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HOST RESPONSE AND INFLAMMATION

# *Lactobacillus rhamnosus* L34 Attenuates Gut Translocation-Induced Bacterial Sepsis in Murine Models of Leaky Gut

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**ABSTRACT** Gastrointestinal (GI) bacterial translocation in sepsis is well known, but the role of *Lactobacillus* species probiotics is still controversial. We evaluated the therapeutic effects of *Lactobacillus rhamnosus* L34 in a new sepsis model of oral administration of pathogenic bacteria with GI leakage induced by either an antibiotic cocktail (ATB) and/or dextran sulfate sodium (DSS). GI leakage with ATB, DSS, and DSS plus ATB (DSS+ATB) was demonstrated by fluorescein isothiocyanate (FITC)-dextran translocation to the circulation. The administration of pathogenic bacteria, either *Klebsiella pneumoniae* or *Salmonella enterica* serovar Typhimurium, enhanced translocation. Bacteremia was demonstrated within 24 h in 50 to 88% of mice with GI leakage plus the administration of pathogenic bacteria but not with GI leakage induction alone or bacterial gavage alone. *Salmonella* bacteremia was found in only 16 to 29% and 0% of mice with *Salmonella* and *Klebsiella* administrations, respectively. *Klebsiella* bacteremia was demonstrated in 25 to 33% and 10 to 16% of mice with *Klebsiella* and *Salmonella* administrations, respectively. *Lactobacillus rhamnosus* L34 attenuated GI leakage in these models, as shown by the reductions of FITC-dextran gut translocation, serum interleukin-6 (IL-6) levels, bacteremia, and sepsis mortality. The reduction in the amount of fecal *Salmonella* bacteria with *Lactobacillus* treatment was demonstrated. In addition, an anti-inflammatory effect of the conditioned medium from *Lactobacillus rhamnosus* L34 was also demonstrated by the attenuation of cytokine production in colonic epithelial cells *in vitro*. In conclusion, *Lactobacillus rhamnosus* L34 attenuated the severity of symptoms in a murine sepsis model induced by GI leakage and the administration of pathogenic bacteria.

**KEYWORDS** *Lactobacillus rhamnosus* L34, gastrointestinal leakage, antibiotics, dextran sulfate solution, murine model

Sepsis is a syndrome of dysregulated host responses to systemic infection, independent of the organisms, resulting in organ dysfunction (1). Gastrointestinal (GI) leakage occurs in sepsis, due in part to the disruption of the actin cytoskeleton and tight junctions of intestinal epithelial cells, resulting in circulatory exposure to pathogens and pathogen-associated molecular patterns (PAMPs) (2–5). In mice, this can be modeled by the administration of low-dose dextran sulfate sodium (DSS), diluted into drinking water, for 1 week to induce gastrointestinal leakage. Subtle histological damage of the gastrointestinal tract without overt colitis occurs (3). Bacteremia is not induced in the DSS model (3). In addition, prolonged antibiotic (ATB) administration alters the gut microbiota ("dysbiosis"). This induces GI permeability barrier impairment as well (6). In mouse models, antibiotic-induced gut bacterial translocation in the

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absence of bacteremia occurs with as little as a single dose of antibiotic. This is nicely demonstrated by mesenteric lymph node culture (7). Similarly, we recently demonstrated gut leakage after antibiotic administration, and gut bacterial translocation, in a mouse model of *Clostridium difficile* infection (2). As GI leakage induced by both DSS and antibiotics does not result in overt bacteremia, we hypothesized that the burden of intestinal pathogenic bacteria might be another factor promoting bacteremia.

Indeed, some bacteria develop specific strategies to disrupt and penetrate tight junctions (an important component of gut permeability) (8). Among several bacteria, *Salmonella* and *Klebsiella* were demonstrated to be bacteria with and without direct tight junction disruption abilities, respectively (9–11). In addition, the incidence of sepsis from salmonellosis and *Klebsiella* bacteremia in Southeast Asia is increasing (12). Accordingly, *Salmonella enterica* serovar Typhimurium or *Klebsiella pneumoniae* was administered concomitantly with gut leakage induction.

Colonization resistance, the normal gut microbiota control of intestinal pathogenic bacteria, is an established concept, and probiotics are beneficial for several conditions (13). As such, the benefit of probiotics in sepsis has been demonstrated in endotoxin and intra-abdominal infection sepsis models (14–17). However, the benefit of probiotics in gut bacterial translocation in a model where sepsis originated directly from gut leakage has not been tested. Probiotic use in patients with sepsis is still controversial, at least in part due to the different characteristics of patients with sepsis of different etiologies (18). Hence, evaluation of probiotic-based therapeutic strategies in different animal models is needed to design appropriate translational studies of such patients.

We recently demonstrated that *Lactobacillus rhamnosus* strain L34 attenuates interleukin-8 (IL-8) production both in intestinal epithelial cells after stimulation with *Clostridium difficile* (19) and in a mouse model of *C. difficile* infection (2). Accordingly, we evaluated this approach in new murine sepsis models.

## RESULTS

**Pathogenic bacteria enhance gastrointestinal leakage and cause mortality in all gut leakage models (antibiotic cocktail, dextran sulfate solution, and antibiotics with dextran sulfate administration).** GI leakage was demonstrated by the appearance of fluorescein isothiocyanate (FITC)-dextran in blood 3 h after oral administration as well as the spontaneous elevation of serum (1→3)-$\beta$-D-glucan (BG) levels. Both blood FITC-dextran and serum BG levels in GI leakage models with ATB or a DSS solution or with antibiotics with dextran administration (DSS+ATB) were higher than those for saline controls (normal saline solution [NSS]) (Fig. 1A and B). Interestingly, GI leakage measured by serum BG levels, but not FITC-dextran levels, was more severe in the DSS+ATB group than in the group given ATB alone. Although the administration of bacteria alone did not induce GI leakage (Fig. 1C), the severity of leakage was maintained in the ATB model and increased in the DSS and DSS+ATB models (Fig. 1D to F). Without the introduction of bacteria, the intensity of GI leakage was reduced after antibiotic administration ceased (Fig. 1D).

In addition, the administration of bacteria also increased the mortality rate for GI leakage-induced mice (Fig. 2A and B), possibly due to bacteremia (Fig. 2C to F). Despite similar mortality rates among gut leakage sepsis models, the DSS+ATB and DSS models showed a significantly higher intensity of bacteremia than did the ATB model at 6 h post-*Salmonella* gavage (Fig. 2C) and at 24 h post-*Klebsiella* gavage (Fig. 2F). Bacteremia was nondetectable in all mice with the administration of bacteria alone without gut leakage induction (Fig. 2C and D) or DSS administration alone without bacterial gavage (data not shown). However, approximately 50 to 88% of the mice with induced leaky gut and with the introduction of bacteria showed bacteremia 6 and 24 h after bacterial gavage (Fig. 2C to F). Blood bacterial analysis demonstrated polymicrobial bacteremia in all mice. *Salmonella* bacteremia was present in only 16 to 29% of mice with induced leaky gut and with *Salmonella* administration, despite positive fecal cultures for *Salmonella* spp. for all mice 24 h after *Salmonella* gavage (see Fig. 5). *Salmonella* bacteremia did not occur in mice administered *Klebsiella* spp. (Fig. 2H). On the other hand,

