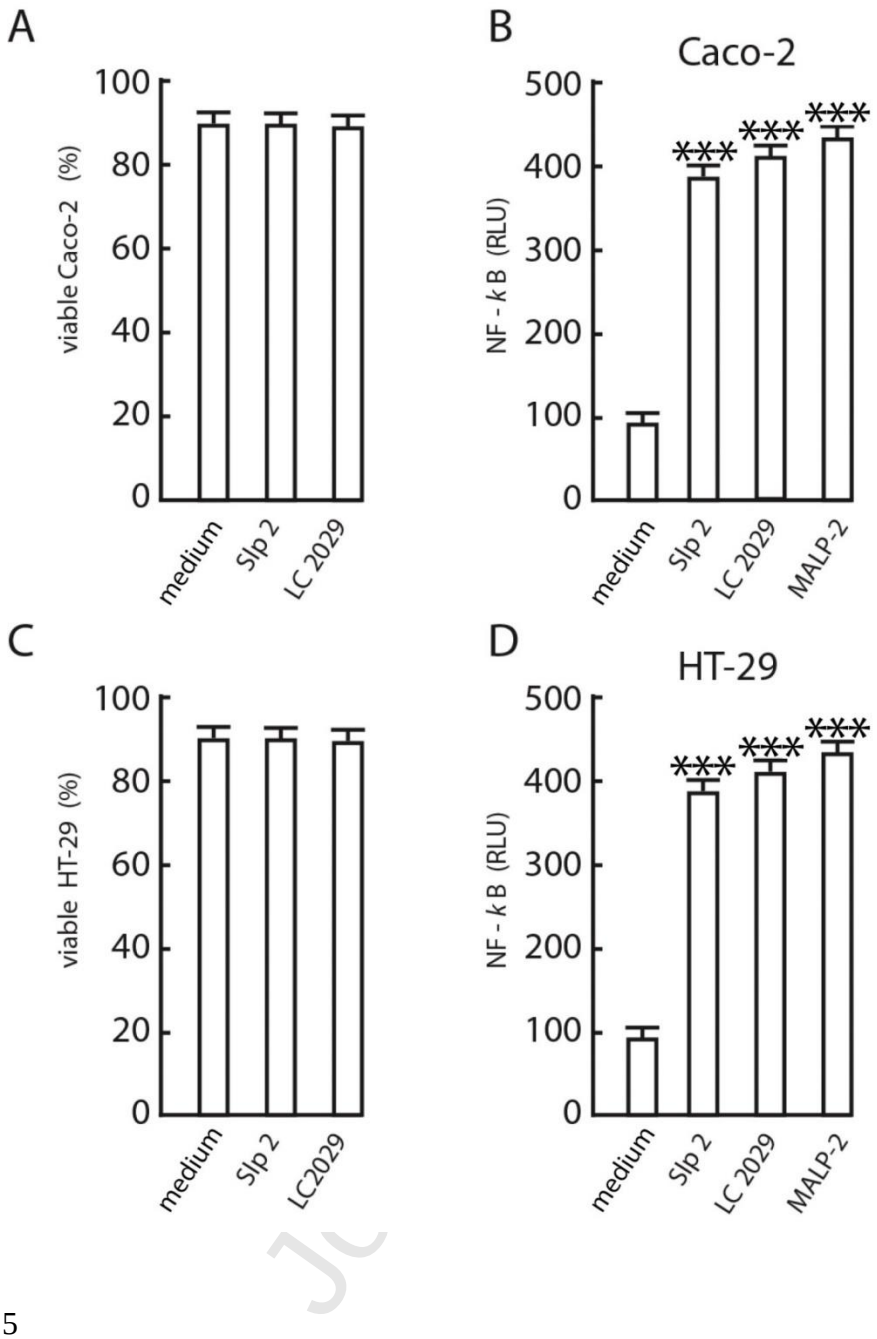
A
viable Caco-2 (%)
100
80
60
40
20
0
medium
Slp 2
LC 2029
B
Caco-2
NF - k B (RLU)
500
400
300
200
100
0
***
***
***
medium
Slp 2
LC 2029
MALP-2
C
viable HT-29 (%)
100
80
60
40
20
0
medium
Slp 2
LC2029
D
HT-29
NF - k B (RLU)
500
400
300
200
100
0
***
***
***
medium
Slp 2
LC 2029
MALP-2

