S-layer protein 2 of vaginal *Lactobacillus crispatus* 2029 enhances growth, differentiation, VEGF production and barrier functions in intestinal epithelial cell line Caco-2

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Abstract

We have previously demonstrated the ability of the human vaginal strain *Lactobacillus crispatus* 2029 (LC2029) for strong adhesion to cervicovaginal epithelial cells, expression of the surface layer protein 2 (Slp2), and antagonistic activity against urogenital pathogens. Slp2 forms regular two-dimensional structure around the LC2029 cells, is secreted into the medium and inhibits intestinal pathogen-induced activation of caspase-9 and caspase-3 in the human intestinal Caco-2 cells. Here, we elucidated the effects of soluble Slp2 on adhesion of proteobacteria pathogens inducing necrotizing enterocolitis (NEC), such as *Escherichia coli* ATCC E 2348/69, *E. coli* ATCC 31705, *Salmonella enteritidis* ATCC 13076, *Campylobacter jejuni* ATCC 29428, and *Pseudomonas aeruginosa* ATCC 27853 to Caco-2 cells, as well as on growth promotion, differentiation, vascular endothelial growth factor (VEGF) production, and intestinal barrier function of Caco-2 cell monolayers. Slp2 acts as anti-adhesion agent for NEC-inducing proteobacteria, promotes growth of immature Caco-2 cells and their differentiation, and enhances expression and functional activity of sucrase, lactase, and alkaline phosphatase. Slp2 stimulates VEGF production, decreases paracellular permeability, and increases transepithelial electrical resistance, strengthening barrier function of Caco-2 cell monolayers. These data support the important role of Slp2 in the early postnatal development of the human small intestine enterocytes.

Key words: S-layer, lactobacilli, enterocytes

1. Introduction

Surface-layer proteins (Slps) create regular two-dimensional cell envelope structures found in archaea and many bacteria [1]. Bacterial surface layers are composed of single protein subunits and have significant strength, flexibility, semi-permeability, resembling chain mail [2,3]. Some lactobacilli are associated with the host mucosal surface, such as the gastrointestinal tract (GIT) and urogenital tract [4]. Protective functions of Slps in these bacteria provide resistance to colonization of host tissues by pathogenic microorganisms [5]. The variability of the sequences and the functions of the surface layer (S-layer) in lactobacilli depend not only on the species, but also on the strain [6,7]. This variability is one of the reasons for the significant strain differences in the biological properties of lactobacilli. *Lactobacillus* species predominates in healthy vaginal microbiota and forms a critical line of defense against genitourinary pathogens [8–10]. The beneficial function of vaginal lactobacilli is to maintain an environment that limits the growth of pathogens [11]. Decreased levels of vaginal lactobacilli lead to an increased risk of bacterial vaginosis (BV), vulvovaginal yeast candidiasis (VVC), and urinary tract infections (UTIs) [12]. Healthy vaginal microbiota is typically dominated by *Lactobacillus* species, such as *L. crispatus, L. jensenii,* and *L. gasseri* [9,13]. *L. crispatus* appears to be substantially prevailing over the other hydrogen peroxide producing *Lactobacillus* species [14]. A type I microbiota with the dominance of the *L. crispatus* species is important for maintaining of healthy birth tract [9]. Furthermore, vaginal colonization with probiotic human-derived *L. crispatus* Slp-positive strain CTV-05 has been successful [15]. The latest study using CTV-05 showed that approximately 50% of the patients who were administrated CTV-05, still kept this strain in the vagina after 24 weeks of administration [16]. Verstraelen and colleagues reported that vaginal strains *L. crispatus* promote the stability of the normal vaginal microbiota during pregnancy [17]. *L. crispatus* strain JCM 7696 accelerated re-epithelization of the experimentally damaged monolayer of vaginal epithelial MS74 cells *in vitro* [18]. *L. crispatus* strain M247, showing a cell aggregation phenotype, was isolated from a fecal sample taken from a weaning baby [19]. The strain adhered to ileal mucus and Caco-2 cells *in vitro.* Three human trials with M247 were performed, and the strain could be recovered from either fecal samples or biopsies taken from the colon. Slp-positive M247 strain increased toll-like receptor 2 (TLR2) mRNA and protein levels, while it reduced toll-like receptor 4 (TLR4) mRNA and protein levels in murine colonic mucosa, whereas Slp-negative isogenic mutant MU5 was ineffective [20]. Inhibition of the degree of TLR4 signaling limits the necrotizing enterocolitis (NEC) incidence in neonatal mice [21].

Slp-positive *L. crispatus* 2029 (LC2029) strain was isolated upon investigation of the spectrum of vaginal lactobacilli from healthy women of reproductive age and was selected as probiotic candidate for prophylactics and treatment of genitourinary infections [22]. The Slp is involved in homeostatic interaction with cervicovaginal epithelial cells and participates in their effective colonization by vaginal LC2029 strain. Slp is responsible for the LC2029 strain high affinity binding to type IV collagen as one

of the main components of extracellular matrix on cervical HeLa and vaginal VK2/E6E7 epithelial cells (Kd = $(8.0\pm0.7)\times10^{-10}$ M). Slp producing LC2029 strain has antagonistic activity increasing colonization resistance to genitourinary tract infections by BV and VVC associated pathogens [22]. LC2029 strain has two homologous S-layer genes, *slp*1 and *slp*2 [23]. High expression of only the *slp*2 gene was detected. Slp2 forms regular two-dimensional structure around the LC2029 cells and is also secreted into the medium [24].

Early bacterial populations of neonates depend on the environmental factors, such as mode of delivery, formula feeding vs. breast feeding, and other [25]. Recent studies have suggested that transfer of bacteria from a mother to infant during vaginal birth is fundamental for the formation of the early infant intestinal microbiota and later disease risk [26,27]. During 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 [28]. The functions of the vertically transferred mother's vaginal lactobacilli and their components in the mother-child microecological environment need further study. It is known that in prematurely born infants the intestinal microbiota contains a high level of Proteobacteria responsible for necrotizing enterocolitis (NEC) development [29,30]. NEC remains the leading cause of death from gastrointestinal disease in premature infants [31].

