

Probiotics improve survival of septic rats by suppressing conditioned pathogens in ascites

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Abstract

AIM: To investigate the benefits of probiotics treatment in septic rats.

METHODS: The septic rats were induced by cecal ligation and puncture. The animals of control, septic model and probiotics treated groups were treated with vehicle and mixed probiotics, respectively. The mixture of probiotics included *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. We observed the survival of septic rats using different amounts of mixed probiotics. We also detected the bacterial population in ascites and blood of experimental sepsis using cultivation and real-time polymerase chain reaction. The severity of mucosal inflammation in colonic tissues was determined.

RESULTS: Probiotics treatment improved survival of the rats significantly and this effect was dose dependent. The survival rate was 30% for vehicle-treated septic model group. However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly compared

with septic model group (80% and 55% vs 30%, $P < 0.05$). The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group (5.20 ± 0.57 vs 9.81 ± 0.67 , $P < 0.05$). The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group (33.3% vs 100.0%, $P < 0.05$). The population of *Escherichia coli* and *Staphylococcus aureus* in ascites of probiotics treated group were decreased significantly compared with that of septic model group (3.93 ± 0.73 vs 8.80 ± 0.83 , $P < 0.05$; 2.80 ± 1.04 vs 5.39 ± 1.21 , $P < 0.05$). With probiotics treatment, there was a decrease in the scores of inflammatory cell infiltration into the intestinal mucosa in septic animals (1.50 ± 0.25 vs 2.88 ± 0.14 , $P < 0.01$).

CONCLUSION: *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens in septic rats. Probiotics improve survival of septic rats by suppressing these conditioned pathogens.

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Key words: Sepsis; Probiotics; Pathogens; *Escherichia coli*; *Staphylococcus aureus*

Core tip: We observed the survival of septic rats treated with different amounts of mixed probiotics. The data indicated that conditioned pathogens such as *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens of septic rats in our study. Probiotics improve the survival of septic rats by suppressing the conditioned pathogens.

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INTRODUCTION

Sepsis is the systemic inflammatory response to infection and one of the most common causes of death in critically-ill patients^[1]. Each year, more than 750000 clinical cases of death occur due to sepsis, and the mortalities from severe sepsis were 20%-30% in the period of 1979-2000 in the United States^[2,3]. Microbial infection initiates and promotes systemic inflammatory responses by increasing cytokines release and neutrophils recruitment in target organs and inducing systemic inflammatory response syndrome and multiple organ dysfunction syndrome^[4]. It has been demonstrated that intestinal microbes play an important role in sepsis^[5]. Cecum is a pouch of large intestines connecting the terminal ileum to the ascending colon and home to a large number of anaerobic and aerobic microbes^[6]. Cecal ligation and puncture (CLP) of rats produce cecal ischemia and polymicrobial infection^[7]. The bacteria of colonic contents will spill into the abdomen, and produce severe peritonitis and bacteremia^[8]. So the CLP has been used as a classic animal model of sepsis^[9-11].

There is a complex microbial population in intestinal tract, some of which are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host^[12]. The products of probiotics include mucin, organic acids, branched chain fatty acids, H₂, CO₂, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis^[13,14]. The probiotics include *Bifidobacteria*, *Lactobacilli*, *Enterococci*, *Streptococci*, *Propionibacteria*, *Bacillus*, and yeasts. A variety of species of probiotics have been shown to benefit human gastrointestinal health^[15-17]. However, the mechanisms of probiotics in improving survival in sepsis are unclear. In this study, we sought to address this question in a septic model of Wistar rats.

