


# Colonization of preterm gnotobiotic piglets with probiotic *Lactobacillus rhamnosus* GG and its interference with *Salmonella* Typhimurium

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## Summary

A balanced microbiota of the gastrointestinal tract (GIT) is a prerequisite for a healthy host. The GIT microbiota in preterm infants is determined by the method of delivery and nutrition. Probiotics can improve the GIT microbiota balance and suitable animal models are required to verify their harmlessness. Preterm gnotobiotic piglets were colonized with *Lactobacillus rhamnosus* GG (LGG) to evaluate its safety and possible protective action against infection with an enteric pathogen, *Salmonella* Typhimurium (ST). Clinical signs (anorexia, somnolence, fever and diarrhea), bacterial interference and translocation, intestinal histopathology, transcriptions of claudin-1, occludin and interferon (IFN)- $\gamma$ , intestinal and systemic protein levels of interleukin (IL)-8, IL-12/23 p40 and IFN- $\gamma$  were compared among (i) germ-free, (ii) LGG-colonized, (iii) ST-infected and (iv) LGG-colonized and subsequently ST-infected piglets for 24 h. Both LGG and ST-colonized the GIT; LGG translocated in some cases into mesenteric lymph nodes and the spleen but did not cause bacteremia and clinical changes. ST caused clinical signs of gastroenteritis, translocated into mesenteric lymph nodes, the spleen, liver and blood, increased claudin-1 and IFN- $\gamma$  transcriptions, but decreased occludin transcription and increased local and systemic levels of IL-8 and IL-12/23 p40. Previous colonization with LGG reduced ST colonization in the jejunum and translocation into the liver, spleen and blood. It partially ameliorated histopathological changes in the intestine, reduced IL-8 levels in the jejunum and plasma and IL-12/23 p40 in the jejunum. The preterm gnotobiotic piglet model of the vulnerable preterm immunocompromised infant is useful to verify the safety of probiotics and evaluate their protective effect.

**Keywords:** bacterial interference, gnotobiotic piglets, *Lactobacillus rhamnosus* GG, preterm, *Salmonella* Typhimurium

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## Introduction

Preterm infants are usually defined as infants born before 37 weeks' completed gestation. They are vulnerable due to underdevelopment of their organs and they show higher morbidity and mortality in comparison to their full-term counterparts [1]. A balanced gastrointestinal tract (GIT) microbiota has normally been established by the first year of life [2]. Its formation is highly influenced by the method of delivery. Babies born by vaginal delivery are colonized

by maternal vaginal and fecal microbiota immediately during birth [3] while colonization of babies delivered by cesarean section (CS) is impacted by the microbiota of hospital surroundings [4]. This hospital microbiota can cause nosocomial infections due to their frequent resistance to a broad spectrum of antibiotics [5]. Preterm infants are highly susceptible to dysbiosis and infections that can culminate in early (EOS) or late onset of sepsis (LOS) [6,7]. Probiotics are microbes that are commonly beneficial to their host [8] and their application influences and supports

the establishment of a balanced microbiota [2,9]. *Lactobacillus rhamnosus* GG (LGG) belongs to a group of the most frequently used probiotic bacteria applied as a therapeutic agent [10]. It was originally isolated by Goldin and Gorbach from human feces in 1983 and later used successfully for the treatment of relapsing *Clostridium difficile* colitis [11]. Many reports detail the beneficial effects of LGG to host health [10]. It was also shown to be effective in preterm infants in improving necrotizing enterocolitis (NEC) [12]. Despite the widely accepted beneficial role of probiotics, it is necessary to be careful regarding their application to immunocompromised preterm infants [13]. Consequently, suitable *in vivo* animal models are required to study and evaluate possible probiotic actions of candidate probiotics to verify their safety for the immunocompromised host. The pig shows closer physiological, anatomical and genetic similarities to the human compared to other animal models [14]. They have been used as the animal model of human gastrointestinal illnesses [15] and infectious diseases [16]. Preterm piglets are used as animal models of the preterm infant gastrointestinal tract [17] and necrotizing enterocolitis (NEC) [18]. The preterm germ-free piglet model has been established recently to study preterm immunocompromised host–bacteria interactions [19].

In contrast to probiotic *L. rhamnosus* GG, *Salmonella* Typhimurium is an obligatory human and animal gastrointestinal pathogen. It causes self-limiting enterocolitis in healthy humans [20], but it can provoke life-threatening systemic illness in immunocompromised individuals [21,22].

The aim of this work was to introduce a preterm gnotobiotic piglet model to evaluate the effect of probiotics, including their safety, on the immunocompromised host and their possible efficacy in protecting against infection with enteric pathogens. Possible amelioration of infection with non-typhoidal *S. Typhimurium* after previous colonization with *L. rhamnosus* GG was assessed in preterm gnotobiotic piglets. Clinical signs (anorexia, somnolence and diarrhea), intestinal histopathology, transcriptions of tight junction proteins claudin-1 and occludin in the ileum and the colon and intestinal and plasma levels of chemokine interleukin (IL)-8, proinflammatory cytokine IL-12/23 p40 and interferon (IFN)- $\gamma$  were evaluated. Additionally, a transcription of IFN- $\gamma$  in the ileum and the colon were detected to elucidate its possible participation and dynamics in *Salmonella*-provoked inflammatory response.

## Material and methods

### Ethical statement

All experiments with animals were approved by the Animal Care and Use Committee of the Institute of Microbiology in accordance with European Community laws.

### Bacterial suspensions

Bacterial suspensions comprised *L. rhamnosus* GG (LGG) ATCC 53103 (via a Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic – CCM 7091) and *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 (ST) was from a collection of the microorganisms of the Institute of Microbiology, Novy Hradek, Czech Republic.

Fresh cultures of bacteria were prepared for each experiment by cultivation at 37°C overnight. LGG was cultivated in 10 ml MRS broth (Oxoid, Basingstoke, UK). The cells were harvested by centrifugation at 4000 g for 10 min. The pellet was washed twice with 0.05 M phosphate buffer. ST was cultivated on meat-peptone agar slopes (blood agar base; Oxoid). Both bacteria were resuspended to an approximate density of  $5 \times 10^8$  colony-forming units (CFU)/ml. The number of CFU that was estimated by spectrophotometry at 600 nm was verified by a cultivation method.

### Gnotobiotic piglets

The preterm gnotobiotic piglets were obtained by hysterectomy of pregnant miniature Minnesota-derived sows [23] (Animal Research Institute, Kostelec nad Orlici, Czech Republic) on the 104th day of gestation (average full term in these miniature sows is 112 days), under isoflurane anesthesia (Piramal Healthcare UK Ltd, Morpeth, UK) and reared in fiberglass isolators (Supporting information, Fig. S1), as described elsewhere [19]. Briefly, the piglets were bottle-fed using a cow's milk-based diet (Mlekarna Hlinsko, Hlinsko, Czech Republic). Initial feeding was performed by dripping the diet onto the tongue to provoke a swallowing reflex. The piglets received 50 mg of the iron–dextran complex (Ferribion; Bioveta, Ivanovice na Hane, Czech Republic) and 5 mg of phytomenadione (vitamin K) (Kanavit; HBM Pharma, Martin, Slovakia) intramuscularly 2 h after hysterectomy. Twenty-six preterm gnotobiotic piglets were grouped into four groups (Supporting information, Fig. S2): (i) sterile for the whole experimental period (germ-free; GF,  $n = 6$ ), (ii) orally colonized with  $1 \times 10^8$  CFU of *L. rhamnosus* GG 4 h after hysterectomy (LGG,  $n = 8$ ), (iii) 1-week-old GF piglets orally infected with  $1 \times 10^8$  CFU of *S. enterica* serovar Typhimurium for 24 h (ST,  $n = 6$ ) and (iv) 1-week-old LGG-colonized piglets orally infected with  $1 \times 10^8$  CFU of *S. Typhimurium* for 24 h (LGG+ST,  $n = 6$ ).