Enterocyte maturational delay in preterm infants is one of the main pathogenic mechanisms of NEC [32]. NEC-inducing pathogens significantly increase inflammation in the immature intestine [33], reduce the growth and differentiation of enterocytes, and inhibit the processes of intestinal regeneration [34]. The intestine of preterm infants and also infants with NEC displays impaired barrier function and increased intestinal permeability [35–39]. These pathogens increase carbohydrate malabsorption in the small intestine due to the loss of function of microvillar disaccharidases [40–42], inhibit the production of the intestinal alkaline phosphatase (ALP) by enterocytes of preterm infants [43], and stimulate the apoptosis development [44].

Our previous data was demonstrated that soluble Slp2 inhibits intestinal pathogen-induced activation of caspase-9 and caspase-3 as apoptotic biomarkers in the human intestinal Caco-2 cells [24]. The effects of Slp2 on the human small intestine remain poorly understood. Here, we investigated the effects of soluble Slp2 on adhesive properties of NEC-inducing proteobacteria to Caco-2 cells, growth promotion, differentiation, vascular endothelial growth factor (VEGF) production and intestinal barrier function of Caco-2 cell monolayers.

Altogether, obtained results demonstrate that Slp2, a critical secreted component of vaginal LC2029 strain, acts as anti-adhesion agent for NEC-inducing proteobacteria, promotes growth of immature Caco-2 cells and their differentiation, enhances expression and functional activity of sucrase, lactase and alkaline phosphatase. Slp2 stimulates VEGF production, decreases paracellular permeability and increases transepithelial electrical resistance, strengthening barrier function of Caco-2 cell monolayers.

2. Materials and methods

2.1 Bacterial strains and growth conditions

LC2029 Slp-positive strain was originally isolated from a vaginal smear of a healthy woman of reproductive age [22]. The strain was deposited at the All-Russian Collection of Microorganisms at the G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Federal Research Center "Pushchino Scientific Center for Biological Research, RAS" Pushchino, Moscow Region, Russia under the registration number VKM B-2727D. A complete list of microorganisms used in this study and their growth conditions is provided in **Table 1**.

2.2 Preparation of Slp2, SDS-PAGE and Western blotting analysis

Slp2 was extracted and purified according to [45] with modifications [24]. Briefly, Slp2 was extracted from LC2029 strain by 5M LiCl. The extract was purified by chromatography on cation-exchange column. The purified Slp2 was examined by SDS-PAGE using 12% polyacrylamide gel. Western blotting analysis was performed as described in [46]. 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). 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 was used as a secondary antibody. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) and X-ray film (Phenix Research Products) were used for visualization.

SDS-PAGE and Western blotting analysis of extracted and purified Slp2 from LC2029 strain were shown in **Figure S1**. Molecular weight of purified soluble Slp2 was ~46 kDa.

2.3 In vitro intestinal epithelial Caco-2 model

Immortalized epithelial Caco-2 cells showing marked characteristics of human small intestine enterocytes including the ability to differentiate [47] were used as *in vitro* intestinal epithelial model. Caco-2 cells are the "gold standard" of the *in vitro* intestinal epithelial models [48]. Caco-2 cell culture is most widely used compared to other cell lines to study the properties of various drugs, probiotics and their individual components, in particular Slps, and predict their impact on human health, as well as to assess their activities against pathogens [49–52]. Caco-2 model is recognized as a reliable and relatively simple tool for assessing intestinal permeability [53]. In our experiments, Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin

(100 mg/mL) and kept in CO_2 incubator with 5% CO_2. Medium was replaced daily. Caco-2 cells reached confluence by 15 day, a time when morphological and functional differentiation is complete [54].

2.4 Adhesion of Slp2-treated proteobacteria to Caco-2 cells

To assess the effect of Slp2 from LC2029 on adhesion of proteobacteria to monolayers of Caco-2 cells, 5×10^7 cells/mL suspensions of strains *Escherichia coli* ATCC E 2348/69, *E. coli* ATCC 31705, *Salmonella* Enteritidis ATCC 13076, *Campylobacter jejuni* ATCC 29428, *Pseudomonas aeruginosa* ATCC 27853 were incubated in the presence of Slp2 at concentrations of 0, 10, 50, 100 µg/mL in phosphate buffered saline (PBS) for 30 min. The strains treated with Slp2 were then applied to the monolayers of Caco-2 cells. The plates were incubated for 1 h at 37°C under 5% CO_2. Caco-2 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 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 cell.

2.5 Cell growth determination

Caco-2 cells were seeded onto 96-well microtiter plates (10^4 cells/well) and cultured for 3 days in a DMEM with 10% FBS. After 24 h of serum starvation, cells were exposed to increasing doses of Slp2 for 48 h in DMEM FBS-free. ^{3}H-thymidine (0.5 µCi/well, ICN Biomedicals, Irvine, CA) was added 18 h before harvesting the cells with cell harvester (Skatron Instruments, Lier, Norway). The filters were dried, and beta radioactivity was measured with MINI BETA liquid scintillation spectrometer (LKB, WALLAC). The same experiments were repeated after 6, 10 and 15 days from plating. For cell counts, Caco-2 sells were seeded onto 24-well plates (5×10^4 cells/well), cultured, stimulated under the same experimental conditions, and counted in a Neubauer chamber.

2.6 Sucrase and lactase activity assay

Enzymatic activities of intestinal sucrase and lactase were measured as described in [55]. Briefly, Caco-2 cells were collected after 24 h of Slp2 stimulation, rinsed in cold PBS and scraped into cold maleate buffer 0.1 M pH 6.0. Samples were sonicated three times for 15 s each, using a Labsonic 2000 (Sartorius AG, Germany). Total cell lysates were incubated at 37°C with 50 mM sucrose for 30 min or lactose for 60 min. The concentration of glucose generated by sucrase or lactase was measured using a glucose oxidase assay.