MATERIALS AND METHODS

Animal experiments

Male Wistar rats (8-10 wk old, Animal Center of Academy of Military Medical Sciences, China) were housed on a 12:12 h light-dark cycle under pathogen-free conditions with free access to food and water. We performed CLP, a clinically relevant animal model for human sepsis^[18,19]. The animals were anesthetized by 10% chloral hydrate (3 mL/kg *via* intraperitoneal injection). After a midline incision was made in the abdomen, we isolated the cecum gently and placed a ligature 2.0 cm from the cecal tip using 2-0 silk suture. Ligated cecal stump was punctured by a 12-gauge needle. Colonic contents were extruded into abdominal cavity. We put back the cecum into its normal position and closed the abdomen by suturing muscle and skin, respectively. For control animals, the cecum was isolated without ligating and puncturing. The probiotic mixture consisted of three different viable strains. One

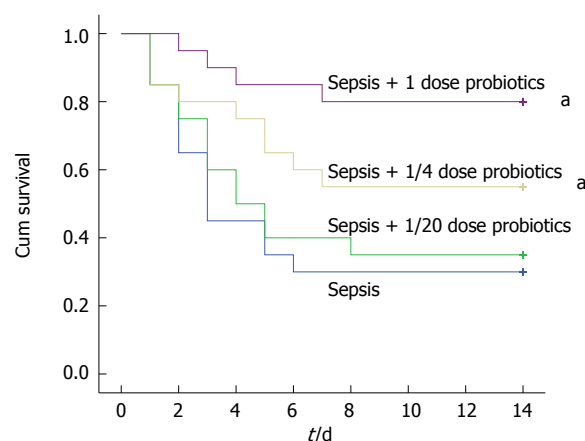


Figure 1 Survival in experimental sepsis. Survival was analysed in Wistar rats subjected to cecal ligation and puncture. Probiotics (1, 1/4 or 1/20 doses) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. All animals were observed for two weeks to compare their survival rates ($n = 20$; $^aP < 0.05$ vs septic model group).

dose of the probiotic mixture contained 1×10^7 CFU *Bifidobacterium longum* (ATCC 15697), 1×10^6 CFU *Lactobacillus bulgaricus* (ATCC 11842) and 1×10^6 CFU *Streptococcus thermophilus* (ATCC 19987). Before administration, the probiotic mixture was reconstituted in sterile water for 10 min at 37 °C. We gave probiotics to animals of treated groups through intragastric administration. The animals of control and septic model groups were treated with vehicle (sterile water). The first administration of probiotics or vehicle was started 6 h after surgery. Thereafter, it was administered once a day for 3 d.

Samples collection

All surviving animals were anaesthetized by 10% chloral hydrate (3 mL/kg, *via* intraperitoneal injection) after a 72 h period of CLP. Samples of blood and ascites were harvested for both anaerobic and aerobic microbial analysis immediately. Another portion of ascites was stored at -80 °C for DNA extraction. Then rats were killed by cervical dislocation, and colonic tissues were collected in neutral buffered formalin for histological analysis.

Microbial analysis of blood and ascites

Serial 10-fold dilutions were made in 0.9% sterile saline. We spread 20 μ L of 10^0 - 10^{-7} dilutions on the nonselective blood-agar (Jinzhang Co, Ltd., Tianjin, China) surface. For anaerobic incubation, the anaerobic blood-agar dishes (Jinzhang Co, Ltd., Tianjin, China) were placed in anaerobic bags (bioMérieux, France) immediately. The time of aerobic incubation was shorter (24 h) than anaerobic (48 h) at 37 °C. The colonies were determined in appropriate dilution, and total viable counts of original samples were calculated. Different colonies were separated and isolated for 2-3 times. We identified bacterial species using colony morphology and Gram's stain. Microstation microbe analysis system (Biolog, Winooski, VT, United States) was used for advanced identification.