### Inspection of gnotobiotic state

Amniotic membranes, umbilical cords, meconium, mouth and isolator surface smears after hysterectomy and later, mouth and surface smears and stool, were cultivated twice a week for the presence of aerobic and anaerobic bacteria

and mold. Additionally, Gram-stained rectal swabs were inspected under a light microscope.

### Blood plasma and intestinal lavages

At the end of the experiment the piglets were euthanized by cardiac puncture exsanguination under general isoflurane inhalation anesthesia. The citrated blood was spun at 1500 g for 15 min, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was added and the plasma was stored at  $-45^{\circ}\text{C}$  until cytokines were measured. Forty cm of the proximal jejunum (marked in the text as jejunum) and the ileum with the distal jejunum (marked in the text as ileum) were filled with 2 ml of Dulbecco's phosphate-buffered saline (DPBS; Life Technologies, Carlsbad, CA, USA). They were gently kneaded and rinsed. The colon was cut into small pieces and was rinsed with 4 ml of DPBS. Parts of these intestinal lavages were used immediately for CFU counting. Other parts with added a protease inhibitor cocktail (Roche Diagnostics) were spun at 2500 g, pressed through 0.20  $\mu\text{m}$  acetate cellulose filter (Sartorius AG, Goettingen, Germany), and the aliquots were stored at  $-45^{\circ}\text{C}$  until following analyses.

### CFU counting

The intestinal lavages before addition of the protease inhibitor cocktail (Roche) were log<sub>10</sub> diluted in phosphate-buffered saline (PBS) and cultivated in 90 mm Petri dishes – LGG on MRS agar (Oxoid) with acetic acid at  $37^{\circ}\text{C}$  for 72 h and ST on McConkey agar (Merck, Darmstadt, Germany) at  $37^{\circ}\text{C}$  for 24 h. The CFU from dishes containing 20–200 colonies were counted. Mesenteric lymph nodes, liver and spleen were homogenized in deionized water in a 2 ml Eppendorf tube by shaking with 3.2 mm stainless steel beads in a TissueLyser LT beadbeater (Qiagen, Hilden, Germany) for 3 min at 50 Hz. The tissue homogenates were cultivated in the same way as the intestinal lavages.

### Histopathological evaluation

Terminal ileum samples were fixed in Carnoy's fluid for 30 min, dehydrated and embedded in paraffin. Five  $\mu\text{m}$  tissue sections were cut on a Leica microtome RM2245 (Leica Microsystems, Wetzlar, Germany), stained with hematoxylin and eosin and examined under an Olympus BX 40 microscope with a Olympus Camedia C-2000 digital camera (Olympus, Tokyo, Japan). Sections were evaluated in a blinded fashion. Averages of 30 evenly spaced radial lamina mucosalis propria widths per each piglet were determined. Ten measurements for each parameter were taken per piglet to assess ileal villus length and crypt depth. A histopathological score to evaluate differences between the piglet groups was suggested: (i) submucosal

edema (0–2 score points), (ii) polymorphonuclear neutrophils (PMNs) infiltration into the lamina propria (0–2 score points), (iii) villus atrophy (0–3 score points), (iv) exudate in lumen (0–2 score points), (v) vessels dilatation (0–2 score points), (vi) inflammatory cellularity in lymphatic vessel lumen (0–2 score points), (vii) hyperemia (0–2 score points), (viii) hemorrhage (0–2 score points), (ix) peritonitis (0–1 score points) and (x) erosion of the epithelial layer (0–3 score points). The total score of 0–21 points was obtained.

### Purification of total RNA and reverse transcriptions

One to 2 mm-thin cross-sections of the terminal ileum and transversal colon were stored in RNAlater at  $-20^{\circ}\text{C}$  until the purification of total RNA was performed. A 10 mg sample was moved from RNAlater to a 2 ml Eppendorf tube containing 600  $\mu\text{l}$  RLT buffer of the RNeasy Mini Plus kit (Qiagen) with addition 1/1000 of anti-foaming reagent DX (Qiagen) and 2 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA). The tissue was homogenized at 50 Hz for 5 min in TissueLyser LT beadbeater (Qiagen). Other purification steps followed the manufacturer's instructions. Five hundred ng of total RNA with ratio absorbances  $A_{260}-A_{320}/A_{280}-A_{320} \geq 2.0$  measured in DEPC-treated 10 mM Tris-HCl buffer pH 7.5 were reverse-transcribed. The transcriptions with an initial DNA wipe-out buffer incubation was performed at  $42^{\circ}\text{C}$  for 20 min and finished by heating at  $95^{\circ}\text{C}$  for 3 min using the QuantiTect Reverse Transcription kit (Qiagen). The synthesized cDNA was diluted 1 : 10 by PCR quality water (Life Technologies, Carlsbad, CA, USA) and these prepared templates were stored at  $-25^{\circ}\text{C}$  until the following real-time-polymerase chain reaction (RT-PCR).

### RT-PCR

A Universal Probe Library locked nucleic acids (LNA) probe-based RT-PCR system (www.universalprobelibrary.com) was used. Two  $\mu\text{l}$  of cDNA template were added to 18  $\mu\text{l}$  of the FastStart Universal Probe Master (Roche Diagnostics) containing 100 nM LNA probe (Roche Diagnostics) and 500 nM each of the forward and reverse primers (Table 1) to quantify specific sequences in the cDNA templates. The mixtures were incubated and measured on an iQ cycler (Bio-Rad, Hercules, CA, USA) equipped with the iQ5 Optical System software version 1.0 (Bio-Rad). Initial heating for 10 min at  $95^{\circ}\text{C}$  followed by 45 cycles at  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 60 s were used to measure  $C_q$  in duplicate. The obtained values for claudin-1, occludin and IFN- $\gamma$  were normalized using  $\beta$ -actin and cyclophilin A and their relative expressions were calculated by Genex 6 software (Multid AB, Gothenburg, Sweden).

**Table 1.** Locked nucleic acids based real-time polymerase chain reaction systems used in analyses

Gene	GenBank access	Forward primer		#LNA probe
		Reverse primer		
$\beta$ actin	U07786	TCCCTGGAGAAGAGCTACGA		9
Cyclophilin A	NM214353	AAGAGCGCCTCTGGACAC		48
Claudin-1	NM001244539	CCTGAAGCATACGGGTCTT		3
Occludin	U79554	AAAGACCACATGTTTGCCATC		42
Interferon- $\gamma$	NM_213948	CACCACTTTGCAAGCAACC		21
		TGGCCACAAAGATGGCTATT		
		AAAGAGCTCTCTCGACTGGATAAA		
		AGCAGCAGCCATGTACTCTTC		
		TGGAAAGAGGAGAGTGACAAAAA		
		GAATGGCCTGGTTATCTTTGA		

### Luminex xMAP technology

IL-8 (attraction and activation of PMNs [24]), IL-12/23 p40 (early sepsis marker in the pig [25]) and IFN- $\gamma$  (combat intracellular infections [26]) in the intestinal lavages and plasma were measured by a paramagnetic sphere-based Luminex xMAP technology using a Porcine ProcartaPlex kit (Affymetrix, Santa Clara, CA, USA). The spheres were labeled according to the manufacturer's instructions and were washed on a Hydroflex washer with a magnetic plate (Tecan, Groedung, Austria). The cytokines were measured on Bio-Plex Array System and evaluated by Bio-Plex Manager version 4.01 software (Bio-Rad).