Figure 5

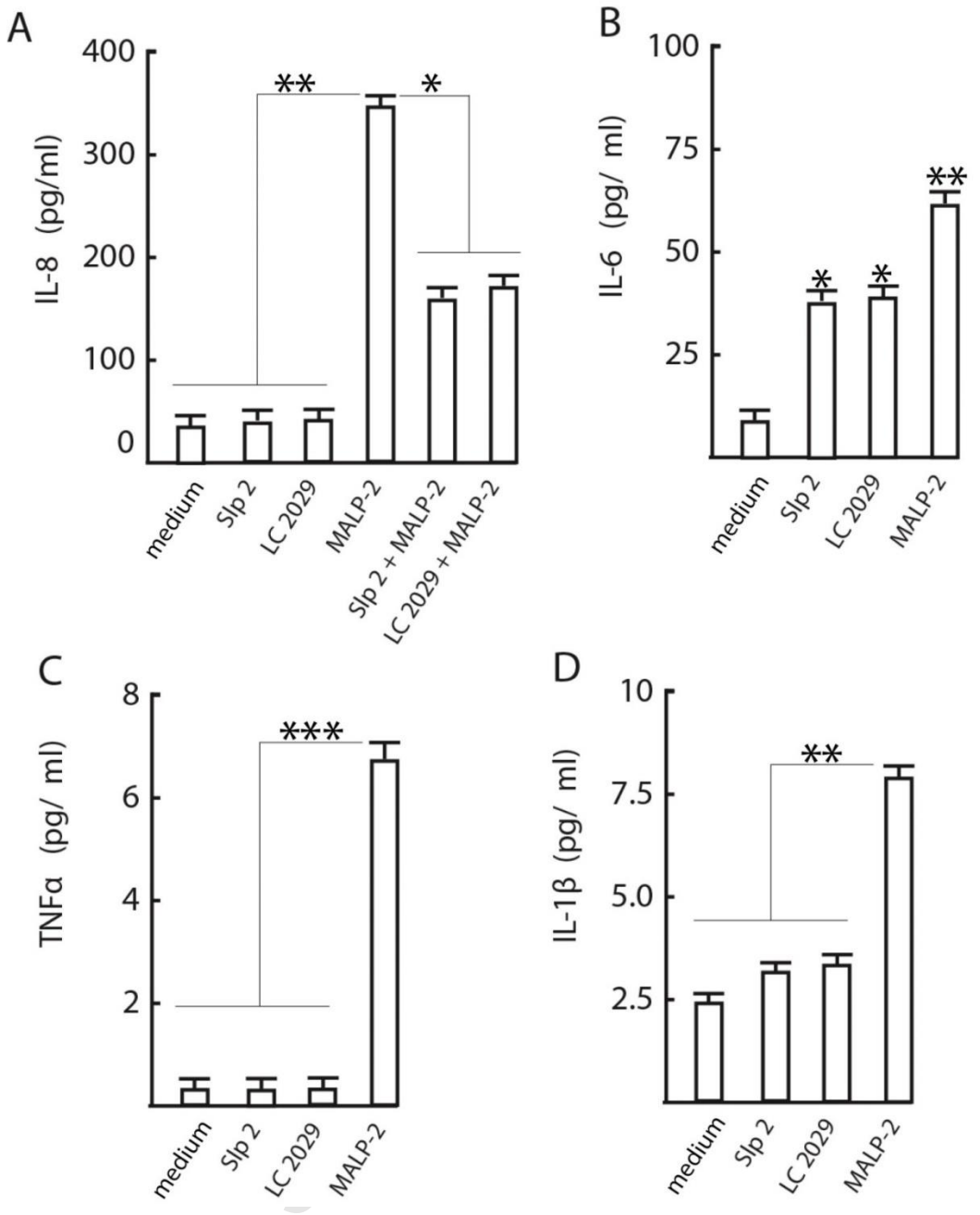
A
IL-8 (pg/ml)
400
300
200
100
0
**
*
medium
Slp 2
LC 2029
MALP-2
Slp 2 + MALP-2
LC 2029 + MALP-2
B
IL-6 (pg/ ml)
100
75
50
25
*
*
**
medium
Slp 2
LC 2029
MALP-2
C
TNFα (pg/ ml)
8
6
4
2
***
medium
Slp 2
LC 2029
MALP-2
D
IL-1β (pg/ ml)
10
7.5
5.0
2.5
**
medium
Slp 2
LC 2029
MALP-2