Table 1 Comparison of bacterial spectrum and total viable count in ascites between septic model group and probiotics treated group

Group	Bacterial spectrums in ascites	Total viable counts (Log ₁₀ cells/mL ascites)
Septic model group	<i>Escherichia coli</i> , <i>Enterococcus faecalis</i> , <i>Staphylococcus aureus</i> , <i>Enterococcus avium</i> , <i>Streptococcus viridans</i> , <i>Streptococcus agalactiae</i> , <i>Micrococcus luteus</i> , <i>Enterococcus gallinarum</i> , <i>Enterococcus durans</i> , <i>Enterococcus malodoratus</i> , <i>Streptococcus ferus</i> , <i>Morganella morganii</i> , <i>ss morganii</i> , <i>Acinetobacter radioresistens</i> , <i>Streptococcus criceti</i> , <i>Lactobacillus reuteri</i> , <i>Veillonella criceti</i> \ <i>ratti</i> , <i>Desulfovibrio fructosivorans</i> , <i>Clostridium oroticum</i> , <i>Lactobacillus bifermentans</i>	9.81 ± 0.67
Probiotics treated group	<i>Escherichia coli</i> , <i>Enterococcus faecalis</i> , <i>Staphylococcus aureus</i> , <i>Enterococcus malodoratus</i> , <i>Morganella morganii</i> <i>ss morganii</i> , <i>Enterococcus durans</i> , <i>Streptococcus viridans</i> , <i>Prevotella dentioola</i> , <i>Desulfovibrio fructosivorans</i> , <i>Bacteroides ovatus</i> , <i>Prevotella nigrescens</i>	5.20 ± 0.57 ^a

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. The bacterial spectrum of ascites was lower in probiotics treated group than in septic model group. The total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group ($n = 18$; ^a $P < 0.05$ vs septic model group).

Quantitative real-time polymerase chain reaction

Bacterial genomic DNA was extracted from ascites of rats using the QIAamp DNA mini kit (Qiagen, Hilden, United States) according to the manufacturer's protocol. We obtained 16S rRNA sequences of bacteria from the Ribosomal Database: (<http://rdp.cme.msu.edu/>), and designed primers for the specific bacterial strain using Primer 5.0 software package. The genomic DNA was used as template for the amplification of specimen and control standard bacterial strain through real-time polymerase chain reaction (PCR). PCR cycles were as follows: initial denaturation at 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s. PCR primers were: *Escherichia coli* forward: 5'-CATGCCGCGT-GTATGAAGAA-3' and reverse: 5'-CGGGTAACGT-CAATGAGCAAA-3'; *Enterococcus faecalis* forward: 5'-CAGCAGTAGGGAATCTTCGGCAATG-3' and reverse: 5'-AGCCTCAGCGTCAGTTACAGACCAG 3'; *Staphylococcus aureus* forward: 5'-CGTCAGCTCGT-GTCGTGAGATGTTG-3' and reverse: 5'-GCGGTT-

Table 2 Comparison of bacterial spectrum and total positive rate of hemoculture between septic model group and probiotics treated group

Group	Bacterial spectrums of hemoculture	Total positive rate of hemoculture
Septic model group	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i> , <i>Curtobacterium pusillum</i> , CDC group II-E subgroup A	100%
Probiotics treated group	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i>	33.3% ^a

Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of blood were harvested for both anaerobic and aerobic culture. The bacterial spectrum of hemoculture was lower in probiotics treated group than in septic model group. The total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group ($n = 18$; ^a $P < 0.05$ vs septic model group).

TCGCTACCCTTTGTATTTGT-3'. The real-time PCR was performed using FastStart SYBR Green Master (Roche, Basel, Switzerland) and IQ5 PCR system (BIO-RAD, Hercules, CA, United States).

Histological examination of intestinal inflammation

The colonic tissues of at least four rats in each group were fixed in neutral buffered formalin, and processed for histological analysis. The sections of colonic tissues were stained by haematoxylin-eosin. Colonic sections were assessed for the severity of mucosal inflammation based on the following: infiltration of neutrophils and mononuclear cells into the intestinal mucosa (0, scant to normal; 1, minimal to mild; 2, mild to moderate; 3, moderate to severe; 4, severe inflammation)^[20,21], and four fields of each sample were assessed. Moreover, epithelial thickness was measured under microscope (Leica, Frankfurt, Germany).