### Statistical analysis

Differences between  $\log_{10}$  bacterial CFU of LGG (LGG versus LGG+ST) and ST (ST versus LGG+ST) were counted using the unpaired two-tailed *t*-test. Relative transcriptions of claudin-1, occludin and IFN- $\gamma$  were compared among piglet groups using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons *post-hoc* test and concentrations of IL-8, IL-12 p40 and IFN- $\gamma$  with Kruskal-Wallis test with Dunn's multiple comparison *post-hoc* test. The statistical analyses and graphs were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and statistical significances were assessed at  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$  ( $\log_{10}$  CFU) or  $P < 0.05$  (multiple comparisons).

### Results

The preterm piglets had closed eyes on the day of hysterectomy and usually opened them on the third day of life (Supporting information, Fig. S2). They also showed other signs of immaturity, as described elsewhere [19].

### Bacterial colonization, interferences and translocation

*L. rhamnosus* GG (LGG) and *S. Typhimurium* (ST) colonization of the intestine and their translocation and

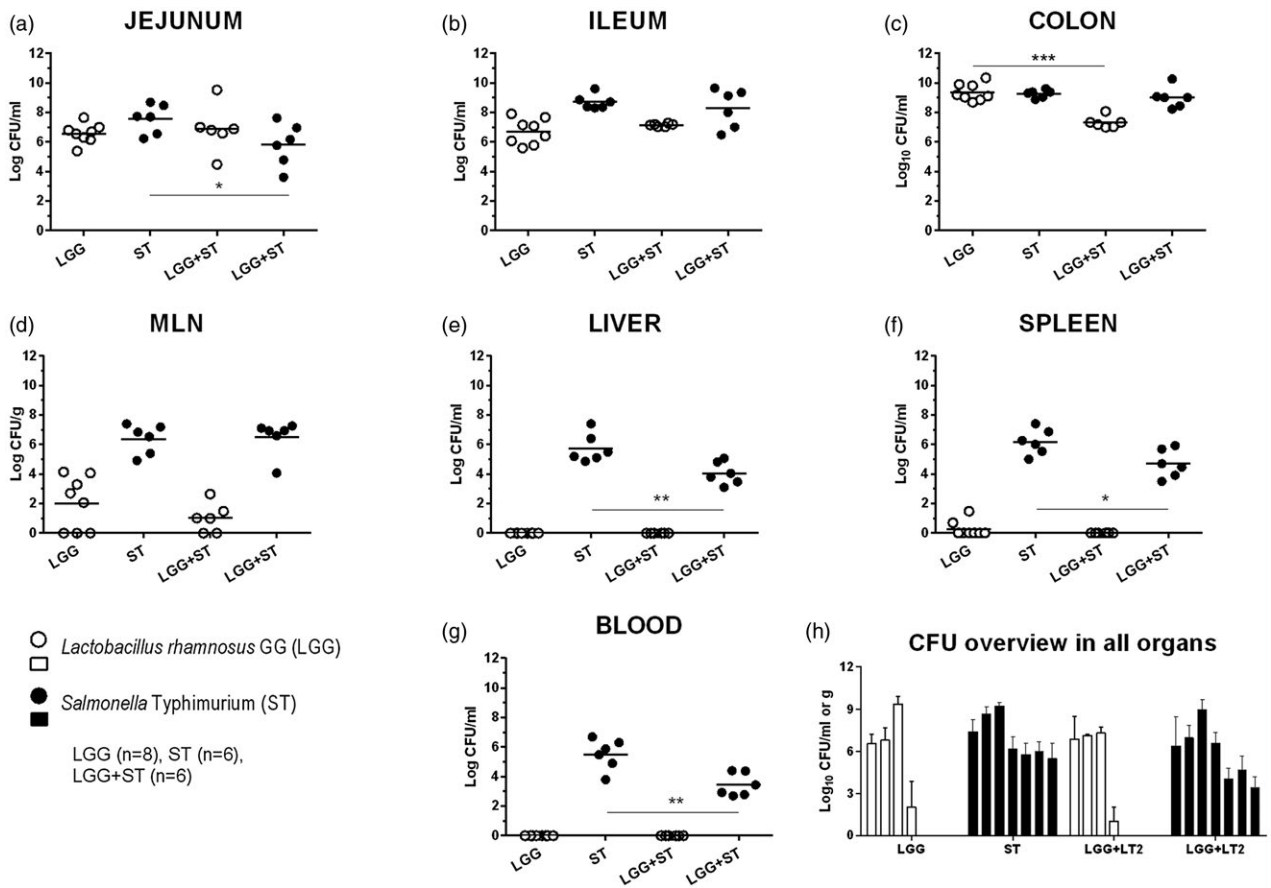
interferences were evaluated by the cultivation methods (Fig. 1). Individual piglet CFU in the jejunum, ileum, colon, mesenteric lymph nodes (MLN), liver, spleen and blood are represented by spots, and the group mean is depicted by the horizontal line (Fig. 1a–g). A summary overview of both bacteria CFUs in all organs are shown for each group as columns with mean + standard deviation (s.d.) (Fig. 1h).

Both LGG and ST showed good ability to multiply in the intestine of the gnotobiotic piglets (Fig. 1a–c). LGG decreased ST growth in the LGG+ST piglets in the jejunum (Fig. 1a,  $*P < 0.05$ ) but not in the ileum and colon (Fig. 1b,c). In contrast, the LGG CFU were diminished in the ST presence in the LGG+ST group in the colon (Fig. 1c,  $***P < 0.001$ ). LGG translocated to MLN, but not in all LGG-colonized piglets (Fig. 1d). LGG did not spread to the liver (Fig. 1e) and blood (Fig. 1g), while it was cultivated from the spleen in two of eight piglets in the LGG group (Fig. 1f). ST translocated to MLN (Fig. 1d), liver (Fig. 1e) and spleen (Fig. 1f) and caused bacteremia (Fig. 1g) in both *Salmonella*-infected ST and LGG+ST groups. ST CFU were diminished in the presence of LGG in the liver (Fig. 1e,  $**P < 0.01$ ), spleen (Fig. 1f,  $*P < 0.05$ ) and blood (Fig. 1g,  $**P < 0.01$ ). The summary of these results is shown in Fig. 1h.