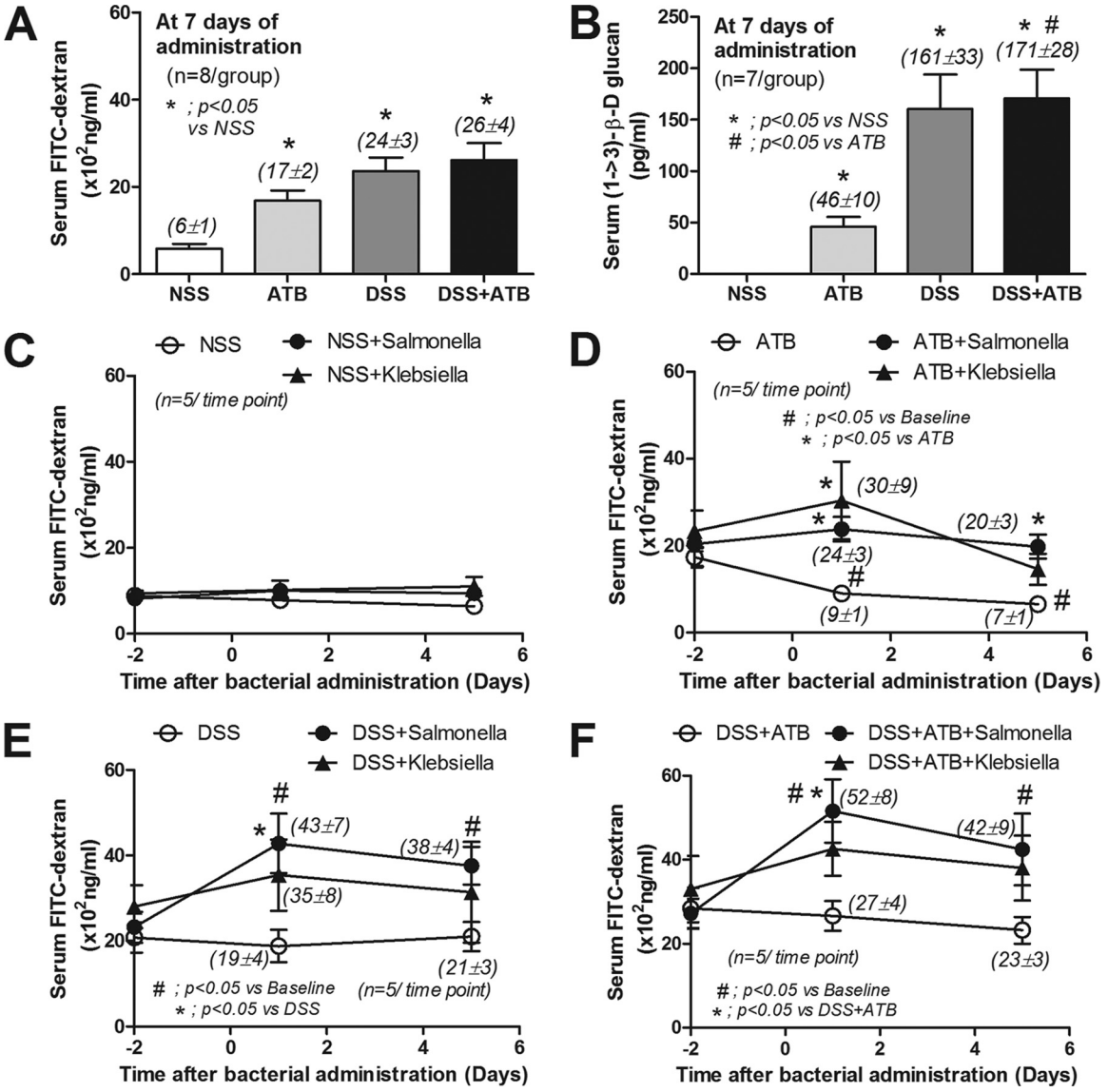


**FIG 1** (A and B) Gastrointestinal permeability barrier defect as determined by FITC-dextran translocation (A) and spontaneous elevation of serum (1→3)-β-D-glucan (BG) levels (B) in mice administered normal saline (NSS), an antibiotic cocktail (ATB), a dextran sulfate sodium (DSS) solution, and DSS with ATB (DSS+ATB). (C to F) Time course of gut leakage for each model with oral administration of bacteria (*Salmonella enterica* serovar Typhimurium or *Klebsiella pneumoniae*) 2 days before (day −2), on the day of (day 0), and after (days 1 and 5) administration. FITC-dextran was orally administered 3 h before blood collection at each time point. Values are means ± standard errors.

with *Klebsiella* administration, the percentage of mice with *Klebsiella* bacteremia at 24 h was higher than that for mice with *Salmonella* administration (Fig. 2G and H). However, fecal *Klebsiella* species burdens could not be demonstrated due to the limitations of the special isolation method used. Additionally, polymicrobial bacteria were present in mesenteric lymph node cultures 24 h after the administration of bacteria in all models with antibiotic and/or DSS administration (data not shown).

***Lactobacillus rhamnosus* L34 attenuates sepsis severity and fecal burdens of pathogenic bacteria in gut leakage-induced sepsis models.** Because the differences between the DSS and DSS+ATB models were nonsignificant regarding mortality rates and bacteremia intensities (Fig. 2), only the DSS model was used for additional experiments involving *L. rhamnosus*. Interestingly, *Lactobacillus rhamnosus* L34 improved survival in the DSS model with either *Salmonella* or *Klebsiella* gavage and in the ATB model with *Klebsiella* gavage (Fig. 3A to C and 4A to C). Twenty-four hours after

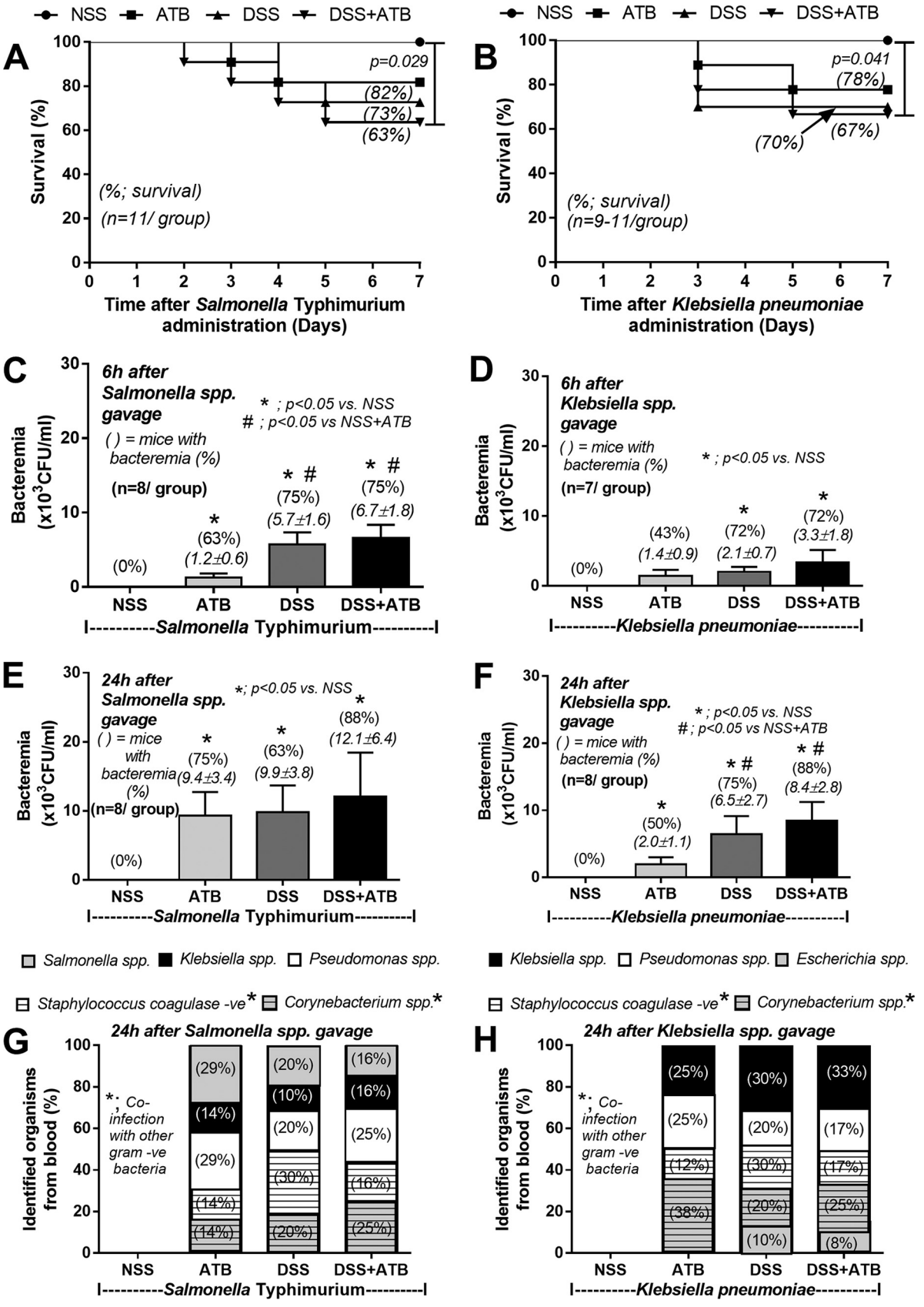


**FIG 2** Survival analysis, bacteremia 6 h and 24 h after bacterial gavage, and organisms identified from blood in gut leakage models of ATB, DSS, and DSS+ATB with oral administration of *Salmonella enterica* serovar Typhimurium (A to G) or *Klebsiella pneumoniae* (E to H). Values are means ± standard errors. −ve, negative.