Figure 6

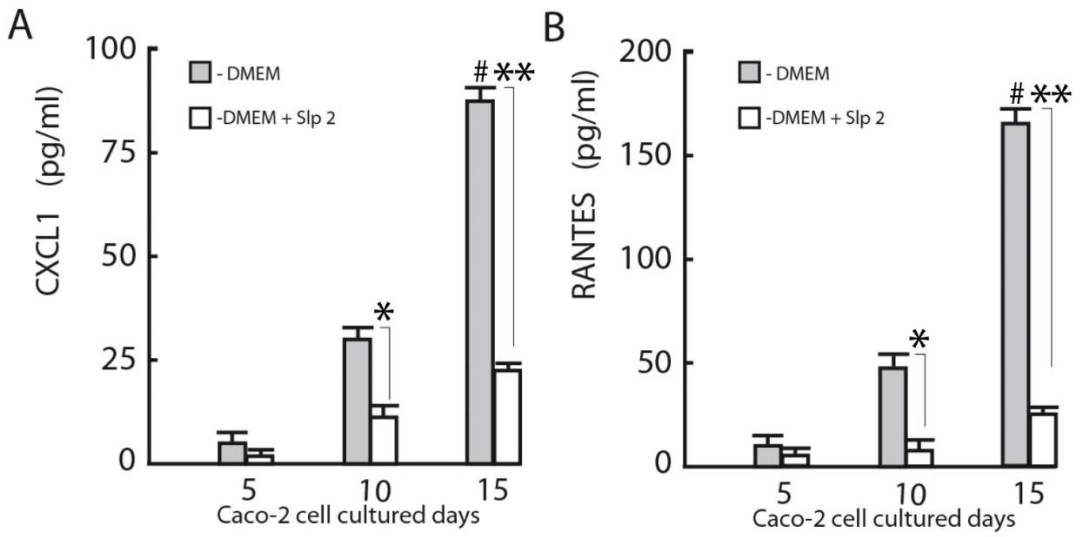

Figure 7

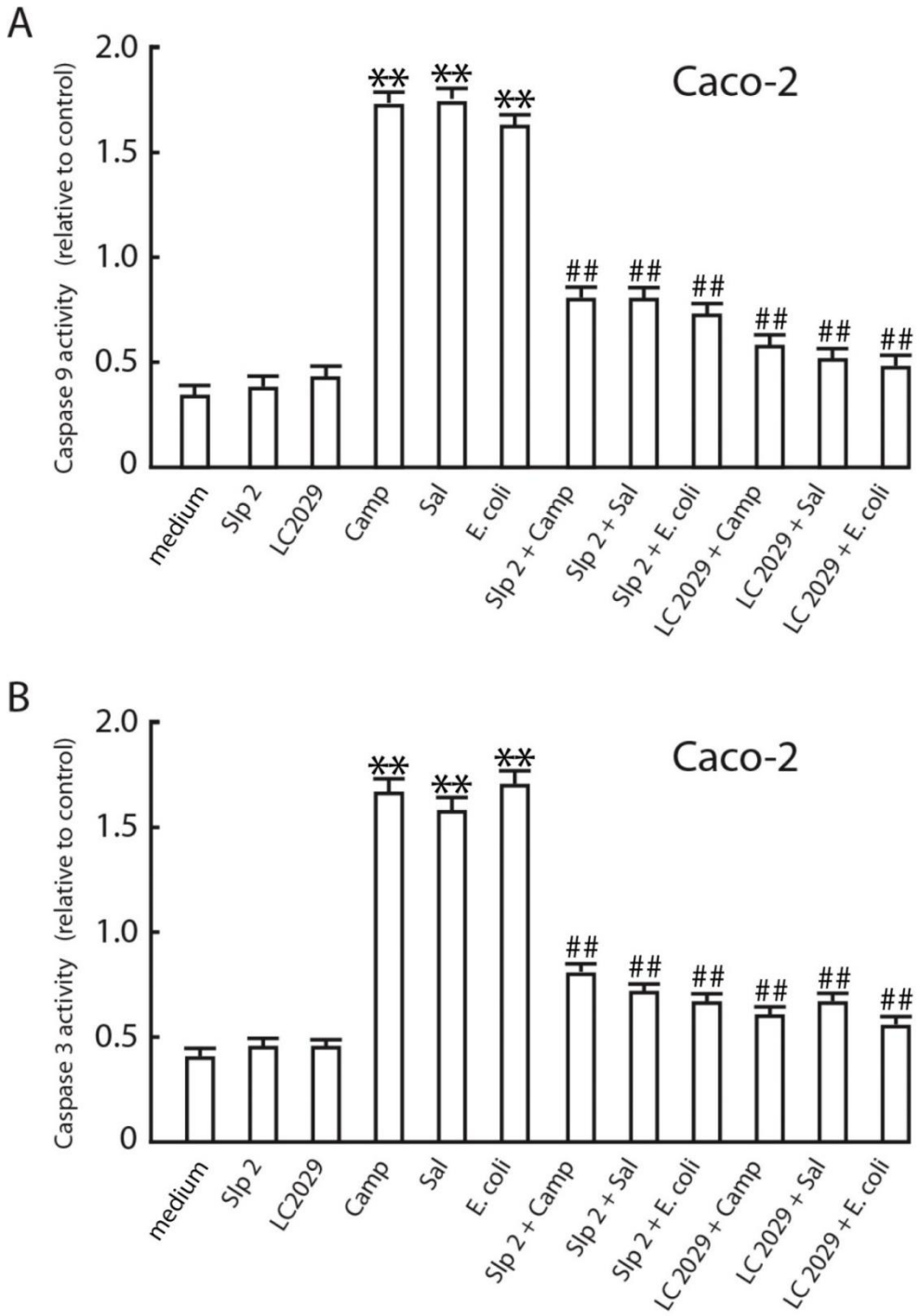
A
Caspase 9 activity (relative to control)
2.0
1.5
1.0
0.5
0
Caco-2
**
**
**
##
##
##
##
##
##
medium
Slp 2
LC2029
Camp
Sal
E. coli
Slp 2 + Camp
Slp 2 + Sal
Slp 2 + E. coli
LC 2029 + Camp
LC 2029 + Sal
LC 2029 + E. coli
B
Caspase 3 activity (relative to control)
2.0
1.5
1.0
0.5
0
Caco-2
**
**
**
##
##
##
##
##
##
medium
Slp 2
LC2029
Camp
Sal
E. coli
Slp 2 + Camp
Slp 2 + Sal
Slp 2 + E. coli
LC 2029 + Camp
LC 2029 + Sal
LC 2029 + E. coli