2.7 Alkaline phosphatase activity assay

Intestinal alkaline phosphatase (ALP) activity was measured using kit for ALP determination according to the manufacturer's instructions (Olvex Diagnosticum, Russia). Briefly, after 24 h of culturing of confluent Caco-2 cells in the presence of different concentrations of Slp2 (0.1, 1, 10, 25, 50, 75 and 100 µg/mL), ALP activity was measured using the p-nitrophenyl phosphate (pNPP) method. Fifty microliters of the cell cytosol were incubated in a 96-well plate with 50 µL pNPP for 30 min at 37°C, after which ALP activity was measured. The amount of liberated pNPP was determined spectrophotometrically at 405 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.8 RNA extraction and reverse transcription

Caco-2 cells were collected after 24 h of Slp2 stimulation and total RNA has been extracted from Caco-2 cells by TRIzol reagent protocol (Invitrogen). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. Reverse transcription of RNA was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

2.9 Real-time quantitative polymerase chain reaction analysis

Analysis of real-time quantitative polymerase chain products (qRT-PCR) was performed according to the recommendations supplied by Applied Biosystems (available at: http://europe.appliedbiosystems.com). Primers for sucrase, lactase and ALP genes were purchased from Applied Biosystems. PCR was conducted in 25-µL reaction mixtures containing about 40 ng of cDNA as template. Reactions were run in 96-well optical reaction plates using an Applied Biosystems 7300 Real-Time PCR System. Thermal cycles were set at 95°C (10 min) and then 35 cycles at 95°C (15 s) and 60°C (1 min) with auto ramp time. For data analysis, the threshold line was set automatically, and it was in the linear range of the amplification curves for all mRNA in all experimental runs. All reactions were performed in triplicates. The abundance of target mRNA was calculated relative to a reference mRNA (GAPDH). Relative mRNA expression levels were calculated based on Ct values [56]. The confidence interval was fixed at 95%.

2.10 ELISA of VEGF

The immature Caco-2 cells were cultured for 2 days (preconfluent condition) to form a cell layer of 80-90%. The cell culture supernatants were collected at 24 hours after culturing the Caco-2 cell layers in the presence of different Slp2 concentrations and centrifuged at 1000 g for 15 minutes. The supernatants were stored at −80°C. The VEGF concentrations in the culture medium were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according

to the manufacturer's instructions. The VEGF concentrations in each well were measured at 450 nm using a microplate reader (Microplate Absorbance Reader, Bio-Rad, USA).

2.11 Transepithelial electrical resistance measurement

Caco-2 cells were seeded onto apical inserts (AP) (Millicell-24, Millipore Burlington, MA, USA) at a density of 10^5 cells/insert (0.5 mL) and allowed to differentiate over the next 15 days. Medium was placed in the basolateral well (BL) (1.5 mL) and was replaced daily. Transepithelial electrical resistance (TEER) was determined with a Millicell-ERSR voltmeter (Millipore Burlington, MA, USA) according to the manufacturer's instructions. Monolayers treated DMEM alone (zero dose of Slp2) were used as controls.

2.12 Caco-2 cell monolayer permeability determination

Caco-2 cells were cultured for 15 days on a DMEM medium to obtain a confluent monolayer. Medium was replaced daily. Permeability of confluent Caco-2 cell monolayers was determined according to [56]. At 15 days of cell culture, the growth media were replaced with Hanks balanced salt solution (HBSS) for 2 h to facilitate protein/peptide depletion. For transport, HBSS in the apical chamber was replaced with 400 µL of 2 mg/mL sodium fluorescein, and fresh HBSS (600 µL) was added to the basolateral chamber. The concentration of sodium fluorescein was measured in both the AP and BL layers with a microplate reader (Infinite M200 pro, Tecan, Männedorf, Switzerland) at 30, 60, and 120 min. The excitation and emission wavelength were 495 nm and 520 nm respectively. The apparent permeability coefficient (P_{app}) was used to evaluate the AP to BL paracellular permeability of Caco-2 cell monolayers and calculated as follows: $P_{app} = (dQ/dt)\cdot(1/(A\cdot C_0))$, where dQ/dt is the permeability rate derived from the slope of the line, A is the surface area of the membrane, and C_0 was the initial drug concentration in the AP compartment.

2.13 Statistical analysis

The results were analyzed using a one-way analysis of variance (ANOVA) and represented as the means ± standard errors of the means (SEM) of six independent experiments, tested in triplicate. Significance was evaluated by *t*-test. Results were considered significant at $p<0.05$.

3. Results and discussion

3.1 Effect of Slp2 on the adhesion of proteobacteria strains to Caco-2 cells

Slp2 isolated from vaginal lactobacilli strain LC2029 very efficiently inhibited the adhesion of proteobacteria strains to intestinal Caco-2 cells. As shown in **Table 2**, the adherence of proteobacteria strains (*E. coli* E 2348/69 (EPEC), *E. coli* 31705 (ETEC), *Salmonella* Enteritidis 13076, *C. jejuni*

29428, *P. aeruginosa* 27853) to Caco-2 cells was inhibited in a dose-dependent manner by the addition of Slp2 in the medium. At the same time, Caco-2 cultivated in the presence of Slp2 increased the adhesion of *Bifidobacterium longum* subspecies *infantis* BLI 2780 to the enterocytes of these line [24]. Slp2 blocks the adhesion of proteobacteria to enterocytes and, as a result, inhibits the colonization of enterocytes and the participation of these bacteria in the formation of the parietal intestinal microbiota. Adhesion and colonization are prerequisites for the establishment of bacterial pathogenesis, involving proteobacteria [57]. Adherence of proteobacteria to intestinal epithelial monolayer diminishes its barrier function [58–60]. In 10-12% preterm infants, proteobacteria induce the development of NEC [29,30]. Among the microorganisms that populate the intestines of a newborn in the first hours after birth, *E. coli* dominates. This representative of the normal intestinal microflora makes up 96% of its aerobic component. On days 5-7, aerobic microorganisms, multiplying with the use of oxygen, create an anaerobic environment in the intestine and the expansion of the anaerobic component of the intestinal microbiota begins [61]. It is mainly represented by such essential microbes in enzymatic activity as bifidobacteria. The main food of the newborn is human milk, which contains prebiotic oligosaccharides. Several chromosomal loci of bifidobacteria reflect potential adaptation to the infant host including clusters encoding catabolic genes, extracellular solute binding proteins and permeases predicted to be active on milk oligosaccharides [62]. However, some strains of *E. coli* (non-pathogenic or pathogenic) can grow in anaerobic conditions and disrupt healthy parietal (membrane or contact) intestinal microbiota created by bifidobacteria. In preterm infants, this can lead to the development of NEC [30]. Membrane or contact digestion was first described in [63]. During the period of the child breastfeeding, membrane (contact) digestion is dominant.