Statistical analysis

Data were expressed as the mean ± SD. All statistical analyses were performed using SPSS 17.0 software package. Survival analysis was shown in Kaplan-Meier survival curves. Survival comparisons between two subgroups were performed by the log-rank test. Differences between two groups were analysed using unpaired *t* test for continuous variables and the χ^2 test for nominal variables. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Probiotics improve the survival of rats with experimental sepsis

One hundred male Wistar rats were divided into five groups (control group, septic model group and three sepsis plus treatment groups) for survival analysis. We gave probiotic mixture (1, 1/4 or 1/20 doses) to animals in three treated groups by intragastric administration (once a day for 3 d). The animals of control and septic model

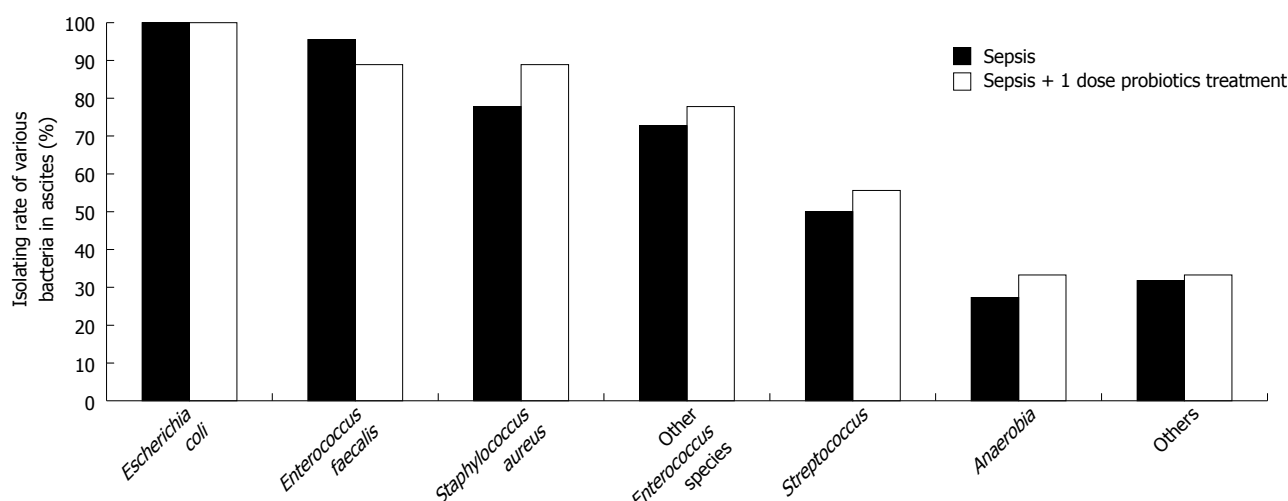


Figure 2 Comparison of isolating rate of various bacteria in ascites between sepsis and probiotics treated group. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Samples of ascites were harvested for both anaerobic and aerobic culture. There was no statistical significance in isolating rates between two groups for all bacterial species ($P > 0.05$). In addition, "other *Enterococcus* species" include *Enterococcus avium*, *Enterococcus gallinarum*, *Enterococcus durans* and *Enterococcus malodoratus*. "Anaerobia" include *Lactobacillus reuteri*, *Veillonella cricetratti*, *Desulfovibrio fructosivorans*, *Clostridium oroticum*, *Lactobacillus bifementans*, *Prevotella dentioola*, *Bacteroides ovatus* and *Prevotella nigrescens*. "Others" include *Micrococcus luteus*, *Morganella morganii ss morganii* and *Acinetobacter radioresistens*.

groups were treated with vehicle only. We observed all animals for two weeks. The animals in control group survived normally. The majority of rats who had CLP showed clear signs of sepsis such as piloerection, lethargy, malaise and forming ascites. Probiotics attenuated the clinical manifestations of sepsis. Probiotics treatment also improved survival significantly and this effect was dose dependent. The survival rate was the lowest (30%, 6/20 rats) in the vehicle-treated septic model group. There was no protective effect using 1/20 dose probiotics (survival rate was 35%, 7/20 rats). However, 1 and 1/4 doses of probiotics treatment increased survival rate significantly (80%, 16/20 rats and 55%, 11/20 rats) compared with vehicle treated septic model group ($P < 0.05$) (Figure 1).