### Histopathological evaluation

Both the enterocytes in the ileum of the GF piglets ( $n = 6$ ) (Fig. 2a,b) and the LGG piglets ( $n = 8$ ) (Fig. 2c,d) showed long villi with the abundant presence of vacuolated enterocytes. The villus height was comparable, the crypts were deeper but statistically non-significant and the muscular layer was thicker ( $P < 0.01$ ) in LGG than in the GF group (Supporting information, Fig. S3). Villus atrophy in *Salmonella*-infected piglets (ST and LGG+ST, both  $n = 6$ , Fig. 2e–h) resulted in the markedly lowered presence of the vacuolated enterocytes in the villi. Villi disappeared completely in some parts of the ileum of these piglet groups, but crypts retained their appearance, comparable





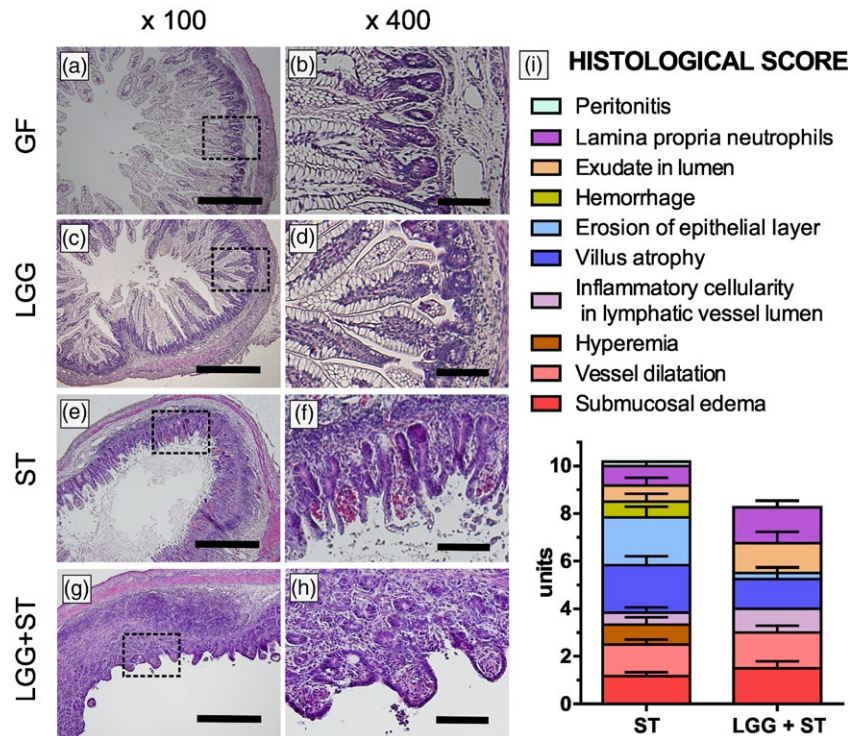
**Fig. 1.** Bacterial counts in the intestine, mesenteric lymph nodes, liver, spleen and blood. *Lactobacillus rhamnosus* GG (LGG) and *Salmonella* Typhimurium (ST) colony-forming units (CFUs) were counted in the jejunum (a, proximal jejunum), the ileum (b, a distal part of the jejunum and the whole ileum), the colon (c), mesenteric lymph nodes (d, MLN), the liver (e), the spleen (f) and blood (g). LGG is depicted as white circles (○, a–g) or columns (□, h) and ST as black circles (●, a–g) or columns (■, h). Differences between LGG CFUs (LGG,  $n = 8$  versus LGG+ST,  $n = 6$ ) and ST CFUs (ST,  $n = 6$  versus LGG+ST,  $n = 6$ ) were evaluated by unpaired two-tailed *t*-test and statistical differences were marked with asterisks as statistically significant (\* $P < 0.01$ ), \*\* $P < 0.01$  and \*\*\* $P < 0.01$ ). A summary CFU overview is shown on a column graph (h) as mean + standard deviation (s.d.) (without statistical significance).

with the ileum of GF and LGG piglets. The cellularity of the lamina propria and submucosa was increased in the LGG group compared to the GF. No inflammation in the preterm GF piglets and piglets colonized with LGG for 7 days was found on hematoxylin and eosin histological ileum slides. In contrast, the histopathological analysis of the ileal sections in the piglets infected with *Salmonella* (ST, Fig. 2e,f and LGG+ST, Fig. 2g,h) showed acute inflammation. Using our scoring system, the total histological score was 10.1 for the ST group and 8.2 for the LGG+ST group (Fig. 2i). Both groups showed submucosal edema, PMN infiltration into the lamina propria, villus atrophy, exudate in the lumen, vessel dilatation and inflammatory cellularity in the lymphatic vessel lumen. Additionally, typical features of the ST group were hyperemia, hemorrhage and multiple erosion, but without intensive PMN

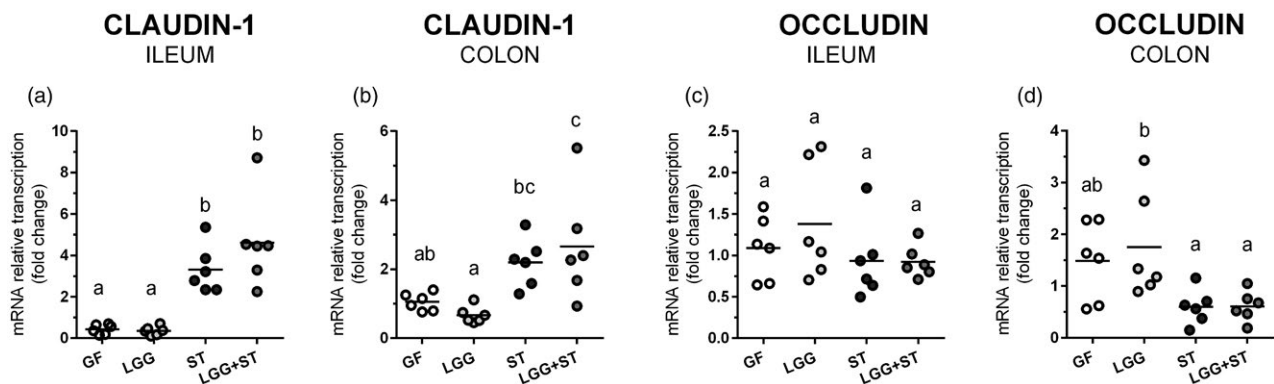
infiltration (Fig. 2e,f), that was typical for the LGG+ST group (Fig. 2g,h). Erosion of the intestinal epithelium was only sporadic in the LGG+ST group.

### Transcriptions of tight junction proteins claudin-1 and occludin in the intestine

The transcription of claudin-1 in the ileum was low and comparable in GF and LGG piglets (Fig 3a,b). Both groups of *Salmonella*-infected piglets (ST and LGG+ST) showed statistically significant increased transcriptions (Fig 3a). In the colon, the transcription was higher in the GF than in the LGG group, and the statistically significant differences were between the LGG and ST groups but not between the GF and ST groups. The occludin transcription (Fig 3c,d) showed an opposite trend than claudin-1 – higher transcription was in the GF and LGG



**Fig. 2.** Representative hematoxylin and eosin-stained cross-sections of the ileum in the preterm gnotobiotic piglets and a histological score. Representative hematoxylin and eosin-stained cross-sections of the ileum in preterm gnotobiotic piglets: 1-week-old germ-free piglets (GF; a,b), piglets colonized with *Lactobacillus rhamnosus* GG for 1 week (LGG; c,d), 1-week-old piglets infected with *Salmonella* Typhimurium for 24 h (ST; e,f) and piglets colonized with LGG for 1 week and infected with ST for 24 h (LGG+ST; g,h) were compared. Bars represent 500  $\mu$ m (a,c,e,g) and 100  $\mu$ m (b,d,f,h). Histological score from the ileum of six piglets per group is depicted (i).