3.2 Growth of Caco-2 cells treated with Slp2

Preconfluent culture of Caco-2 cells (3 days post-plating) were exposed 24 h to different concentrations (0.1 - 100 µg/mL) of Slp2. ^{3}H-thymidine incorporation was increased in Caco-2 cells in the presence of Slp2 (concentrations 1-100 µg/mL). ^{3}H-thymidine incorporation increased in a dose-dependent fashion peaking at 75-100 µg/mL (**Figure 1A**). The experiments were repeated using cell count to monitor cell proliferation. Slp2 stimulated proliferation of Caco-2 cells at concentration 0.1-100 µg/mL in a dose-dependent fashion peaking at 10-100 µg/mL (**Figure 1B**). Using uptake of ^{3}H-thymidine method the cell growth was evaluated in Caco-2 cells at 3, 10, 15 days after plating. Caco-2 cells were exposed 24 h to 1 µg/mL and 100 µg/mL of Slp2. The proliferation of Caco-2 cells induced by Slp2 was maximal at 100 µg/mL in preconfluent culture (3 days), but it was progressively lost in older cells (**Figure 1C**). It is known that the growth of enterocytes changes immediately after birth and reaches a peak in the newborn when stimulated by human milk growth factors [64,65]. Our data shows that Slp2 also exhibits the properties of an enterocyte growth factor.

3.3 Cell differentiation of Caco-2 cells treated with Slp2

The small intestine enzymes sucrase, lactase, and ALP are biomarkers of enterocyte differentiation [66–69]. Therefore, differentiation of Caco-2 cells treated with Slp2 was assessed by measuring of sucrase, lactase and ALP activities. Slp2 induced an increase in the sucrase activity (**Figure 2A**) and lactase activity (**Figure 2B**) in the concentration range of 10-100 ng/mL ($p<0.05$). The activities of sucrase and lactase were dose-dependent from 1 to 10 ng/mL of the Slp2 concentration, and reached the upper limit after 25 ng/mL. Stimulation of disaccharidase activities depended on the time of Slp2 addition. Exposure of more immature cells to Slp2 corresponded to the maximal effect. We added Slp2 (100 ng/mL) to the cells at 3,6,10, and 15 days post-plating in parallel experiments. The effect was maximal in cells exposed to Slp2 on day 3 post-plating for sucrase activity and in those exposed to Slp2 on day 6 post-plating for lactase activity (**Figure 2C**).

In fully differentiated Caco-2 cells (15 days after plating) Slp2 increased activity of ALP in the concentration range of 10-100 µg/mL (**Figure 3A**). The activity of ALP was dose-dependent from 0.1 to 10 µg/mL of the Slp2 concentration, and reached the upper limit after 25 µg/mL. In parallel experiments we added Slp2 (10 µg/mL) to Caco-2 cells at 3, 6, 10, and 15 days after plating. The cells were cultured in the presence of Slp2 for 24 h, then the activity of ALP was evaluated. Fully differentiated Caco-2 cells on day 15 after plating showed higher ALP activity (**Figure 3B**). We determined effect of maximally effective Slp2 concentration (from previous experiments) on the levels of mRNAs encoding sucrase, lactase, and ALP. The experiments for sucrase were performed using Caco-2 cells on 3 days after plating. The experiments for lactase were performed using Caco-2 cells on 6 days after plating. The experiments for ALP were performed using Caco-2 cells on 15 days after plating. Slp2 was introduced into the medium and Caco-2 cells were cultivated for 24 h. After Slp2 stimulation, the cells were collected and the total RNA was extracted. The increase in the sucrase mRNA expression (**Figure 4A**) and lactase mRNA expression (**Figure 4B**) was observed at Slp2 concentration of 1 ng/mL ($p<0.05$). Increase of ALP mRNA expression (**Figure 4C**) was observed at Slp2 concentration of 1 µg/mL ($p<0.05$). These data show that Slp2 has the ability to stimulate transcription of genes encoding sucrase, lactase and ALP. The genes encoding enterocyte differentiation markers sucrase, lactase and ALP are the target genes for Slp2, which directly promotes enterocyte differentiation.

These results showing the Slp2-driven modulation of the disaccharidase (sucrase and lactase) and ALP activities support the important role of Slp2 in the early intestinal development of newborns. Lactase and sucrase are intestinal microvillus membrane hydrolases responsible for the carbohydrate digestion. In the premature infants (26–34 weeks' gestation), lactase activity reaches about 30% of that of the term infants (maturational delay) [70]. Infants with lactose malabsorption are more prone to develop diarrhea [41]. Sucrase is a unique enzyme of the intestinal epithelium due to its high prevalence and its wide

substrate specificity for digestion of different dietary carbohydrates [42]. Therefore, deficiencies in the sucrase function can substantially disrupt the intestinal physiology, a fact associated with the gastrointestinal symptoms, weight loss, and immunological disorders mediated by altered gut microbiota. Intestinal ALP is expressed in the brush border of enterocytes, where it plays a key role in the gut defense [71]. This phosphatase can also be secreted in both the intestinal lumen and bloodstream [72,73]. In contrast to other phosphatases, intestinal ALP does not dephosphorylate proteins but removes phosphates present on the lipid A moiety (which allows lipopolysaccharide (LPS) to bind TLR4), reduces LPS toxicity, and cancels properties of LPS as an agonist of TLR4 [74]. Intestinal ALP expression in primary intestinal epithelial cells and Caco-2 cells markedly reduces LPS-induced nuclear factor-kappa B (NF-κB) responses and inflammation [75]. Oral administration of intestinal ALP impairs colitis induction in response to Dextran Sulfate Sodium (DSS) in the wild-type mice, but not in TLR4-deficient ($TLR4^{-/-}$) mice [76]. Intestinal ALP plays a crucial role in the regulation of gut microbiota function [77]. Upregulated ALP can selectively increase the growth of LPS-suppressing bacteria (*Bifidobacterium* genus), while reducing LPS-producing proteobacteria [78]. Having the capacity to inactivate LPS *in vivo*, ALP is vital in preventing the translocation of LPS, the pro-inflammatory stimulus originated from NEC-inducing proteobacteria [79,80]. Several molecules have been described to be involved in the induction of ALP activity, such as hyaluronan from human milk [81], omega-3, whey protein, and the short chain fatty acid-butyrate [78,82,83]. The presence of human milk oligosaccharides (HMOs) in human milk promote the growth of *Bifidobacterium* genus that have a role in neonatal intestinal maturation and immune tolerance [84] and LPS-suppressing bacteria [78]. During carbohydrate fermentation of HMOs, bifidobacteria enhance the butyrate-producing bacteria by producing acetate that is used as a co-substrate for butyrate synthesis [85]. Butyrate has been shown to increase ALP activity [82]. We found that Slp2 increases the activity of intestinal ALP (**Figure 3A**) in Caco-2 enterocytes and inhibits the adhesion of NEC-inducing pathogens to these intestinal cells (**Table 2**). At the same time, Slp2 stimulates the adhesion of bifidobacteria on Caco-2 enterocytes, ensuring the formation of normal parietal microflora of the newborns [24]. Therefore, this could be a mechanism behind the bifidobacteria correlation with Slp2 and ALP activities.