Probiotics inhibit bacteria in blood and ascites of rat experimental sepsis

The consequence of survival analysis indicated that 1 dose probiotics treatment was more effective than other doses. Therefore, we divided 80 male Wistar rats into three groups (control group, 8 rats; septic model group, 50 rats; and sepsis plus 1 dose probiotics treated group, 22 rats). Probiotics or vehicle were given to the animals through intragastric administration (once a day for 3 d), respectively. All animals (8 rats) in the control group survived normally. Forty-four percent of animals (22 rats) were alive in septic model group, and 81.8% of animals (18 rats) were alive in probiotics treated group. We harvested samples after a 72 h period of CLP. The microbial composition of blood and ascites were analysed. No bacteria were determined in blood and ascites of control group. The bacterial spectrum of ascites (Table 1) and blood (Table 2) was lower in probiotics treated group than in septic model group. There was no statistical significance in isolating rates between two groups for all

bacterial species ($P > 0.05$, Figure 2). However, the total viable counts of bacteria in ascites decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 1). Similarly, the total positive rate of hemoculture decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Table 2).

The consequence of bacterial cultivation indicated that *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus* were predominant microbial population in ascites of sepsis. For this reason, we detected the population of these bacteria in ascites using quantitative real-time PCR. The data indicated that all population of these bacteria decreased significantly in probiotics treated group compared with septic model group ($P < 0.05$, Figure 3).

Probiotics improve colonic mucosal inflammation of experimental sepsis

With probiotics treatment, there was a decrease in the infiltration of neutrophils and mononuclear cells into the intestinal mucosa in septic animals ($P < 0.05$, Figure 4). No apparent differences of epithelial cell hyperplasia were found between the rats in probiotics treated group and septic model group (data not shown).

DISCUSSION

Despite the development of antibiotics and other intensive care treatment, sepsis has a high mortality. CLP of rats is one of animal models of human sepsis. Because colonic contents are extruded into abdominal cavity, various microbes proliferate in ascites immediately. Therefore, the bacteria from feces cause polymicrobial infection, bacteremia and lethal peritonitis^[22,23]. In this study, we treated the experimental septic rats with mixture of