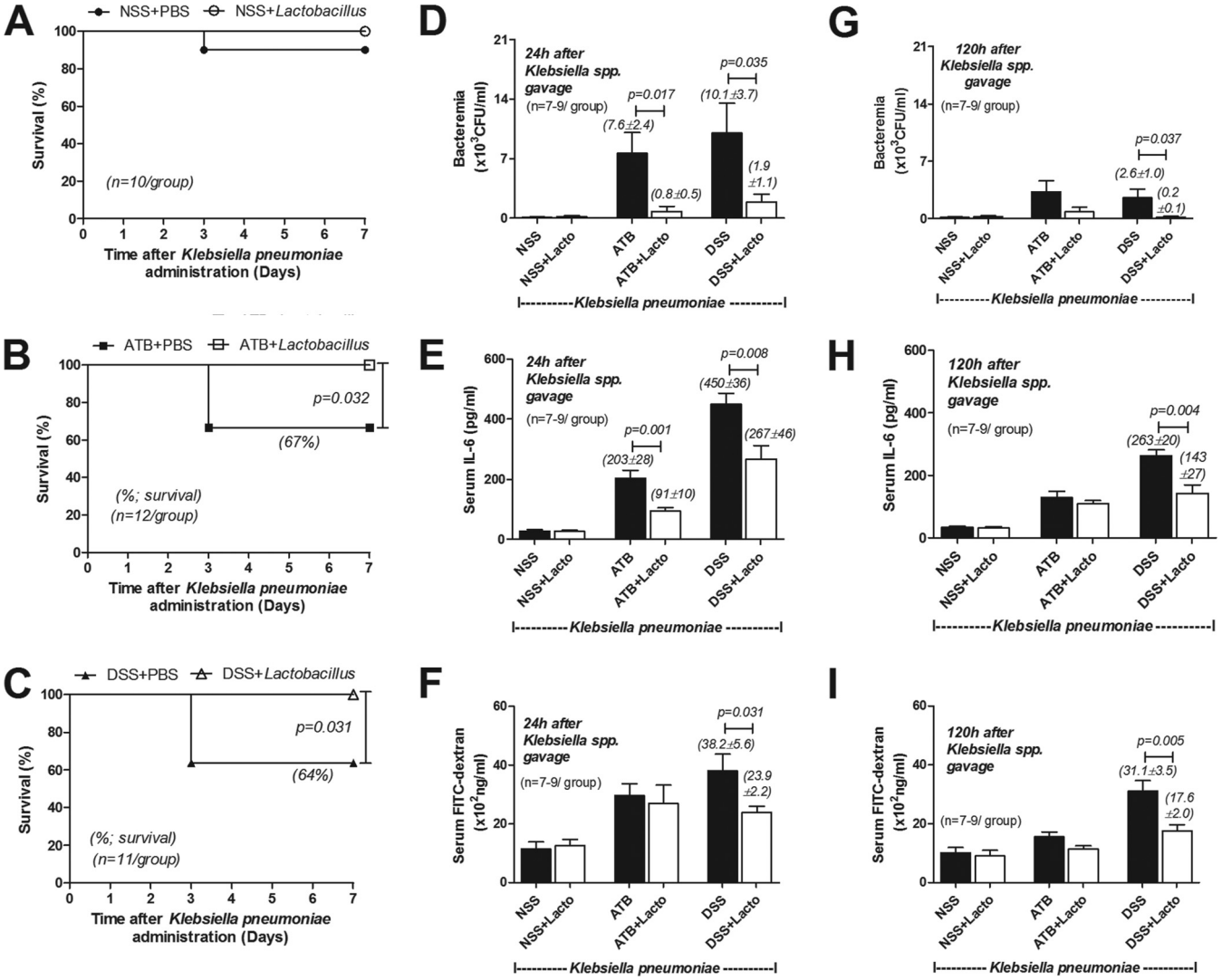


**FIG 3** (A to C) Survival analysis with control normal saline solution (NSS) (A) and in gut leakage models of ATB (B) and DSS (C) with oral administration *Lactobacillus rhamnosus* L34 (Lacto) 1 day prior to administration of *Klebsiella pneumoniae*. (D to I) Bacteremia, serum IL-6 levels, and gut leakage measured by using FITC-dextran 24 h (D to F) and 120 h (G to I) after *Klebsiella pneumoniae* administration. Values are means ± standard errors.

bacterial gavage, *Lactobacillus* administration attenuated bacteremia and serum IL-6 levels in both the ATB (Fig. 3D and E) and DSS (Fig. 4D and E) models but attenuated the severity of gut leakage (measured by FITC-dextran levels) only in the DSS model (Fig. 3F and 4F). In parallel, *Salmonella* burdens in fecal contents 24 h after *Salmonella* administration were also reduced with *Lactobacillus* species prophylaxis (Fig. 5 and 6). Gut leakage improvement by *Lactobacillus* spp. was due, at least in part, to the reduction of the burdens of pathogenic bacteria in feces. On the other hand, 120 h after the administration of bacteria, *Lactobacillus* spp. attenuated bacteremia, serum IL-6 levels, and gut leakage severity only in the DSS model with the administration of both bacteria (Fig. 3G to I and 4G to I) and attenuated only serum IL-6 levels in the ATB model with *Salmonella* administration (Fig. 4H). In parallel, *Lactobacillus* spp. attenuated *Salmonella* burdens in fecal contents 120 h after *Salmonella* administration only in the DSS model but not in the ATB model, possibly due to the spontaneous reduction of *Salmonella* fecal burdens in the ATB model due to the presence of antibiotics (Fig. 5 and 6).

**Anti-inflammatory properties of *Lactobacillus*-conditioned medium.** Previously, anti-inflammatory properties of the conditioned medium of *L. rhamnosus* L34 were

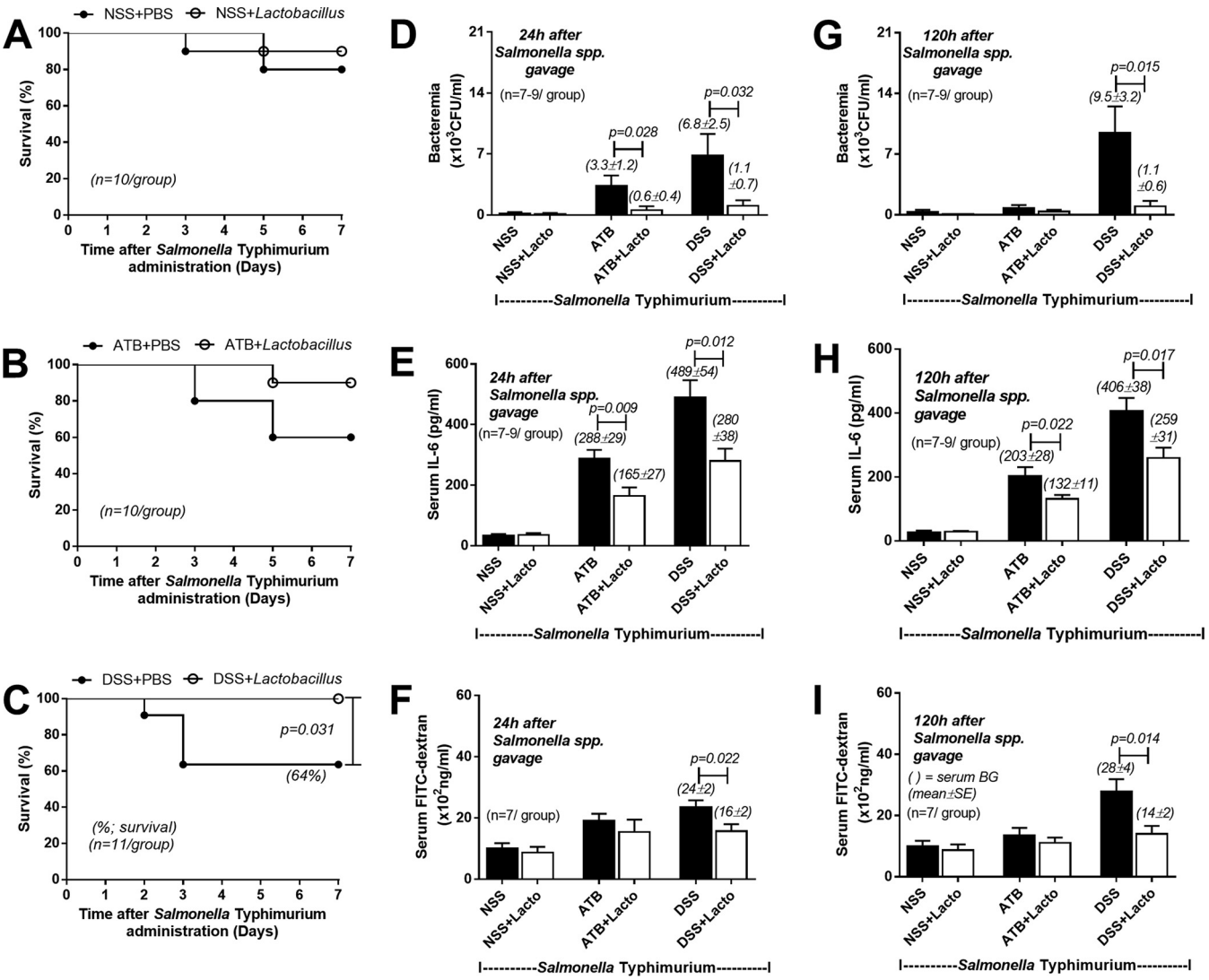


**FIG 4** (A to C) Survival analysis with control NSS (A) and in gut leakage models of ATB (B) and DSS (C) with oral administration of *Lactobacillus rhamnosus* L34 1 day prior to administration of *Salmonella enterica* serovar Typhimurium. (D to I) Bacteremia, serum IL-6 levels, and gut leakage measured by FITC-dextran levels 24 h (D to F) and 120 h (G to I) after *Klebsiella pneumoniae* administration. Values are means ± standard errors.

demonstrated *in vitro* with the attenuation of proinflammatory cytokine production in Caco-2 and HT-29 cells after *C. difficile* stimulation (19). We tested this effect on the *S. enterica* serovar Typhimurium and *K. pneumoniae* administration models. *Lactobacillus*-conditioned medium (LCM) was found to attenuate inflammatory cytokine (IL-8) production by Caco-2 and HT-29 cells after stimulation with both bacteria (Fig. 7A to D). To explore the chemical nature of the active molecule from *L. rhamnosus* L34, the anti-inflammatory properties of LCM after several treatments was determined. Because IL-8 is one of the predominant cytokines produced from epithelial cells and cytokine production by HT-29 cells is prominent (19, 20), IL-8 from Caco-2 and HT-29 cells was used as a representative of proinflammatory activity. Interestingly, the molecules active against *Salmonella* and *Klebsiella* were heat stable (Fig. 8A and B) and contained a polysaccharide structure because the anti-inflammatory property was neutralized only by the amylase enzyme (Fig. 8C and D). In addition, the molecular mass of the active molecules should be >50 kDa, as determined by the loss of IL-8 suppression in the fraction size that was <50 kDa (Fig. 8E and F). In parallel, the molecule active against *Klebsiella* showed the same properties as those of the agents active against *Salmonella* (Fig. 9) but also included lipid structure molecules, as demonstrated by the neutraliza-