3.4 VEGF production in Caco-2 cells treated with Slp2

Caco-2 cells in preconfluent culture (2 day of after plating) were exposed to the different concentrations of Slp2 (0.1-100 ng/mL). Under the influence of Slp2 the level of vascular endothelial growth factor (VEGF) in Caco-2 cell culture supernatants increased in a dose-dependent manner (**Figure 5**). The minimum dose of Slp2 that stimulated VEGF production by Caco-2 cells was 1 ng/mL ($p<0.05$). It is known that in preterm infants the development of NEC is accompanied by inhibition of the process of re-epithelization in the damaged intestine. Impaired healing is due to a lack of growth factors in the area

of enterocytes damage [86,87]. VEGF is a potent survival factor of vascular endothelium, pulmonary, intestinal and vaginal epithelial cells [18,88].

3.5 Effect of Slp2 on Caco-2 cell monolayers resistance and permeability

Caco-2 monolayers in confluent culture (15 day after plating) treated with Slp2 in the concentration range of 10-100 µg/mL showed increased TEER values ($p<0.05$) compared with the untreated cells (**Figure 6A**). TEER is a classic indicator of the strength of tight junctions (TJs) and reflects the ionic conductance of the paracellular pathway in cell monolayers [89]. In according with the data obtained by us in this work, Slp2 is involved in the regulation of the intestinal epithelium barrier functions. Intestinal barrier integrity is a key feature in the health of humans, particularly newborns, because the immature gastrointestinal system is still developing [90]. The results in **Figure 6B** show the transport of sodium fluorescein across Caco-2 cell monolayers in the presence and absence of Slp2. The apparent P_{app} value of the control Caco-2 cell monolayers show a significant increase at 60 min. A longer incubation time of 120 min further increased the P_{app} value (**Figure 6B**). Caco-2 cell monolayers exposed to Slp2 had a lower P_{app} value, especially when 100 µg/mL Slp2 was used ($p<0.01$). These results suggest that Slp2 decreases the transport of sodium fluorescein dose-dependently in the Caco-2 cell monolayers, thereby improving the physical epithelial barrier. Slp2 can be considered as one of the components of a complex multilevel barrier system, which is necessary for maintaining the dynamic balance between the organism and intestinal tract. The intestinal mucosa forms a physical and metabolic barrier against the diffusion of pathogens, toxins, and allergens from the lumen into the circulatory system [91]. Intestinal permeability of term infants decreases in the first days of life [92,93]. This is associated with growth factors in human milk [92]. Preterm infants have impaired epithelial barrier function compared to term infants [94,95], which is thought to contribute to the pathogenesis of NEC [36–39,96]. Our data suggest that Slp2 has the properties of a growth factor that reduces intestinal permeability.

4. Conclusion

In this study, we have discovered new properties of soluble Slp2 from vaginal *L. crispatus* LC2029 strain on intestinal Caco-2 cells. Slp2 provides a physical barrier to protect intestinal Caco-2 cells from adhesion by NEC-inducing pathogens. Anti-adhesive activity of soluble Slp2 indicates its participation in regulation of parietal (membrane or contact) intestinal microbiota in the first days of life. Slp2 is also involved in the intestinal cell proliferation and differentiation. Slp2 enhances the activities of sucrase, lactase and ALP at both the mRNA and protein levels. This protein stimulates sucrase and lactase activity on subconfluent Caco-2 cells at an early phase of differentiation. Slp2 stimulates ALP activity on confluent Caco-2 cells at the late phase of differentiation. Slp2 enhances production of VEGF, decreases paracellular permeability and increases of TEER, strengthening barrier functions of Caco-2

cell monolayers. Together, the data indicate feasibility of using soluble Slp2 for comprehensive personalized prevention of NEC development in very low birth weight premature infants especially in caesarean-born cases. Our further *in vitro* research will be devoted to studying the effects of Slp2 on the expression of TJ proteins in primary human enterocytes. At the next stage, the acceleration of small intestine development and microbiome remodeling after Slp2 treatment in newborn mice will be studied.

Abbreviations

ALP – alkaline phosphatase

AP – apical inserts

BL – basolateral well

BV – bacterial vaginosis

DMEM – Dulbecco's modified Eagle's medium

DSS – Dextran Sulfate Sodium

ELISA – enzyme-linked immunosorbent assay

EPEC – enteropathogenic *Escherichia coli*

ETEC – enterotoxigenic *Escherichia coli*

FBS – fetal bovine serum

GIT – gastrointestinal tract

HBSS – Hanks balanced salt solution

HMOs – human milk oligosaccharides

KLH – keyhole limpet hemocyanin

LC2029 – *Lactobacillus crispatus* 2029

LPS – lipopolysaccharide

NEC – necrotizing enterocolitis

NF-κB – nuclear factor-kappa B

WB – Western blotting

P_{app} – apparent permeability coefficient

PBS – phosphate buffered saline

pNPP – p-nitrophenyl phosphate

qRT-PCR – real-time quantitative polymerase chain reaction

SEM – standard errors of the means

S-layer – surface-layer

Slp – S-layer protein

UTIs – urinary tract infections

TEER – transepithelial electrical resistance

TJ – tight junction

TLR4 – toll-like receptor 4

VEGF – vascular endothelial growth factor

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.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. **(A, B)** Effects of Slp2 on Caco-2 cell growth. Caco-2 cells were cultured with increasing concentrations (0.1-100 µg/mL) of Slp2. Incorporation of ^{3}H-thymidine **(A)** and cell count **(B)** were evaluated. Control corresponds to zero dose of Slp2. Data are means ± SEM of six independent experiments, tested in triplicate. **(C)** Effects of Slp2 on Caco-2 cell growth at different stages of differentiation. Cell growth was evaluated in Caco-2 cells at 3, 10, 15 days after plating using method of ^{3}H-thymidine incorporation. Caco-2 cells were cultured with 1 µg/mL (-■-) and 100 µg/mL (-●-) of Slp2. Data are expressed as percentage *vs* basal conditions (zero dose of Slp2) and are means ± SEM of six independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$; **$p<0.01$).