**Fig. 3.** Transcriptions of tight junction proteins claudin-1 and occludin in the ileum and the colon. Claudin-1 and occludin mRNAs in the terminal ileum and the transversal colon were normalized to  $\beta$ -actin and cyclophilin A and their relative transcriptions are depicted. The preterm germ-free (GF), *Lactobacillus rhamnosus* GG-colonized (LGG), *Salmonella* Typhimurium-infected (ST) and *L. rhamnosus* GG-colonized and *S. Typhimurium*-infected (LGG+ST) piglets were compared. One-way analysis of variance (ANOVA) with Tukey's multiple comparison *post-hoc* test was used to compare the groups and the values are presented as individual dots and mean (horizontal line). Statistical differences  $P < 0.05$  are marked with different letters above the dots and the same letter indicates no significant differences. Six samples in each group were analyzed.

groups and lower in *Salmonella*-infected piglets. These changes were without statistical significance among groups in the ileum (Fig. 3c), but statistically significant decreases were found between LGG and both *Salmonella*-infected groups in the colon (Fig. 3d).

#### Local and systemic levels of IL-8, IL-12/23 p40 and IFN- $\gamma$

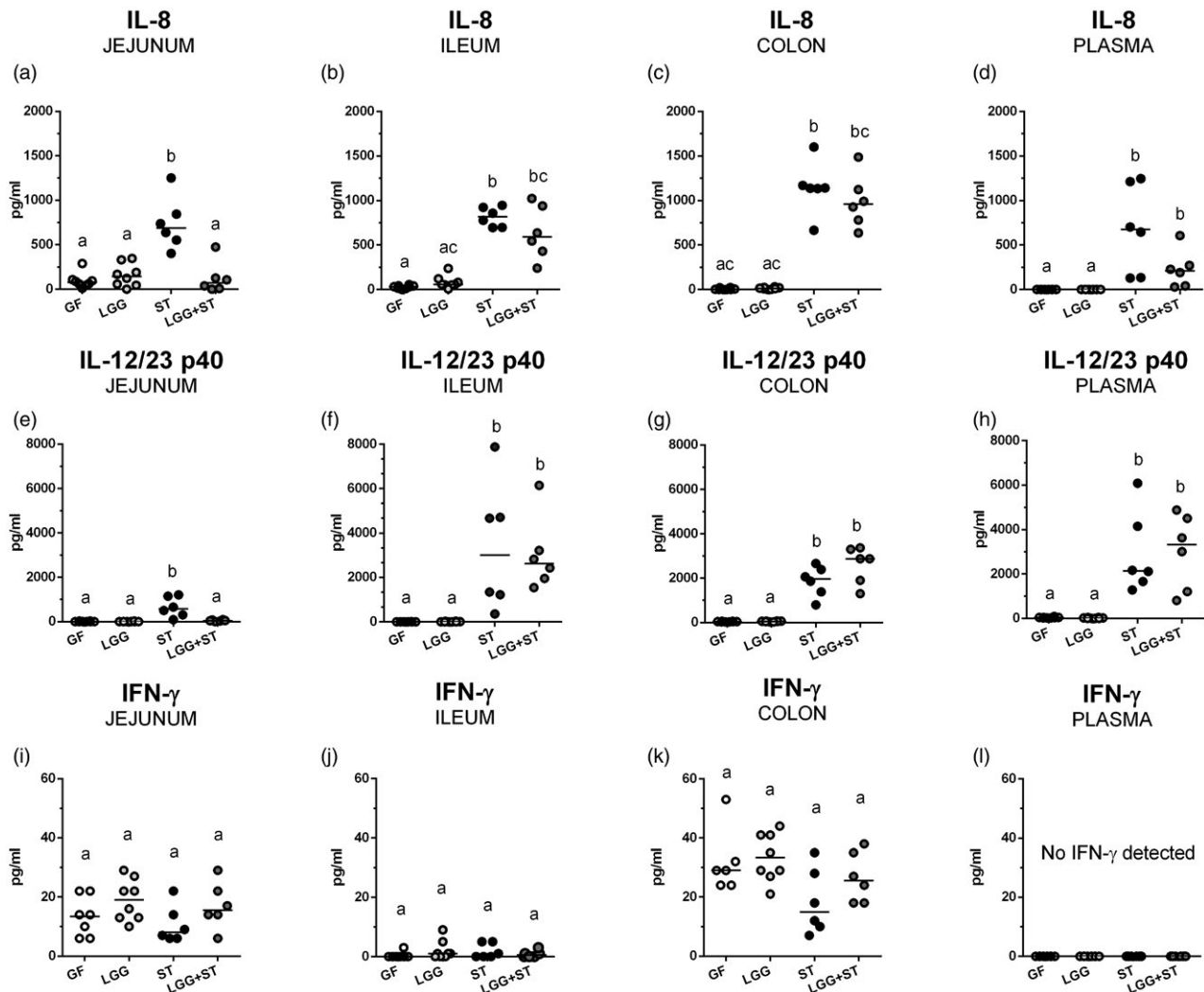
Values of IL-8, IL-12/23 p40 and IFN- $\gamma$  were compared among all groups in the intestinal lavages and plasma.

Although we compared differences among all the groups, in the following text we mainly report crucial changes of GF *versus* ST and ST *versus* LGG+ST to simplify the description of the results obtained.

IL-8 medians in the jejunum (Fig. 4a), ileum (Fig. 4b) and colon (Fig. 4c) in GF and LGG groups were lower than 200 pg/ml or were undetectable in plasma (Fig. 4d). Infection with *S. Typhimurium* in the ST group statistically significantly induced production of IL-8 in the jejunum, ileum, colon and plasma (Fig. 4a–d). The preliminary colonization of the gastrointestinal tract with LGG in the LGG+ST piglets decreased IL-8 values in the intestine and plasma compared with the ST group, but these

decreases were statistically significant in the jejunum (Fig. 4a) only.

IL-12/23 p40 levels were minute in the GF and LGG groups (Fig. 4e–h). They were statistically significantly increased in the *Salmonella*-infected (ST) jejunum (Fig. 4e), ileum (Fig. 4f), colon and plasma (Fig. 4g,h). Previous colonization with LGG protects piglets infected with *Salmonella* (LGG+ST) against this increase in the jejunum (Fig. 4e). This IL-12/23 p40 lowering effect of LGG was absent in other parts of the intestine and plasma, where the levels of this cytokine subunit were significantly higher in the ileum (Fig. 4f), the colon (Fig. 4h) and plasma (Fig. 4g) if compared to the GF group.



**Fig. 4.** Protein levels of interleukin (IL)-8, IL-12/23 p40 and interferon (IFN)- $\gamma$  in the jejunum, ileum, colon and plasma. Levels of IL-8 (a–d), IL-12/23 p40 (e–h) and IFN- $\gamma$  (i–l) in the jejunum (a,e,i), ileum (b,f,j), colon (c,g,k) and plasma (d,h,l) of the preterm germ-free (GF,  $n = 6$ ), *Lactobacillus rhamnosus* GG-colonized (LGG,  $n = 8$ ), *Salmonella Typhimurium*-infected (ST,  $n = 6$ ) and *L. rhamnosus* GG-colonized and *S. Typhimurium*-infected (LGG+ST,  $n = 6$ ) piglets were compared. Kruskal–Wallis test with Dunn’s multiple comparison *post-hoc* test was used to compare the groups. The results are presented as individual dots and median (horizontal line). Statistical differences  $P < 0.05$  are denoted with different letters above the dots, and the same letter indicates no significant differences.