Figure 8

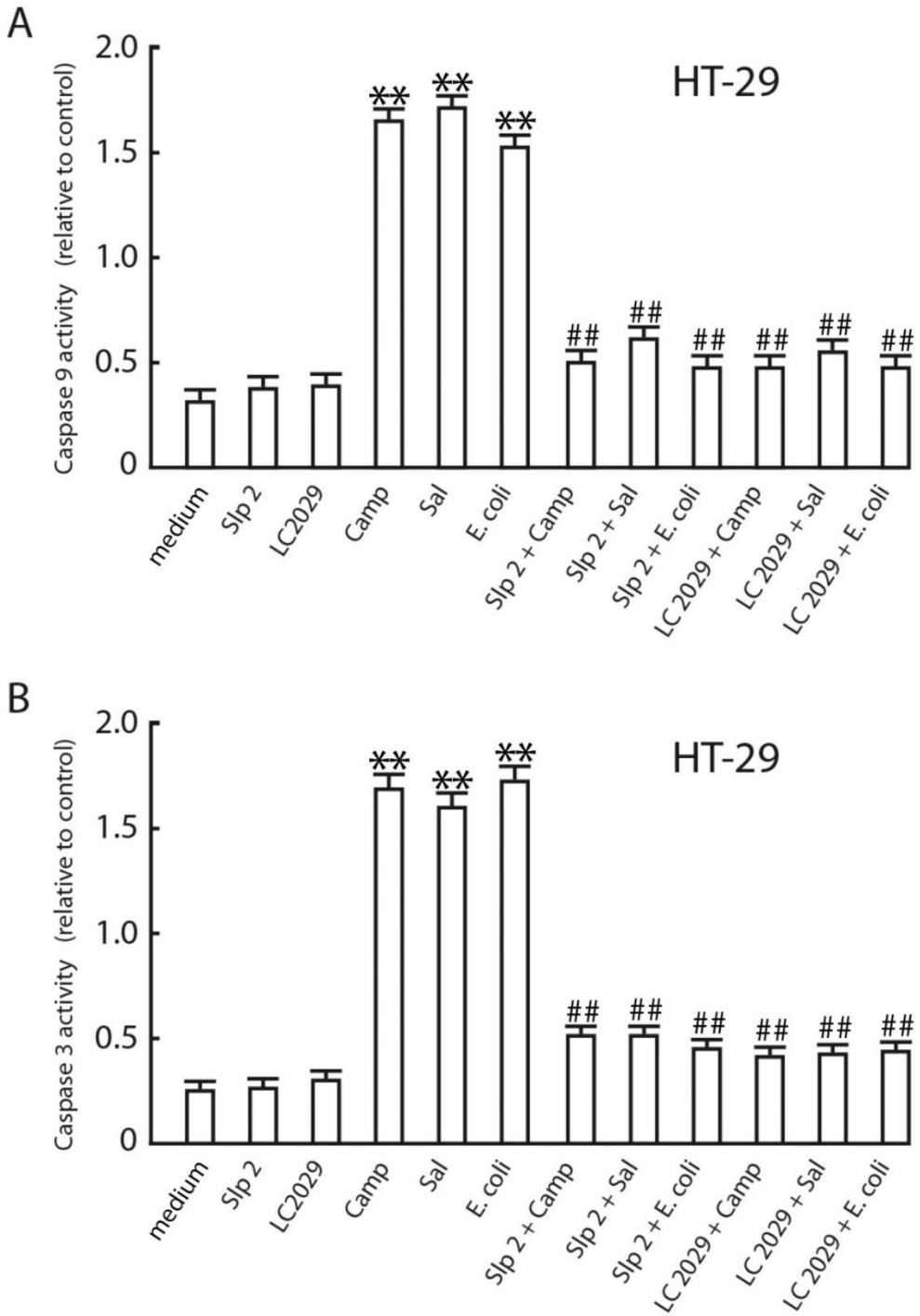
A
HT-29
Caspase 9 activity (relative to control)
2.0
1.5
1.0
0.5
0
**
**
**
##
##
##
##
##
##
medium
Slp 2
LC2029
Camp
Sal
E. coli
Slp 2 + Camp
Slp 2 + Sal
Slp 2 + E. coli
LC 2029 + Camp
LC 2029 + Sal
LC 2029 + E. coli
B
HT-29
Caspase 3 activity (relative to control)
2.0
1.5
1.0
0.5
0
**
**
**
##
##
##
##
##
##
medium
Slp 2
LC2029
Camp
Sal
E. coli
Slp 2 + Camp
Slp 2 + Sal
Slp 2 + E. coli
LC 2029 + Camp
LC 2029 + Sal
LC 2029 + E. coli

Figure 9

Credit Author Statement

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Vyacheslav M. Abramov: Conceptualization, Supervision, Project administration, Resources, Writing – original draft

Igor V. Kosarev: Investigation, Validation, Visualization, Writing – original draft

Tatiana V. Priputnevich: Investigation, Validation, Visualization

Andrey V. Machulin: Investigation, Validation, Visualization, Writing – review & editing

Valentin S. Khlebnikov: Investigation, Validation, Visualization, Writing – review & editing

Sergey Yu. Pchelintsev: Investigation, Validation, Visualization, Writing – review & editing

Raisa N. Vasilenko: Investigation, Validation, Visualization, Writing – review & editing

Vadim K. Sakulin: Investigation, Validation, Visualization, Writing – review & editing

Natalia E. Suzina: Investigation, Validation, Visualization, Writing – review & editing

Irina O.Chikileva: Investigation, Validation, Visualization, Writing – review & editing

Evgenia I. Derysheva: Investigation, Validation, Visualization, Writing – review & editing

Vyacheslav G. Melnikov: Investigation, Validation, Visualization, Resources

Ilya N. Nikonov: Investigation, Validation, Visualization, Writing – review & editing

Vladimir A. Samoilenko: Investigation, Validation, Visualization, Writing – review & editing

Eduard E. Svetoch: Investigation, Validation, Visualization, Writing – review & editing

Gennady T. Sukhikh: Conceptualization, Writing – review & editing

Vladimir N. Uversky: Supervision, Visualization, Validation, Writing – review & editing

Andrey V. Karlyshev: Supervision, Investigation, Validation, Visualization, Writing – original draft