Figure 2. **(A, B)** Activity of sucrase **(A)** and lactase **(B)** in Caco-2 cells treated with different doses of Slp2. Caco-2 cells were cultured with increasing concentrations of Slp2 at 3 **(A)** and 6 **(B)** days after plating and sucrase **(A)** and lactase **(B)** activity was evaluated. Control corresponds to zero dose of Slp2. Data are expressed as nanomoles of glucose generated per minute and normalized for milligram of protein content. **(C)** Modulation of disaccharidase activities in Caco-2 cells by Slp2 at different stages of differentiation. Disaccharidase activities were evaluated in Caco-2 cells at 3, 6, 10, and 15 days after plating. Slp2 (100 ng/mL) induced significant stimulation of sucrase (-■-) and lactase (-●-) activities 3 and 6 days after plating respectively. Control corresponds to zero dose of Slp2. Data are means ± SEM of six independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$).

Figure 3. **(A)** Activity of ALP in Caco-2 cells treated with different doses of Slp2. Confluent Caco-2 cells were cultured with increasing concentrations of Slp2 at 15 days after plating and ALP activity was evaluated. Control corresponds to zero dose of Slp2. **(B)** Modulation of ALP activity by Slp2 in Caco-2 cells at different stages of differentiation. ALP activity was evaluated in Caco-2 cells at 3, 6, 10, and 15 days after plating. Slp2 (10 µg/mL) induced significant stimulation of ALP activity at 15 days after plating. Control corresponds to zero dose of Slp2. Data are means ± SEM of six independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$).

Figure 4. Relative concentration of mRNA for sucrase **(A)**, lactase **(B)** and ALP **(C)** in Caco-2 cells. Relative concentrations of sucrase **(A)**, lactase **(B)** and ALP **(C)** mRNA were determined by real-time quantitative PCR. GAPDH was used as an endogenous RNA control to normalize for differences in the amount of total RNA. Control corresponds to zero dose of Slp2. Data are expressed as relative mRNA

expression and are means ± SEM of three independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$).

Figure 5. Effects of Slp2 on production of VEGF by Caco-2 cells. Control corresponds to zero dose of Slp2. Data are means ± SEM of six independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$; **$p<0.01$).

Figure 6. Effects of Slp2 on TEER values **(A)** of Caco-2 cells and their P_{app} values **(B)**. Control corresponds to zero dose of Slp2. Data are means ± SEM of six independent experiments, tested in triplicate. Asterisks indicate significantly different from control (*$p<0.05$; **$p<0.01$).

Table 1. Microorganisms used in this study

Microorganism	Strain	Growth conditions
Lactobacillus crispatus	VKM[a] B-2727D	MRS[1] 37°C in CO_2 incubator, 10% CO_2 or anaerobically 48 h
Salmonella Enteritidis	ATCC[b] 13076	BHI[2] or LB[3] 37°C aerobically 18 h
Escherichia coli	ATCC E 2348/69 (EPEC[c])	The same
Escherichia coli	ATCC 31705 (ETEC[d])	The same
Campylobacter jejuni	ATCC 29428	BHI 37°C 48 h microaerobically (5% O_2, 10% CO_2, 85% N_2)
Pseudomonas aeruginosa	ATCC 27853	MH[4] 37°C aerobically 24 h

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

[c]Enteropathogenic *Escherichia coli*

[d]Enterotoxigenic *Escherichia coli.*

[1] Man-Rogosa-Sharpe (MRS) broth or agar containing plates (HiMedia, India).

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

[3] Luria-Bertrani medium (LB) broth or agar (HiMedia, India).

[4] Mueller-Hinton medium (MH) broth or agar (Merck, Germany).

Table 2. Slp2 reduction of the NEC-inducing proteobacteria strains adhesion to Caco-2 cells.

Proteobacteria strains	Adhesion bacteria per Caco-2 cells			
	Concentration of Slp2 in medium, µg/mL			
	0	10	50	100
Escherichia coli ATCC E 2348/69	34.7 ± 2.5	4.8 ± 0.3*	1.1 ± 0.5**	0.90 ± 0.06***
Escherichia coli ATCC 31705	31.5± 2.4	4.5 ± 0.6*	1.2 ± 0.4**	0.80 ± 0.04***
Salmonella Enteritidis ATCC 13076	26.1 ± 1.8	5.2 ± 0.4*	1.0 ± 0.3**	0.70 ± 0.04***
Compylobacter jejuni ATCC 29428	32.6 ± 1.9	6.1 ± 0.7*	1.1 ± 0.4**	0.80 ± 0.05***
Pseudomonas aeruginosa ATCC 27853	28.3 ± 2.6	4.3 ± 0.5*	1.3 ± 0.5**	0.60 ± 0.04***

*$p<0.05$ adhesion of strains (*Escherichia coli* ATCC E 2348/69, *Escherichia coli* ATCC 31705, *Salmonella* Enteritidis ATCC 13076, *Compylobacter jejuni* ATCC 29428, *Pseudomonas aeruginosa* ATCC 27853) to Caco-2 alone vs. adhesion to Caco-2 + Slp2 (10 µg/mL); **$p<0.01$ adhesion of strains to Caco-2 alone vs. adhesion to Caco-2 + Slp2 (50 µg/mL); ***$p<0.001$ adhesion of strains to Caco-2 alone vs. adhesion to Caco-2 + Slp2 (100 µg/mL). Date are presented as the means ± SEM of six independent experiments, tested in triplicate.