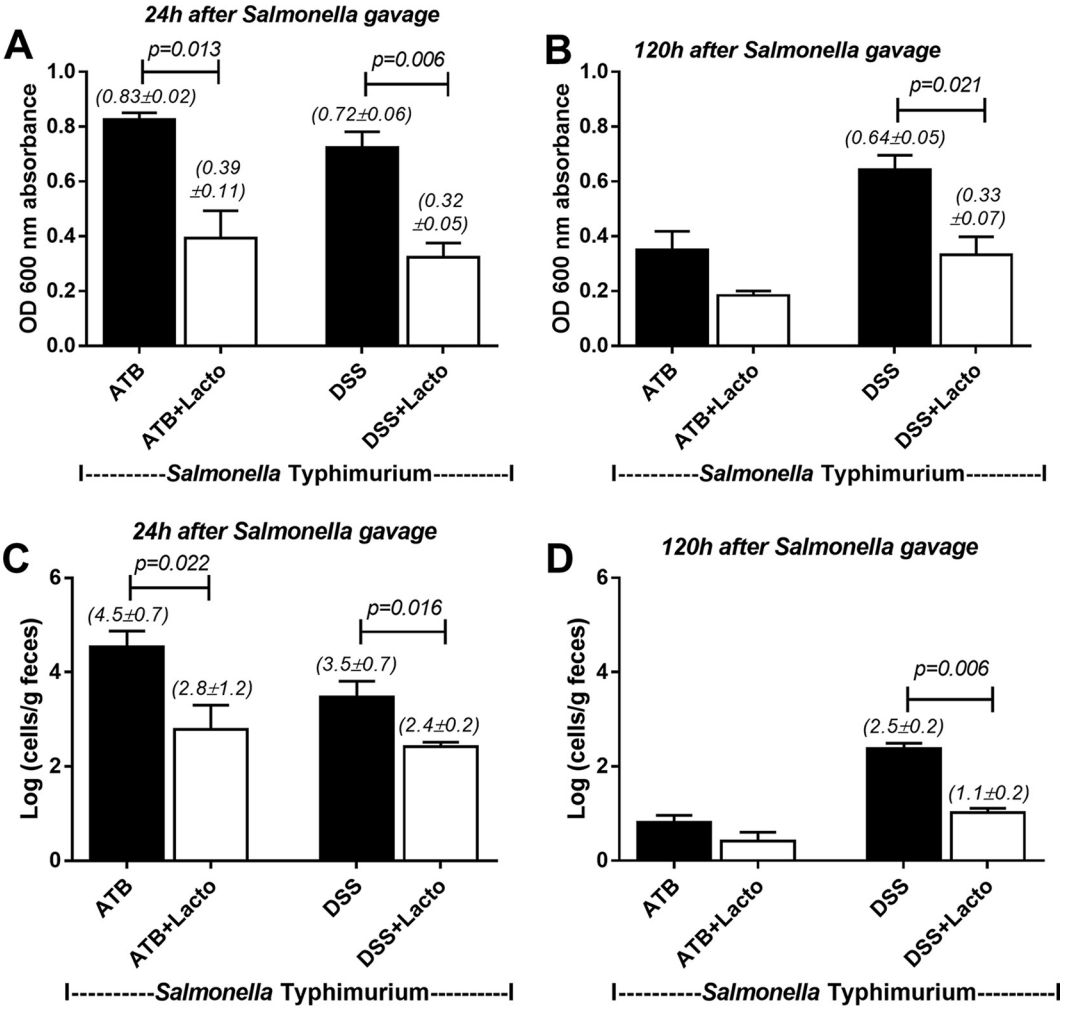


**FIG 5** (A and B) Fecal burdens of *Salmonella enterica* serovar Typhimurium measured by turbidity in selenite cystine broth, a selective enrichment medium, 24 h (A) and 120 h (B) after *Salmonella enterica* serovar Typhimurium administration in gut leakage models of ATB and DSS with oral administration of *Lactobacillus rhamnosus* L34 1 day prior to administration of *Salmonella enterica* serovar Typhimurium. (C and D) Semiquantitative analysis of enumeration of fecal bacteria by real-time PCR targeting *Salmonella enterica* serovar Typhimurium. Values are means ± standard errors.

tion of anti-inflammatory properties by the lipase enzyme (Fig. 9C and D). Moreover, LCM-treated cells with *Salmonella* or *Klebsiella* activation expressed less phosphorylated NF-κB (p-NF-κB) and Toll-like receptor 4 (TLR4) at 15 and/or 30 min of bacterial activation (Fig. 10), suggesting an inhibition of TLR4 and NF-κB pathway signaling. Hence, these observations both *in vivo* and *in vitro* support those of previous work and suggest that *L. rhamnosus* L34 may be of interest for sepsis attenuation studies in additional models and, if found effective, in humans.

## DISCUSSION

Gut barrier impairment in the presence of some common human pathogens was shown to lead to enhanced gut content leakage, bacteremia, proinflammatory responses, and mortality in several murine models. These sepsis models of gut bacterial translocation resemble the "gut origin of sepsis" in critically ill patients. Prophylaxis with *Lactobacillus rhamnosus* L34 administered 1 day prior to gavage with pathogenic bacteria attenuated intestinal burdens of pathogenic bacteria, reduced gut leakage severity, reduced inflammatory responses, and improved survival.

**Sepsis model of gut translocation.** It is of interest that gut leakage induction alone, without oral administration of bacteria, produced (1→3)-β-D-glucanemia and

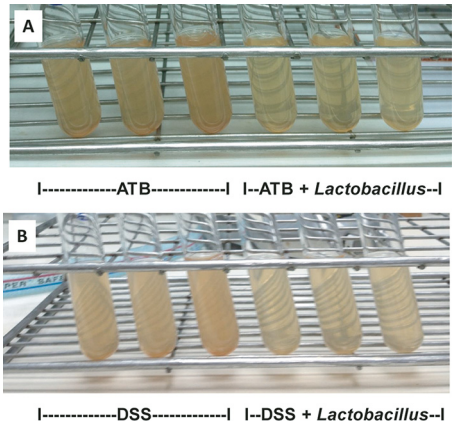


**FIG 6** Representative pictures of the turbidity of selenite cystine broth, a selective enrichment medium. Different levels of growth represent fecal burdens of *Salmonella enterica* serovar Typhimurium in gut leakage models of ATB (A) and DSS (B) with oral administration of *Lactobacillus rhamnosus* L34 1 day prior to administration of *Salmonella enterica* serovar Typhimurium. Only pictures 24 h after *Salmonella enterica* serovar Typhimurium administration are shown.

elevated levels of orally introduced FITC-dextran but not bacteremia. BG is normally found at very low levels in the blood of healthy mammals, and it is thought to enter the circulation due to translocation from the intestinal lumen, where it is present as a foodstuff constituent and a cell wall component of commensal fungi (3). As such, the appearance of elevated levels of serum BG, in the absence of invasive fungal infection, likely represents translocation due to GI barrier injury. Hence, gut content translocation, including microbes and associated molecules, after GI barrier impairment is dependent upon the severity of barrier injury (21). The administration of some bacterial strains leads to bacteremia without the need for gut leakage activation procedures due to direct tissue invasion mechanisms or bacterial toxins (22–24). As such, bacterial properties could affect the severity of the gastrointestinal permeability defect. Indeed, in our study, the induction of leaky gut enhanced bacteremia caused by some, but not all, strains of bacteria, as demonstrated for both *Escherichia coli* ATCC 25922 and *Acinetobacter baumannii* ATCC 19606. Interestingly, antibiotic administration transiently induced leaky gut, as determined by the FITC-dextran translocation assay, but gut patency recovered rapidly, as early as 1 day after antibiotic discontinuation. However, GI permeability was observed to persist with bacterial pathogen gavage for up to at least 5 days after the discontinuation of antibiotics. This supported gut leakage enhancement by the expansion of intestinally pathogenic bacteria or enhanced virulence (25). In addition, pathogenic bacteria enhanced the severity of gut leakage during both DSS administration alone and DSS with antibiotic gavage. Although a single strain of bacteria was administered, bacteremia in all of these mice was polymicrobial. As *Salmonella enterica* serovar Typhimurium does not inhabit the mouse intestine and because a special isolation method is available, increased fecal burdens of *Salmonella* spp. after oral administration can be demonstrated. In contrast, it is difficult to determine if orally administered *Klebsiella pneumoniae* results in increased fecal burdens of *K. pneumoniae* due to its status as an element of the normal mouse gut microbiota. Nevertheless, there was no salmonellosis without *Salmonella* gavage, and the percentage of mice with *Klebsiella* bacteremia was higher in *Klebsiella*-administered mice than in the *Salmonella*-administered group. Accordingly, the oral administration of bacteria affects bacteremia, quantitatively and qualitatively, in this model. It is also interesting to note that only antibiotic-treated mice, but not normal mice, are susceptible to *Salmonella enterica* serovar Typhimurium due to antibiotic-induced gut dysbiosis (26). Hence, the susceptibility of normal mice to *S. enterica* serovar Typhimurium with DSS administration implies the importance of tight junction disruption, in addition to *Salmonella* invasion through intestinal M cells and phagocytic cells, in disease patho-