IFN- $\gamma$  levels reached the highest, but still relatively low values, in the colon (Fig. 4k), lower in the jejunum (Fig. 4i) and lowest in the ileum (Fig. 4j). No statistically significant differences between the groups in any part of the intestine were observed and no IFN- $\gamma$  levels were detected in blood plasma (Fig. 4l).

### Transcriptions of IFN- $\gamma$ in the ileum and the colon

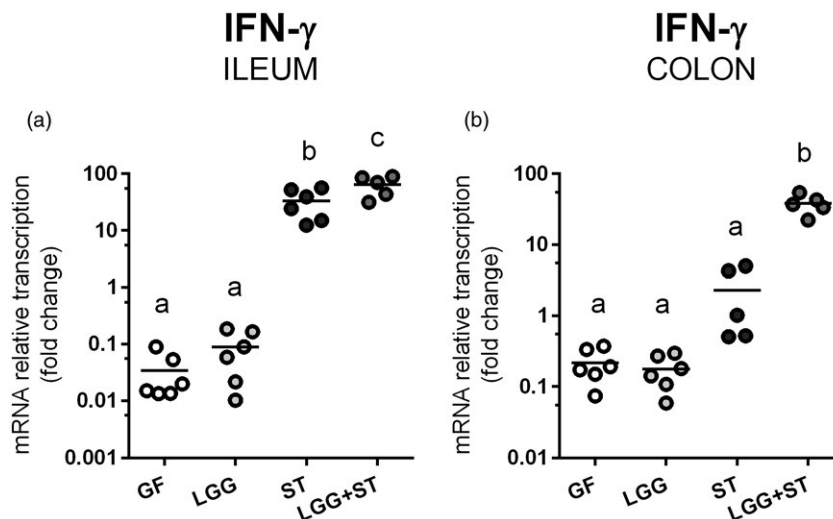
Transcriptions of IFN- $\gamma$  were the lowest in the intestine in the GF and LGG piglets (Fig. 5a,b). The presence of ST alone triggered the IFN- $\gamma$  transcription in the intestine, and previous colonization with LGG potentiated these transcriptions. *Salmonella* increased IFN- $\gamma$  transcriptions against the GF state in the ileum in ST piglets (Fig. 5a). After previous colonization with LGG (LGG+ST) these differences were even more statistically relevant. This previous colonization with LGG also influenced differences between ST and LGG+ST groups in the ileum. The greatest increase after previous colonization with LGG was found in the colon (Fig. 5b). While the differences between the GF or LGG groups to the ST group were not statistically significant in the colon, the differences in the GF or LGG groups to the LGG+ST group were significant.

### Discussion

Preterm infants have underdeveloped organ systems, including those of the immune system. They suffer from severe acute and chronic morbidities, e.g. intraventricular

hemorrhage, necrotizing enterocolitis, bronchopulmonary dysplasia, chronic lung disease, severe visual impairment, hearing impairment, cerebral palsy and cognitive developmental delay [1]. Probiotics are used in preterm infants as prophylaxis of NEC and sepsis [27]. *L. rhamnosus* GG is a useful, time-proven probiotic bacteria [10] but it is able to endanger immunocompromised patients [28] including newborn infants [29]. These events accentuate the need to take into account the increased vulnerability of preterm infants to infections and sepsis.

The epitheliochorial placentation of the pig prevents the prenatal transfer of immunoglobulins, and underdeveloped humoral and cell-mediated immunity have to be supplied via colostrum intake after birth to thrive in conventional conditions [30], while the colostrum-deprived conventional piglets succumb to bacterial sepsis [31]. The preterm piglet model of immunocompromised infants [18] shows similarities in organ development and clinical features with preterm infants on the 75% of gestation that corresponds to 32 weeks in the human [17,32]. Surgically derived piglets thrive in the sterile conditions of a gnotobiotic isolator without the intake of colostrum [19,33]. Germ-free animals show lower colonization resistance due to an absence of balanced microbiota. This all assists in the manifestation of the potential opportunistic pathogenicity of probiotics. Additionally, the immaturity of preterm germ-free piglets [19] contributes to the vulnerability to bacterial attack, as it is known in preterm infants [34]. In contrast to the host-beneficial properties of *L. rhamnosus* GG, the *Salmonella* genus comprises food-borne



**Fig. 5.** Relative expression of interferon (IFN)- $\gamma$  in the ileum and the colon. IFN- $\gamma$  mRNA was normalized to  $\beta$ -actin and cyclophilin A and the relative transcription of the IFN- $\gamma$  gene in the ileum (a) and colon (b) in the preterm germ-free (GF), *Lactobacillus rhamnosus* GG-colonized (LGG), *Salmonella* Typhimurium-infected (ST) and *L. rhamnosus* GG-colonized and *S. Typhimurium*-infected (LGG+ST) piglets were compared. One-way analysis of variance (ANOVA) with Tukey's multiple range *post-hoc* test was used to compare the groups. The results are presented as individual dots and mean (horizontal line). The statistical differences are denoted with different letters above the dots and the same letter indicates no significant differences. Six samples were analyzed in each group.



pathogens [35] that can cause life-threatening illness in immunocompromised hosts [21,22].

The hysterectomy-derived preterm germ-free piglets in our experiments were colonized with *L. rhamnosus* GG (LGG) 4 h after hysterectomy. At this time the piglet intestinal barrier is passable for intact colostral immunoglobulins and cells [30]. We supposed that possible LGG translocation could be facilitated in this early post-natal period, and the manifestation of opportunistic pathogenicity of LGG occurred as described in immunocompromised hosts [28,29]. While one section of the LGG-colonized piglets was kept monocolonized for the entire week-long experiment (LGG), another section was infected with *S. Typhimurium* strain LT2 for 24 h (LGG+ST) at 1 week of age. Our intention in this section of the experiments was to verify if LGG translocates and if previous colonization with LGG could ameliorate signs of salmonellosis.

Mechanisms of LGG actions against *Salmonella* are not fully understood, and bacterial interferences use different principles such as inhibition of adhesion and biofilm formation [36], production of lactic acid [37] and others. Positive effects of LGG against enterocolitis in animal models [38–40] or in infants [9] have been observed. However, other authors did not confirm such beneficial effects. LGG-caused exacerbation of mouse colitis has also been reported [41].

The balanced microbiota in conventional hosts is essential for their health [2] but dysbiosis, for example, caused by antibiotic therapy, can disturb this balance and commensal bacteria can turn into opportunistic pathogens [42]. Antibiotic treatment provoked GIT dysbiosis in C57BL/6 mice, which resulted in the translocation of commensal bacteria into colonic mesenteric lymph nodes [43]. The translocation of LGG into mesenteric lymph nodes, spleen and liver but no bacteremia was described in 6-week-old gnotobiotic piglets which were colonized at 7 days of age with LGG and were challenged with human rotavirus 2 weeks later [44]. In this case, the rotavirus probably disrupted the intestinal barrier which aided the bacterial translocation. Bacterial monocolonization in the gnotobiotic piglets may share some characteristics of the dysbiosis. In our experiments, the LGG successfully colonized the GIT of the preterm monocolonized piglets and its translocation into mesenteric lymph nodes in five of eight piglets, and the spleen in two of eight piglets could suggest the opportunistic pathogenicity of LGG. The lactobacilli were, in the majority of the piglets of the LGG group, successfully trapped in mesenteric lymph nodes which prevented their systemic spread, and no bacteremia was found in any piglet. All these piglets thrived and showed no clinical signs such as anorexia, somnolence, fever and diarrhea.