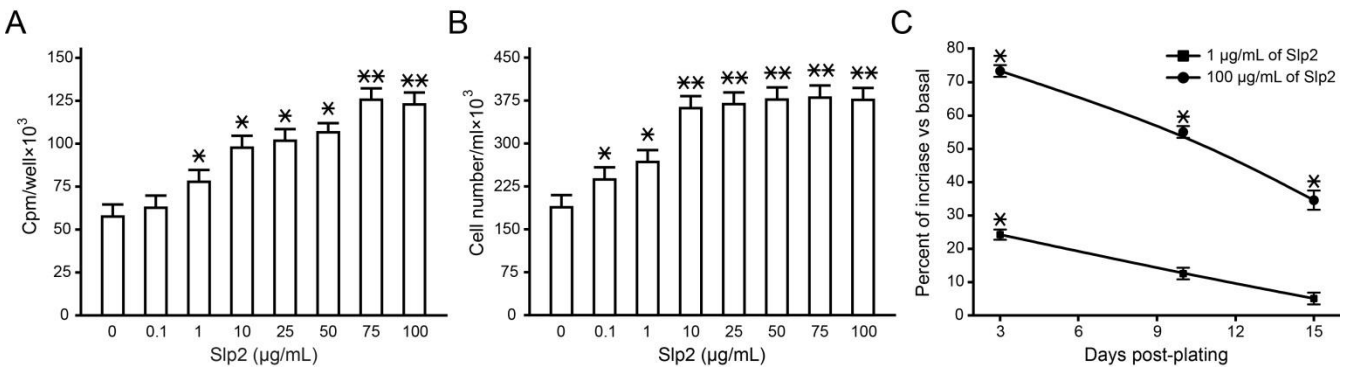
A
Cpm/well×10^3
150
125
100
75
50
25
0
0
0.1
1
10
25
50
75
100
Slp2 (μg/mL)
B
Cell number/ml×10^3
450
375
300
225
150
75
0
0
0.1
1
10
25
50
75
100
Slp2 (μg/mL)
C
Percent of incriase vs basal
80
70
60
50
40
30
20
10
0
3
6
9
12
15
Days post-plating
1 μg/mL of Slp2
100 μg/mL of Slp2

Figure 1

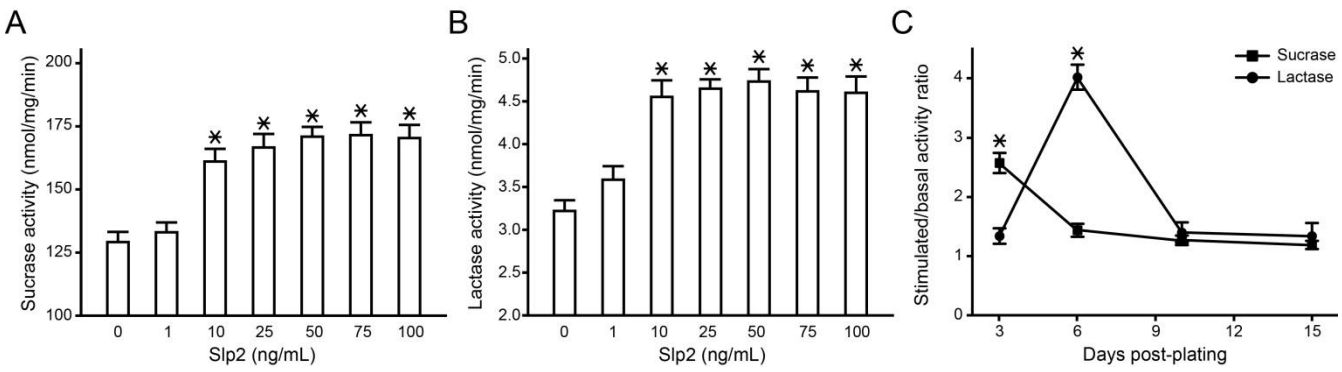
A
Sucrase activity (nmol/mg/min)
200
175
150
125
100
0
1
10
25
50
75
100
Slp2 (ng/mL)
B
Lactase activity (nmol/mg/min)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
1
10
25
50
75
100
Slp2 (ng/mL)
C
Stimulated/basal activity ratio
4
3
2
1
0
3
6
9
12
15
Days post-plating
Sucrase
Lactase

Figure 2

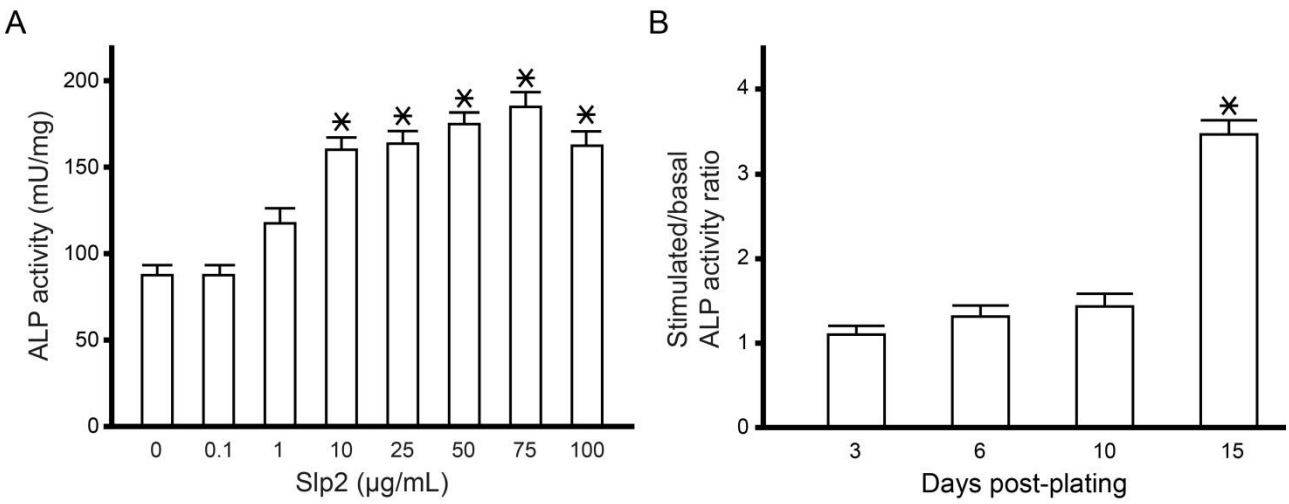
A
ALP activity (mU/mg)
200
150
100
50
0
0
0.1
1
10
25
50
75
100
Slp2 (µg/mL)
B
Stimulated/basal
ALP activity ratio
4
3
2
1
0
3
6
10
15
Days post-plating