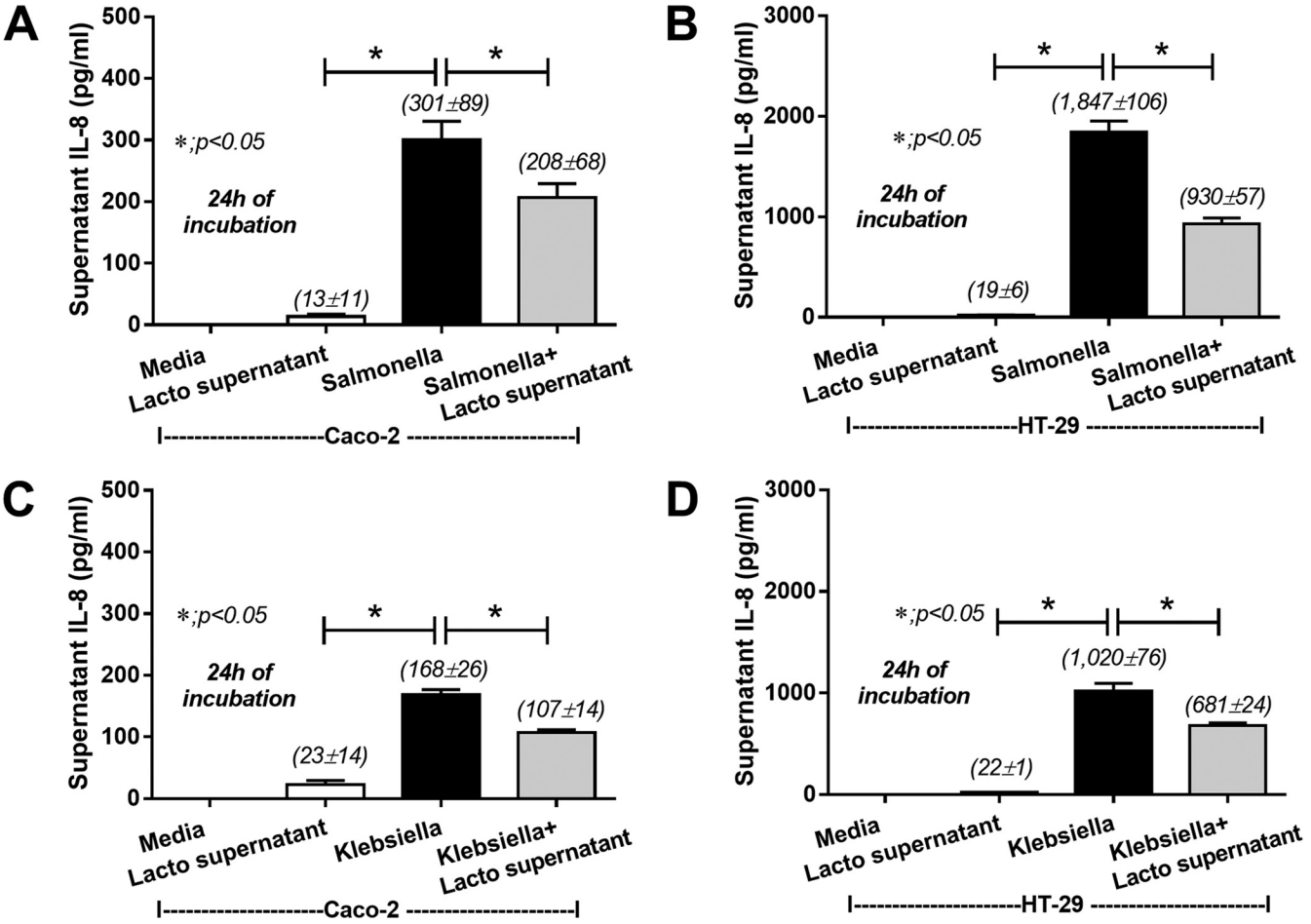


**FIG 7** Cytokines in the supernatants of Caco-2 and HT-29 cells after incubation with *Lactobacillus rhamnosus* L34-conditioned medium, with or without heat-killed bacteria (*Salmonella enterica* serovar Typhimurium or *Klebsiella pneumoniae*). Independent experiments were performed in triplicate.

physiology (27). On the other hand, DSS-administered mice were susceptible to *Klebsiella* bacteremia, despite the nondirect tight junction injury properties of *K. pneumoniae* (10). More studies are needed to explore this topic.

In addition, bacterial gut translocation in our model was demonstrated by the recovery of bacteria from mesenteric lymph nodes. Evidence of bacterial translocation from the gut to the mesenteric lymph node, without bacteremia, in mice after antibiotic administration was reported previously (7). In contrast, we demonstrated that gavage with pathogenic bacteria after oral antibiotic administration induced the presentation of bacteria in both the mesenteric lymph node and blood. This demonstrated that gut-pathogenic bacteria enhanced the severity of bacterial translocation from the gut. These models have the advantages of being facile and not requiring surgical procedures. They may thus be suitable for investigations of the biology of human gut-origin sepsis (5).

**Probiotics in sepsis.** After conditions of a prolonged use of antibiotics, the selection of drug-resistant pathogens, leading to overgrowth, may enhance the degrees of intestinal barrier impairment and sepsis severity (4, 28, 29). It has been demonstrated that certain probiotic organisms reduce the burdens of such pathogens, suggesting potential clinical utility as interventions in sepsis and, particularly, gut-origin sepsis (30). Indeed, the benefit of probiotics has been demonstrated in several studies in a surgically mediated sepsis model, which is used as a representative model of patients with intra-abdominal sepsis (cecal ligation and puncture) (15, 16, 31). In contrast, we examined the effect of probiotic treatment on nonsurgical sepsis models of gut bacterial translocation, potentially mimicking patients with gut-origin sepsis.

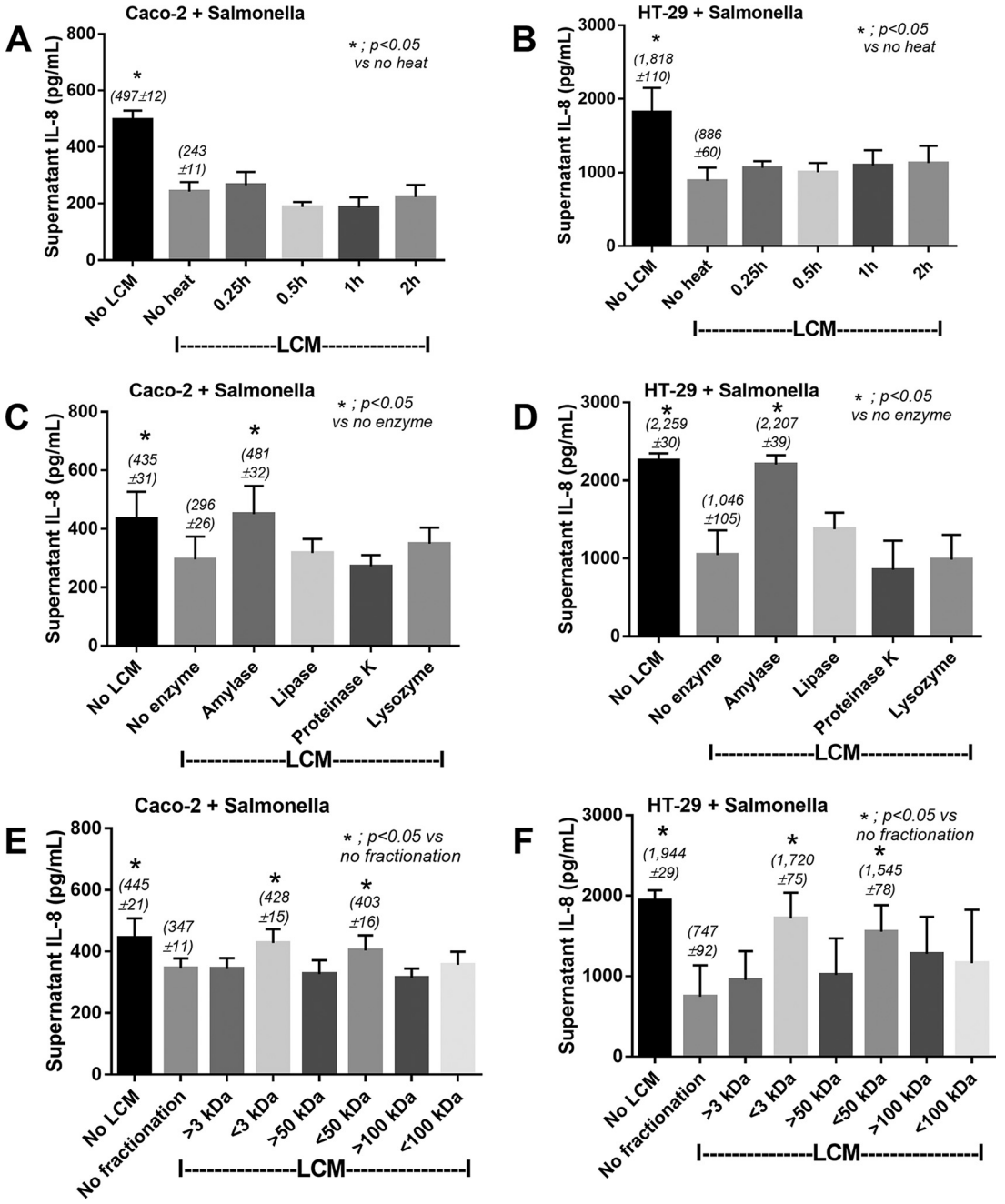


**FIG 8** *Salmonella*-induced IL-8 production from colonic epithelial cells (Caco-2 and HT-29) after incubation with conditioned medium from *L. rhamnosus* L34 (LCM) that was treated by heat exposure (A and B), enzyme digestion (C and D), and fractionation (E and F). Independent experiments were performed in triplicate.