The ingested *Salmonella* adhere in the intestine and multiplies itself [45]. Its translocation can be proceeded by four different mechanisms: (i) via M cells that overlay Peyer's patches [46], (ii) by an internalization through enterocytes [47], (iii) by entering between epithelial cells with intact tight junctions engulfed by macrophages [48] or dendritic cells [49] and (iv) by a paracellular way through disrupted tight junctions [50]. The virulence of the LT2 strain of *S. Typhimurium* used for term germ-free piglets was documented in our previous experiments [51–53]. In the present experiments, *S. Typhimurium* quickly multiplied in the 1-week-old preterm gnotobiotic piglet intestine over 24 h, translocated into all observed organs, caused bacteremia and induced clinical signs of enterocolitis. These *Salmonella*-infected piglets (ST) suffered from anorexia, somnolence, fever and diarrhea. In the piglets previously colonized with LGG and later infected with *Salmonella* (LGG+ST), LGG translocated in the majority of the piglets into mesenteric lymph nodes in a ratio comparable with the LGG group. The translocation of ST in the LGG+ST group was similar in mesenteric lymph nodes to the ST group, but reduced in the spleen, liver and blood. This reduced dissemination of *S. Typhimurium* may be due to immunomodulation by transforming growth factor (TGF)- $\beta$  and IFN- $\gamma$  in mesenteric lymph nodes, as was observed in conventional piglets treated by *L. reuteri* [54].

The GIT microbiota influences the development of the intestine. Conventionalization of germ-free piglets by pig feces triggered changes leading to intestinal histology that was similar to the histology typical for conventional piglets [55]. The *lamina propria*, submucosa cellularity and muscular layer thickness were increased in the LGG group in comparison with the preterm GF piglets, and histology of their ileum resembled the ileum of the term germ-free piglets [19,55]. Quite a different picture was seen in the ileum of the *Salmonella*-infected preterm piglets (ST and LGG+ST) in which inflammatory cellularity in the lymphatic vessel lumen, villus atrophy, exudate in the lumen and PMN infiltration into the *lamina propria* shared characteristics with *Salmonella*-infected term gnotobiotic piglets [52]. Furthermore, the ST group showed hyperemia, the multiple erosion of the intestinal epithelium, hemorrhage and peritonitis that were not obvious in the term *Salmonella*-infected piglets in our former experiments [52]. The histological score we used indicates that LGG ameliorated the deleterious effect of *Salmonella* infection on the intestine of the preterm gnotobiotic piglets. Similarly, previous colonization of gnotobiotic piglets with avirulent *S. enterica* serovar Infantis strain 1326/8 [24] or Typhimurium strain SF1591 [51] protected the piglets against consecutive infections with virulent *S. Typhimurium*. The higher abundance of neutrophils

in the intestine of gnotobiotic piglets induced by avirulent *Salmonella* mediated a protective effect.

A thin layer of enterocytes forms the interface between the intestinal lumen and the host body. The tight junction (TJ) proteins join adjacent enterocytes in their apical part. To estimate possible changes in the intestinal barrier we gave our attention to two TJ proteins with different actions. While claudin-1 seals an epithelial layer for small molecules and protects the host against the loss of electrolytes [56], occludin impedes the paracellular transport of large molecules and cells [57]. *S. Typhimurium* was shown to alter both claudin-1 and occludin to increase its translocation and transepithelial migration of polymorphonuclear neutrophils [58].

Colonization of the preterm germ-free piglets with LGG did not influence transcriptions of claudin-1 and occludin in the intestine compared with the germ-free piglets. Infection with *Salmonella* increased the transcription of claudin-1 but decreased the transcription of occludin. The increased transcription of claudin-1 was probably an attempt to prevent the loss of electrolytes and dehydration of the organism in both *Salmonella*-infected groups (ST and LGG+ST) [56]. The decreased transcription of occludin in *Salmonella*-infected piglets probably reflected a disrupted intestinal barrier for large molecules that can simultaneously facilitate bacterial translocation [58]. To confirm our speculation concerning changes in claudin-1 and occludin, a suitable test of the functionality and the intestinal barrier function [59] should be performed. Because *Salmonella*-infected piglets suffered from anorexia and normal peristalsis was disturbed, their stomachs were full of a precipitated milk diet but the colon was more or less empty. Locally produced biomarkers such as intestinal fatty acid binding protein may provide a suitable leaky gut test in salmonellosis [59,60].

The immune system is developed during ontogeny but in preterm newborns it has not been fully developed [61]. The inflammatory reaction is characterized by movements of leukocytes and fluids from the blood vessels into extravascular space controlled by different mediators. Local and systemic levels of CXCL chemokine interleukin (IL)-8, proinflammatory cytokine IL-12/23 p40 subunit and IFN- $\gamma$  were measured to reveal whether their levels are related to physiological processes or if they exceed their regulatory function and can cause a self-damaging over-exuberant innate immune response called a 'cytokine storm' [62]. IL-8 attracts PMNs from intravascular into the interstitial inflammatory site and activates them [63]. It has been found that infection with *S. Typhimurium* increased transepithelial migration of the PMNs [58]. We detected relatively high levels of IL-8 in the jejunum and lower in the ileum and colon in the preterm GF and LGG piglets. Our finding of intestinal IL-8 occurring in the

ileum of preterm gnotobiotic piglets is seemingly in contradiction with the absence of the histopathological changes in the non-infected piglet groups (GF and LGG). Nevertheless, IL-8 probably plays a role in the ontogeny of the GIT. IL-8 is present in the colostrum and mother's milk and its receptors occur in the human fetal and neonatal intestine [64]. Different IL-8 isoforms were widely expressed in human fetal tissues, although inflammatory changes were not obvious [65]. These IL-8 isoforms during prenatal development minimize IL-8 inflammatory effects, but their isoform changes make it possible to modify its activity after birth [65]. This probably explains the existence of IL-8 without inflammatory findings in the non-infected preterm intestine. In concurrence with our findings, IL-8 was found in the intestinal homogenates from preterm conventional piglets [66]. Extremely low birth weight infants that suffer from NEC showed increased levels of IL-8 [67] and significant activation of IL-8 gene was detected in the preterm piglets suffering from severe NEC [68]. It documents the involvement of IL-8 in pathological processes in the immature intestine. Systemic IL-8 levels were suggested as a potential biomarker for neonatal sepsis in infants [69].