Figure 3

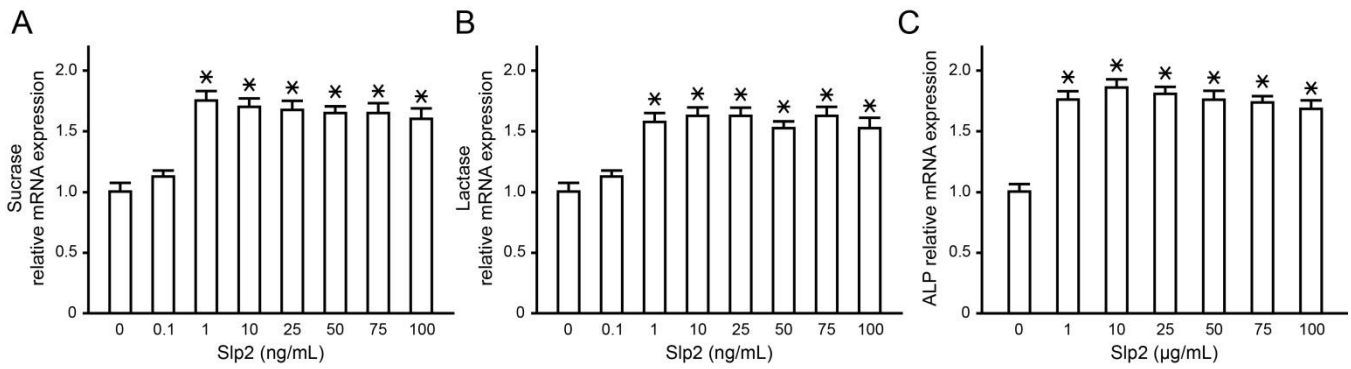
A
Sucrase
relative mRNA expression
2.0
1.5
1.0
0.5
0
0
0.1
1
10
25
50
75
100
Slp2 (ng/mL)
B
Lactase
relative mRNA expression
2.0
1.5
1.0
0.5
0
0
0.1
1
10
25
50
75
100
Slp2 (ng/mL)
C
ALP relative mRNA expression
2.0
1.5
1.0
0.5
0
0
1
10
25
50
75
100
Slp2 (μg/mL)

Figure 4

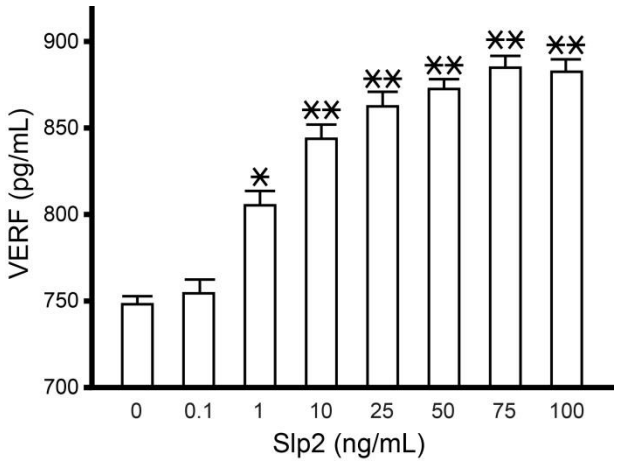

Figure 5

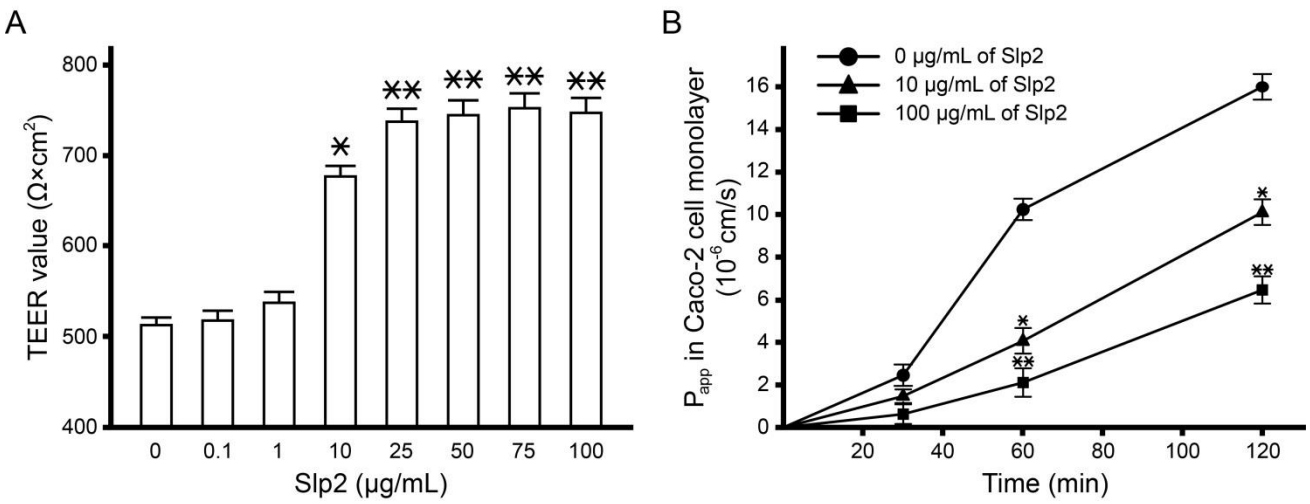
A
TEER value (Ω×cm²)
800
700
600
500
400
0
0.1
1
10
25
50
75
100
Slp2 (μg/mL)
B
0 μg/mL of Slp2
10 μg/mL of Slp2
100 μg/mL of Slp2
P_app in Caco-2 cell monolayer (10⁻⁶cm/s)
16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
Time (min)

Figure 6

Supplementary materials

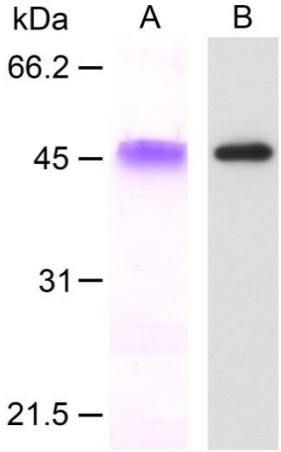

Figure S1. SDS-PAGE and Western blotting analysis of extracted and purified Slp2 from LC2029 strain. **(A)** SDS-PAGE (12% acrylamide) of Slp2 (6 µg/track) after Coomassie Brilliant Blue G-250 staining. **(B)** Western blotting of Slp2 detected by specific anti Slp2 mouse monoclonal antibodies. Sizes of the molecular mass marker proteins are indicated on the left.