In this model, the impact of gut dysbiosis on GI mucosal barrier function and the role of the normal gut flora in the maintenance of GI barrier integrity are critical (32). The effect of probiotic administration on sepsis severity in sepsis models of gut translocation was tested because of (i) the importance of normal gut flora in the pathogenesis of gut-origin sepsis (33), (ii) the controversy over probiotic treatment in sepsis (34), and (iii) the effect of probiotics on improving gut permeability in sepsis, a working hypothesis of probiotic benefit (35, 36). These data lend themselves to being tested in suitable animal models.

Indeed, oral administration of *Lactobacillus rhamnosus* L34 at $1 \times 10^7$ CFU 24 h prior to the administration of pathogenic bacteria attenuated sepsis severity, as demon-

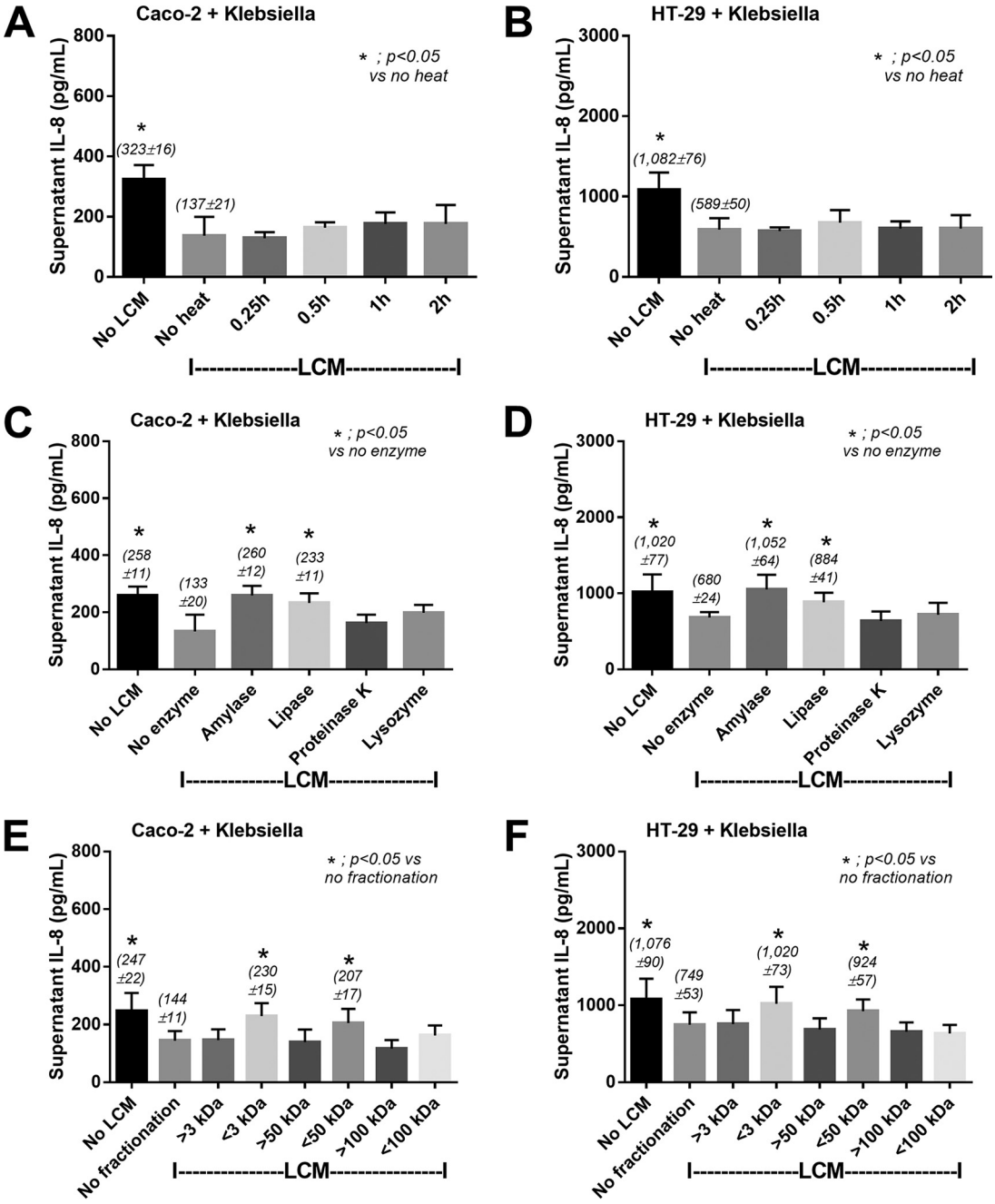


**FIG 9** *Klebsiella*-induced IL-8 production from colonic epithelial cells (Caco-2 and HT-29) after incubation with conditioned medium from *L. rhamnosus* L34 (LCM) that was treated by heat exposure (A and B), enzyme digestion (C and D), and fractionation (E and F). Independent experiments were performed in triplicate.

strated by the improvement of parameters including mortality rates, bacteremia, serum IL-6 levels, and gut leakage. This dose of *L. rhamnosus* L34 was selected because the dosage and qualities of probiotics are important, and a dose of a beneficial probiotic that is too high could be harmful (37–39), as previously demonstrated (2). Due to the availability of selective media for the isolation of *Salmonella* spp., the attenuation of *Salmonella* burdens in the gut with *L. rhamnosus* L34 administration 24 h and 120 h after *Salmonella* administration was demonstrated. In parallel, *L. rhamnosus* L34 also attenuated the severity of gut leakage, as determined by the levels of FITC-dextran, bacteremia, and inflammatory cytokines. Our data supported the concept that probiotics may attenuate sepsis severity through reductions of burdens of pathogenic