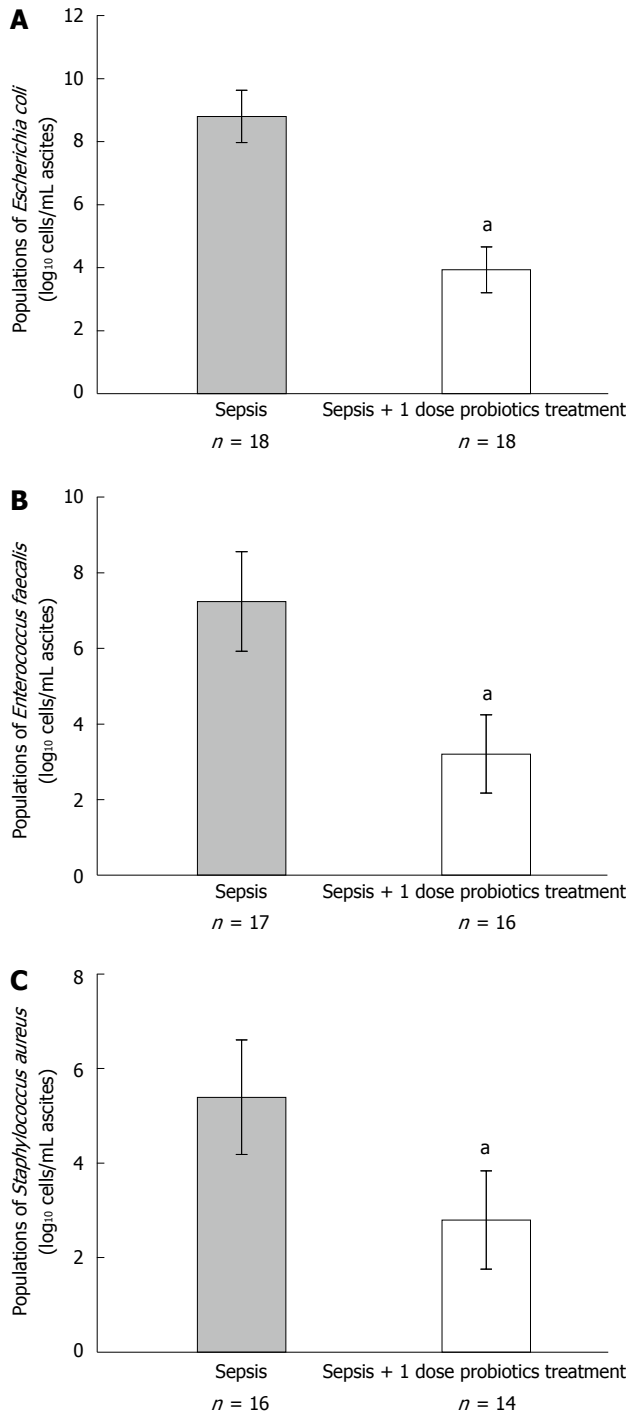


Figure 3 Comparison of predominant bacterial populations in ascites between sepsis and probiotics treated groups. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. Bacterial genomic DNA was extracted and analysed by quantitative real-time PCR as described previously. The population of *Escherichia coli* (A), *Enterococcus faecalis* (B) and *Staphylococcus aureus* (C) were compared between two groups (^a*P* < 0.05 vs septic model group).

three live probiotics. We also analysed the survival of probiotics treated septic animals. It was demonstrated that probiotics improved the survival of rats with experimental sepsis and this effect was dose dependent. No protective effect was observed using the lowest concentration of probiotics (1/20 dose). However, 1 and 1/4