*Salmonella* strongly induced IL-8 levels in the intestine and blood of the ST group. Previous colonization with LGG alleviated these levels only in the jejunum and plasma, and these levels were kept within the range of expected physiological levels comparable with LGG and GF piglets. These findings correspond to the diminished CFUs in the jejunum and blood of LGG+ST piglets. This LGG-ameliorating effect was not found in the ileum and the colon. In contrast to the preterm GF piglets, no IL-8 levels were found in the intestine of the term germ-free piglets and similarly induced IL-8 values were found in the term gnotobiotic piglets infected with *S. Typhimurium* [51,52]. Local induction of IL-8 with *S. Typhimurium* rough mutants in the intestine of gnotobiotic piglets colonized with avirulent *Salmonella* serovars and relationships of IL-8 and PMNs were believed to be responsible for the protective effect against subsequent infection with *S. Typhimurium* virulent strains [24,51] as mentioned above. The induction of intestinal and systemic IL-8 in conventional piglets infected with *S. Typhimurium* has been described by other authors [70,71]. IL-8 was also shown 1, 2 and 6 days post-infection (dpi) in experiments with 1-month-old piglets orally infected with *Salmonella* and the immune response in the jejunum, ileum and colon differed and had various time progressions [70]. This may explain the differences in different parts of the intestine.

Another important part of the innate immune response covers the T helper type 1 (Th1) cell-mediated immune response, that is crucial in host protection against intracellular pathogens. It activates macrophages through

IFN- $\gamma$ -controlled mechanisms. IFN- $\gamma$  stimulates the anti-microbial activity of infected macrophages to kill off intracellular pathogens through inducible nitric oxide synthase produced reactive nitrogen intermediates [72]. Up-regulated transcriptions of IFN- $\gamma$ , IL-8 and IL-12/23 p40 were found in porcine jejunal loops infected with *S. Typhimurium* [73]. IL-12/23 p40 is an IL-12 and IL-23 shared protein subunit that is strongly up-regulated after microbial stimuli [74]. While IL-12 is a heterodimeric cytokine consisting of p40 and p35 subunits, IL-23 consists of p40 and p19 subunits [75]. Additionally, the IL-12/23 p40 homodimer can regulate the inflammatory process as an IL-12 antagonist [76]. IL-12 and IL-23 regulate the host resistance and intestinal inflammation during infection with *Salmonella* [77]. IL-12 is primarily produced by antigen-presenting cells and controls differentiation of naive T-cells into IFN- $\gamma$ -producing Th1 cells. It is a potent inducer of IFN- $\gamma$  production by NK cells and innate lymphoid cells [78]. Similarly, IL-23 is produced by dendritic cells and macrophages [75]. Negligible or undetectable levels of IL-12/23 p40 were present in the intestine and blood of non-infected preterm gnotobiotic piglets (GF and LGG). Infection with *Salmonella* strongly induced IL-12/23 p40, but only in the jejunum was this induction diminished by previous colonization with LGG. This finding agrees with CFUs and IL-8 in the jejunum.

IFN- $\gamma$  shows anti-bacterial properties against intracellular microbes, including *Salmonella*. It activates macrophages, promotes phagocytosis and destroys phagocytosed microbes by free radical-driven toxification of phagosomes and induces the expression of many other genes which participate in anti-microbial strategies [26]. It has been proved that some probiotic bacteria are able to induce IFN- $\gamma$  production that ameliorates *Salmonella* infection [79]. Local or systemic levels of IFN- $\gamma$  were found in the term gnotobiotic [80,81] and conventional piglets [82] infected with *Salmonella* or other enteric bacterial pathogens. We measured local and systemic levels of IFN- $\gamma$  to test if LGG shows an IFN- $\gamma$ -stimulatory effect [79], but no levels of IFN- $\gamma$  were found in the LGG group. Nevertheless, much more surprising was the absence of the stimulatory effect of *Salmonella* on IFN- $\gamma$  levels in the intestine of both groups of the piglets infected with *Salmonella* (ST and LGG+ST) and the absence of IFN- $\gamma$  levels (or their levels under the lower limit of detection) in blood. Low levels of IFN- $\gamma$  or their absence, in contrast to IL-8 and IL-12/23, were the reason that we gave our additional attention to IFN- $\gamma$  transcription, because IFN- $\gamma$  transcription in intestinal tissues in the gnotobiotic piglets after infection with *S. Typhimurium* has been reported [81]. While both *Salmonella*-infected groups showed highly activated gene transcription in the ileum, only piglets previously colonized with LGG showed it in the colon. During infection with *S. Typhimurium*, various transcriptional profiles were found

along the porcine intestine and it was determined that the jejunum, ileum and colon respond differently to infection [71]. In addition to the variable immune response in different parts of the intestine, it is possible that *L. rhamnosus* GG modifies the induction of IFN- $\gamma$  in the colon. This highly differentiated transcription between the piglet groups suggests that IFN- $\gamma$  protein would probably appear later than 24 h post-infection to support IL-8-driven mechanisms in *Salmonella* infection. The local and systemic absence of IFN- $\gamma$  protein contrasted with its highly induced transcription in the ileum and the colon suggests a longer experimental post-infection period in our future experiments to evaluate the involvement of IFN- $\gamma$  in combating *Salmonella* infection.

## Conclusion

We evaluated the effect of the previous colonization of the hysterectomy-derived preterm gnotobiotic piglets with *L. rhamnosus* GG against subsequent infection with *S. Typhimurium*. This colonization with LGG partially ameliorated the deleterious effect of infection with *S. Typhimurium*, as it was evaluated by CFU enumeration, intestinal histology and the local and systemic values of IL-8 and IL-12/23 p40. This is the first use of preterm gnotobiotic piglets as an infectious model of vulnerable immunocompromised preterm infants to verify probiotic safety and its bacterial interference with an enteric pathogen.

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## Disclosure

The authors have no conflicts of interest.

## Author contributions

A. S. planned and participated in experiments, performed microbiological and histological evaluations, arranged figures and wrote the manuscript, V. J. performed experiments and RT-PCR measurements and partially evaluated results, Z. S. designed histological score criteria and performed histopathological evaluation, I. S. planned experiments, performed xMAP technology measurements, statistical evaluations, graphs, arranged figures and wrote the manuscript. All authors approved the submitted version of the manuscript.

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### Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

**Fig. S1. The preterm gnotobiotic piglet.** The hysterectomy-derived, colostrum-deprived preterm gnotobiotic piglets were reared in fiberglass isolators for one week and were fed a cow's milk-based formula via a nipple. The picture depicts a three-day-old preterm gnotobiotic piglet.

**Fig. S2. Schema of the experiment.** The preterm gnotobiotic piglets ( $n = 26$ ) were grouped into four groups: i) sterile for the whole experimental period (germ-free; GF,  $n = 6$ ), ii) orally colonized with  $1 \times 10^8$  CFU of *Lactobacillus rhamnosus* GG 4 hours after hysterectomy (LGG,  $n = 8$ ), iii) one-week-old GF piglets orally infected with  $1 \times 10^8$  CFU of *S. Typhimurium* for 24 hours (ST,  $n = 6$ ), and iv) one-week-old LGG-colonized piglets orally infected with  $1 \times 10^8$  CFU of *S. Typhimurium* for 24 hours (LGG+ST,  $n = 6$ ).

**Fig. S3. Intestinal morphometry in the ileum of the preterm gnotobiotic piglets.** Villus height, crypt depths and muscular thickness were compared between the GF ( $n = 6$ ) and LGG ( $n = 8$ ) groups. Unpaired two-tailed t-test was used to compare the groups. The results are presented as individual dots and mean (horizontal line). The statistical significances are denoted with asterisks ( $*P < 0.05$ ).