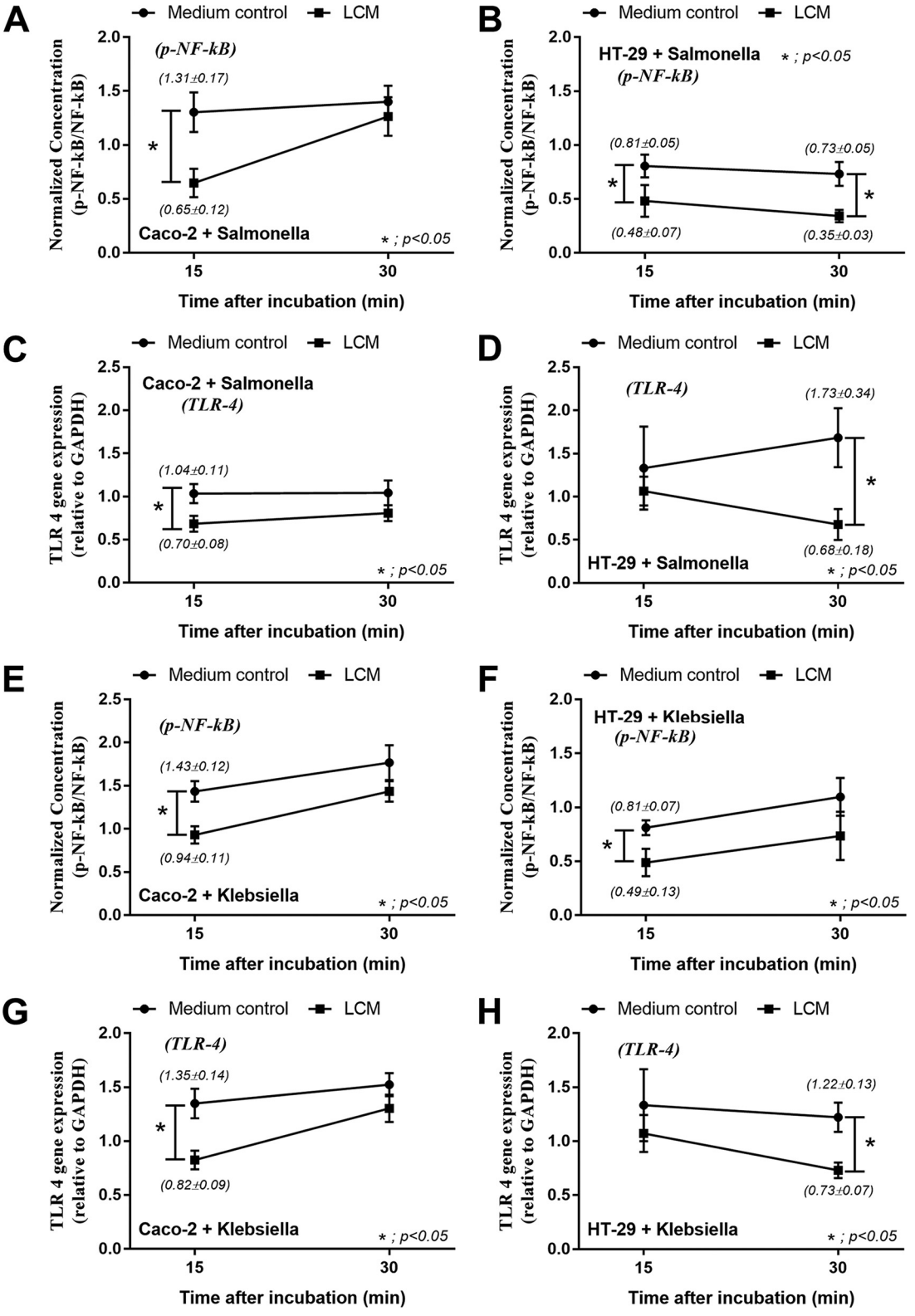


**FIG 10** Induction of phosphorylated NF-κB (p-NF-κB) and TLR4 expression in colonic epithelial cells (Caco-2 and HT-29) after incubation with conditioned medium from *L. rhamnosus* L34 (LCM) and administration of heat-killed *Salmonella* (A to D) and *Klebsiella* (E to H) bacteria. Independent experiments were performed in triplicate.

bacteria in the gut, resulting in less severe gut leakage and inflammation. In addition, we previously demonstrated that the supernatant of *L. rhamnosus* L34 could attenuate *C. difficile*-induced inflammation in Caco-2 cells (19). Extending that work to the models used in this study, the supernatant of *L. rhamnosus* L34 was also shown to attenuate inflammatory cytokine production by Caco-2 cells incubated with heat-killed bacterial preparations of *S. enterica* serovar Typhimurium and *K. pneumoniae*. This implied that *L. rhamnosus* L34 might attenuate sepsis severity through the production of anti-inflammatory molecules. To explore the molecular nature of immunomodulating substances in conditioned medium, several medium treatment protocols were used. Interestingly, the active molecules from the culture medium of *L. rhamnosus* L34 were a heat-stable, amylase-sensitive polysaccharide (against *Salmonella*) and an amylase- and lipase-sensitive polysaccharide/lipid (against *Klebsiella*), with a molecular mass of more than 50 kDa. Indeed, the exopolysaccharides, extracellular polysaccharides attached to the bacterial cell surface or secreted into the extracellular environment, of lactobacilli have been recognized as one of the substances that influence host immune responses (40–46). Soluble immunomodulatory agents from other probiotics have also been mentioned as potential anti-inflammatory substances against several inflammatory conditions (44, 46). Moreover, we demonstrated that the anti-inflammatory effect of the *L. rhamnosus* L34 polysaccharide against *Salmonella* and *Klebsiella* infection was possibly mediated through attenuation of the activations of TLR4 and NF-κB signaling. As such, the active molecules of *L. rhamnosus* L34 might interfere with TLR4, a pattern recognition receptor, of colonic epithelial cells, leading to the immunomodulating effect. More studies on this topic are needed. However, the structures and compositions of polysaccharides and other biological substances are diverse among probiotics, and this variation may contribute to the different immunomodulations of host immune responses. Although purification of bioactive substances was not performed, our initial characterization suggests the importance of the exopolysaccharide and/or lipopolysaccharide of *L. rhamnosus* L34 against bacterial sepsis. Further studies are required to determine the exact molecular substances.

In conclusion, we have explored the potential benefit of *L. rhamnosus* L34 in a new model of sepsis utilizing the administration of pathogenic bacteria along with the oral administration of gut barrier-disrupting materials. It was observed that *L. rhamnosus* L34 reduced the levels of pathogenic bacteria, resulting in less severe gut leakage and inflammatory responses, and probiotic-induced local organism control reduces sepsis severity (15, 47). Other anti-inflammatory mechanisms are possible, and other beneficial bacterial strains might be available. Our data support the potential benefit of probiotics in the prevention of gut-origin sepsis.

## MATERIALS AND METHODS

**Animals and animal models.** U.S. National Institutes of Health (NIH) protocols (NIH protocol no. 85-23, revised 1985) were followed (57). Eight- to ten-week-old male ICR mice (National Laboratory Animal Center, Nakornpathom, Thailand) were used. The animal protocols (SST 10/2557) were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

**Gut bacterial translocation sepsis models and probiotic treatment.** Gut bacterial translocation sepsis models consisted of gut leakage induction with a mixed antibiotic (ATB) and/or a DSS solution prior to the oral administration of pathogenic bacteria. Slightly increased gut leakage after ATB administration was described previously (2). Briefly, 0.5 ml of a cocktail of antibiotics (Sigma-Aldrich, St. Louis, MO, USA) containing gentamicin (3.5 mg/kg of body weight), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg), and vancomycin (4.5 mg/kg) was administered by gavage twice a day for 4 days, followed by a single dose of intraperitoneal clindamycin (10 mg/kg) on the fifth day. After a 2-day antibiotic-free period, mice were administered pathogenic bacteria, *Salmonella enterica* serovar Typhimurium ATCC 13311, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, or *Acinetobacter baumannii* ATCC 19606 (ATCC, Manassas, VA, USA), by gavage at $2 \times 10^{10}$ CFU/mouse in NSS, which also served as a control. Several bacteria were tested to evaluate their gut translocation and sepsis induction potentials. Both *E. coli* and *A. baumannii* could not induce sepsis in ATB- and DSS-induced gut leakage in mice in this model (data not shown).

Although gut leakage with antibiotics mimics the patient situation, gut leakage is transient and not severe. To enhance gut permeability, a DSS-induced gut leakage model was utilized, according to a previously reported method (3). Briefly, 1.5% (wt/vol) dextran sulfate (Sigma-Aldrich, St. Louis, MO, USA)

was diluted into drinking water for 1 week prior to the administration of pathogenic bacteria, *Salmonella enterica* serovar Typhimurium or *Klebsiella pneumoniae*, as mentioned above, and DSS treatment was continued until the end of the experimental period. In the model of DSS with ATB, DSS was diluted in drinking water, and antibiotics were administered as described above for 5 days, with the administration of pathogenic bacteria 2 days after the cessation of antibiotics. For probiotic treatment, mice were administered *Lactobacillus rhamnosus* L34 at $1 \times 10^7$ CFU/mouse by gavage or the phosphate buffer solution (PBS) control 1 day before the administration of pathogenic bacteria by gavage. *L. rhamnosus* L34 from the stock was cultured on deMan-Rogosa-Sharpe (MRS) agar (Oxoid, Hampshire, UK) under anaerobic conditions (10% $CO_2$, 10% $H_2$, and 80% $N_2$) by using gas generation sachets (AnaeroPack-Anaero; Mitsubishi Gas Chemical, Japan) at 37°C for 24 to 48 h before use.

**Gut permeability testing.** Gut permeability was determined by serum measurement of the levels of FITC-dextran, a nonabsorbable high-molecular-weight molecule, after oral administration (48) and by measurement of serum BG titers. Intestinal luminal BG is available due to the gut fungal microbiota and the BG content of mouse chow. Thus, elevated serum BG levels in the absence of fungal infection indicate GI leakage (2, 3). Fifty microliters of blood was collected through the tail vein for BG measurement. A total of 0.5 ml of FITC-dextran (molecular mass, 4.4 kDa) (FD4; Sigma, St. Louis, MO, USA) at 25 mg/ml was administered by gavage, and 50 $\mu$l of blood was collected through the tail vein 3 h later for measurement of FITC-dextran levels. The serum FITC-dextran level was measured fluorospectrometrically (NanoDrop 3300; Thermo Scientific, Wilmington, DE, USA), and the BG level was assayed by using Fungitell (Associates of Cape Cod, Inc., East Falmouth, MA). BG levels of $<$7.8 and $>$523.4 pg/ml were recorded as 0 and 523 pg/ml, respectively, reflecting the lower and upper limits of the assay. Other methods to test GI permeability were not used for several reasons. For example, anuria in sepsis models is a limitation of the urine sucralose assay (49). Additionally, spontaneous Gram-negative bacteremia itself produced gut leakage without the need to perform serum endotoxin analysis (50, 51). Moreover, BG is also more stable than endotoxin due to the presence of endotoxin-degrading enzymes in blood (52). In addition, BG activation of unmodified *Limulus* amebocyte lysate (LAL) reagent can be a confounding factor in LAL-based endotoxin measurements (53).

**Mouse feces and blood sample analyses.** To determine if bacteria administered by gavage were present in feces, fecal samples were collected by placing an individual mouse in an empty cage for 0.5 to 1 h, 1 day after the administration of pathogenic bacteria by gavage. Collected feces were well mixed with PBS. For *Klebsiella pneumoniae* identification, serially diluted fecal samples were spread onto blood agar plates (Oxoid, Hampshire, UK), and the colonies were identified by standard biochemical tests.

For *Salmonella enterica* serovar Typhimurium identification, 1 mg of feces was mixed in NSS, and 50 $\mu$l was inoculated into 5 ml of a selective enrichment medium (selenite cysteine; Difco, Becton Dickinson and Company, Sparks, MD, USA) and incubated under aerobic conditions at 37°C for 24 h. Subsequently, bacterial burdens in the inoculated medium were measured by spectrophotometry (absorbance reader; BioTek, Winooski, VT, USA) as the optical density at 600 nm ($OD_{600}$). The *Salmonella* levels were reported as $OD_{600}$ values. In addition, a correlation between the semiquantitative enumerations of *Salmonella* burdens determined by spectrophotometry and *Salmonella* Typhimurium putative cytoplasmic protein gene (STM4497) levels was demonstrated to support data from this analysis. The protocol for the detection of the *Salmonella* STM4497 gene was used as described previously (54). In brief, fecal samples (0.25 g) from individual mice were processed for total nucleic acid extraction using a High Pure PCR template preparation kit (Roche, USA), and nucleic acids were quantified by NanoDrop spectrophotometry (Thermo Fisher Scientific, Inc., USA). Real-time PCR targeting *Salmonella enterica* serovar Typhimurium was performed with the following set of primers: STM4497M2-F (5′-AAC AAC GGC TCC GGT AAT GAG ATT G-3′) and STM4497M2-R (5′-ATG ACA AAC TCT TGA TTC TGA AGA TCG-3′). Purified genomic DNA of *Salmonella enterica* serovar Typhimurium ATCC 13311 was used for the standard curve. PCR amplification was performed by using LightCycler FastStart DNA Master$^{PLUS}$ SYBR green I (Roche, Germany). The amount of amplified product was measured by the presence of a SYBR green fluorescence signal using LightCycler FastStart DNA Master$^{PLUS}$ SYBR green I (Roche, Germany), and the channel detection wavelength was 530 nm. Quantification of *Salmonella enterica* serovar Typhimurium bacteria was performed by using a standard curve, and values are expressed as numbers of bacteria (CFU).

To further support that the bacterial colonies present on selective enrichment medium were *Salmonella* spp., other selective culture media were also used. Briefly, turbid samples of the culture were swabbed, spotted onto modified semisolid Rappaport-Vassiliadis (MSRV) agar (Difco), and incubated under aerobic conditions at 42°C for 24 to 48 h. This method is based on the rapid detection of motile *Salmonella* bacteria on MSRV agar. Colonies from MSRV agar that resulted from migration from the original deposition spot were streaked onto *Salmonella-Shigella* (SS) agar (Difco), a selective medium that enables the detection of hydrogen sulfide ($H_2S$), and incubated under aerobic conditions at 37°C for 24 h to show the production of $H_2S$ from *Salmonella* colonies. Colonies with $H_2S$ (black colonies) were then identified further by bacterial Gram staining and biochemical reactions (data not shown).

For analysis of bacteria in blood, 20 $\mu$l of blood was spread onto blood agar plates (Oxoid) and incubated at 37°C for 24 h before the enumeration of bacterial colonies. The colonies were subsequently identified by standard biochemical tests. The remaining blood was centrifuged to separate serum and kept at −80°C for the analysis of serum IL-6 levels with an enzyme-linked immunosorbent assay (ELISA) (ReproTech, NJ, USA) according to the manufacturer's instructions.

**Anti-inflammatory effects of *Lactobacillus*-conditioned medium on human colonic epithelial cells.** The anti-inflammatory effects of the conditioned medium of *Lactobacillus rhamnosus* L34 in human colonic epithelial cells against pathogenic bacteria were tested according to methods reported previously (19). In short, human colorectal adenocarcinoma cells (Caco-2 and HT-29) from the American Type

Culture Collection (Manassas, VA, USA) (ATCC HTB-37 and ATCC HTB-38, respectively) were maintained in supplemented Dulbecco's modified Eagle medium (DMEM) and McCoy's 5a modified medium, respectively, at 37°C under 5% $CO_2$ and subcultured before use in the coculture assay. *Salmonella* Typhimurium ATCC 13311 and *Klebsiella pneumoniae* ATCC 13883 were grown on tryptic soy agar (Oxoid, Hampshire, UK) supplemented with 5% sheep blood under aerobic conditions at 37°C for 24 h. The bacteria were heat killed by incubation at 70°C for 45 min, sonicated for 1 h, and used for the activation of colonic epithelial cells. For the preparation of LCM, *L. rhamnosus* L34 cells at an $OD_{600}$ of 0.1 were incubated anaerobically for 48 h. The cell-free supernatants were then collected by centrifugation and filtered (0.22-$\mu$m membrane filter) (Minisart; Sartorius Stedim Biotech GmbH, Göttingen, Germany), and 500 $\mu$l of the preparation was concentrated by speed vacuum drying at 40°C for 3 h (Savant Instruments, Farmingdale, NY). The cell-free concentrated pellets were resuspended in an equal volume of DMEM and McCoy's 5a modified medium and stored at −20°C until use. Caco-2 and HT-29 cells ($5.0 \times 10^4$ cells/well) were then treated with LCM (5%, vol/vol) from *L. rhamnosus* L34 and coincubated with the heat-killed bacteria ($1.5 \times 10^7$ CFU/well) under 5% $CO_2$ at 37°C for 24 h. After this, the culture supernatants were prepared by centrifugation ($125 \times g$ at 4°C for 7 min), and levels of IL-8 were measured by an ELISA (Quantikine immunoassay; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Of note, measurements of IL-8 were used in the *in vitro* experiment because of the predominant IL-8 production, over IL-6 production, by human epithelial cells (19, 20).

**Characterization of the active anti-inflammatory molecules of LCM.** A protocol for the preliminary identification of active substances secreted from LCM was followed (40). The biochemical properties of LCM were observed by the neutralization of LCM anti-inflammatory properties (attenuation in bacterium-induced IL-8 production by colonic epithelial cells) after processing (heat, size separation, and enzyme inactivation) before use. Thermal stability was assessed by exposing LCM to a 100°C water bath for 0.25, 0.5, 1, or 2 h before use. Size separation was performed with centrifugal filters (Amicon Ultra-4; Millipore Ireland BV, Tullagreen, County Cork, Ireland) with molecular mass cutoffs of 3, 50, and 100 kDa, respectively, according to the manufacturer's instructions. The enzyme sensitivity of LCM was tested by incubation with one of various enzymes (Sigma-Aldrich), including $\alpha$-amylase, lipase, lysozyme, and proteinase K. Each enzyme, at a concentration of 1 mg/ml of LCM, was incubated at 37°C (25°C for amylase and lysozyme) for 6 h and heated in a 100°C water bath for 10 min for enzyme inactivation. After this, treated LCM was tested for IL-8-suppressive activity in a coculture assay with human colorectal adenocarcinoma cells (Caco-2 and HT-29 [ATCC HTB-37 and ATCC HTB-38, respectively]), as described above.

**Characterization of the anti-inflammatory cell signaling pathway of LCM.** Because the inhibitory effect of *Lactobacillus*-conditioned medium on the transcriptional factors TLR4 and NF-$\kappa$B has been demonstrated, we followed a protocol described previously (40). Hence, Caco-2 or HT-29 cells ($2.0 \times 10^6$ cells/well) were stimulated with heat-treated bacteria ($6.0 \times 10^8$ CFU/well) in the presence or absence of LCM (5%, vol/vol) for 15 and 30 min and centrifuged to separate the cells. The cellular NF-$\kappa$B level was then measured by Western blotting. Human antibodies against phosphorylated subunit p65–NF-$\kappa$B (p-NF-$\kappa$B) and total p65–NF-$\kappa$B (p-NF-$\kappa$B) (Santa Cruz Biotechnology, CA, USA) were used, and peroxidase signals were measured by using a ChemiDoc XRS system (Bio-Rad, Philadelphia, PA, USA). Densitometric analyses for protein quantification were carried out by using ImageJ 1.45s software. The activity of cellular NF-$\kappa$B was demonstrated by the ratio of p-NF-$\kappa$B (active molecule) to NF-$\kappa$B (total molecule).

In addition, the cellular TLR4 level was determined by quantitative reverse transcription-PCR (qRT-PCR). In short, total RNA of treated Caco-2 or HT-29 cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was prepared from total RNA (50 ng) by using a SuperScript Vilo cDNA synthesis kit (Invitrogen), and qPCR was performed with a LightCycler 2.0 instrument (Roche Diagnostics, Indianapolis, IN, USA). The following primers were used to amplify cDNA fragments: TLR4 forward primer 5′-CAGAACTG CAGGTGCTGG-3′, TLR4 reverse primer 5′-GTTCTCTAGAGATGCTAG-3′, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-GGAAGGTGAAGGTCGGAGTC-3′, and GAPDH reverse primer 5′-TCAGCCTTGACGGTGCCATG-3′ (55). TLR4 gene expression, relative to the value for GAPDH, was calculated according to the $2^{-\Delta\Delta Cp}$ method (56).

**Statistical analysis.** Data were analyzed as means ± standard errors (SE), and the differences between groups were examined for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey's comparison test. For experiments with data collected at multiple time points, repeated-measures ANOVA with Bonferroni *post hoc* analysis was used. Survival analysis was performed by using a log-rank test. For 2-group comparisons, the Student *t* test was used. All statistical analyses, including correlation and receiver-operator curve (ROC) plots, were performed with SPSS 11.5 software (SPSS, IL, USA). A *P* value of $<0.05$ was considered to be statistically significant.

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