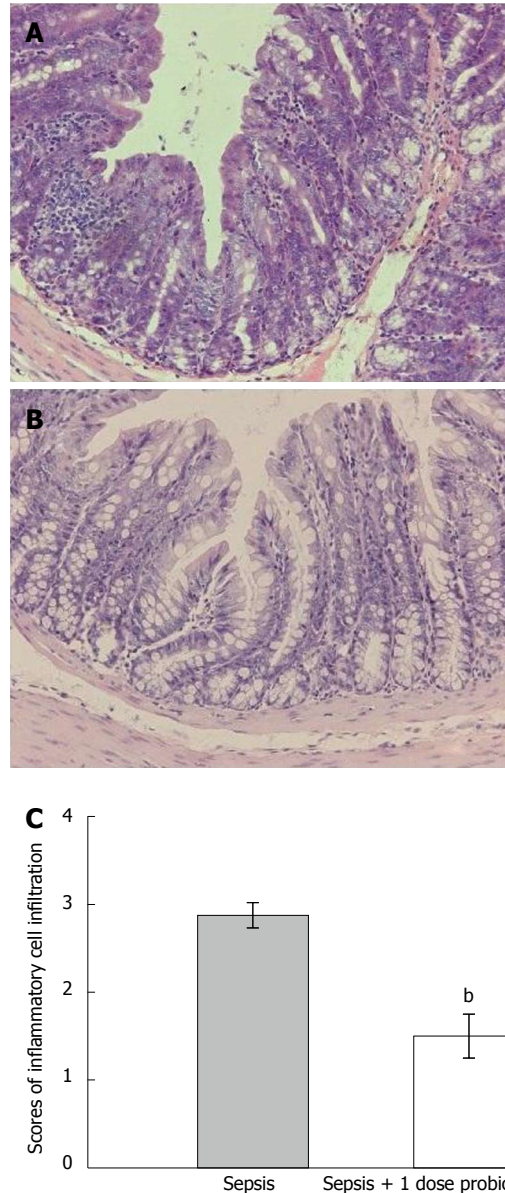


Figure 4 Colonic mucosal inflammation of experimental sepsis. Probiotics (1 dose) or vehicle treatment started 6 h later and thereafter administered once a day for 3 d. We harvested colonic tissues after a 72 h period of cecal ligation and puncture. The sections of colonic tissues were stained by haematoxylin-eosin (× 400). Four fields of each sample were assessed. More neutrophils and mononuclear cells infiltrated into the intestinal mucosa in septic model group (A) than probiotics treatment group (B). We also compared scores of inflammatory cell infiltration between two groups (C) (^b*P* < 0.01 vs septic model group).

doses of probiotics treatment increased survival significantly compared with septic model group. Therefore, we treated septic animals in subsequent experiments using 1 dose of probiotics all the time.

Ascites culture data indicates that more pathogens grew in septic model group (10^9 - 10^{10} cells/mL) than in probiotic treated group (10^4 - 10^5 cells/mL). Both aerobes and anaerobes were detected in ascitic samples, although the majority of microbes were aerobes. Cecum contained anaerobes, facultative aerobes and aerobes. Furthermore, the amounts of anaerobes were greater than those of aerobes^[24,25]. However, in our study, aerobes had been

isolated frequently from septic ascitic samples such as *Escherichia coli* (isolating rate was 100%), *Enterococcus faecalis* (95.5%) and *Staphylococcus aureus* (77.8%). The total isolating rate of anaerobes was less than 30%. The main reason for this phenomenon was “oxygen”. When the operation of CLP was performed in experimental sepsis, the anaerobes were exposed to oxygen directly. Furthermore, some oxygen was stored in abdominal cavity of animal after operation. For these reasons, the majority of anaerobes were killed by oxygen. Thereafter, the aerobes which were minority in original colonic contents proliferated immediately. When we gave probiotics to septic rats, the bacterial spectrum of ascites and blood was lower than in the septic model group. Meanwhile, probiotics decreased total viable counts of pathogens in septic ascites significantly. In addition, the data of hemoculture showed that *Escherichia coli* and *Staphylococcus aureus* usually were detected in septic model group. Probiotics decreased the positive rate of hemoculture in septic rats.

It seemed that *Escherichia coli* and *Staphylococcus aureus* are the primary pathogens of CLP rats in septic model in our study. On one hand, we detected the population of these bacteria in ascites by quantitative real-time PCR. All population of these bacteria decreased significantly in probiotics treated group compared with septic model group. On the other hand, inflammatory response of intestinal mucosa was lessened in probiotics treated group compared with septic model group. All these data indicated that the mixture of probiotics improved the survival in a murine model of polymicrobial sepsis by suppressing the conditioned pathogens. However, the reasons for this suppression are not clear. There are two potential reasons: first, the decreased bacterial number may result from the inhibition of bacterial proliferation; second, a less bacteria infiltration or promoted bacterial killing^[26-30].

Based on what had been mentioned above, we draw a conclusion that conditioned pathogens (*Escherichia coli* and *Staphylococcus aureus*) may be primary pathogens of CLP rats in septic model in our study. Probiotics (*Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) contribute to improving the survival in an animal sepsis model by suppressing the conditioned pathogens.

COMMENTS

Background

Sepsis is the systemic inflammatory response to infection. Microbial infection initiates and promotes systemic inflammatory responses. A variety of species of probiotics have been shown to benefit human gastrointestinal health. However, the mechanisms of probiotics in improving sepsis are unclear. In this study, the authors sought to address this question in the septic model of Wistar rats.

Research frontiers

Recently, more and more gastrointestinal diseases have been treated using probiotics. The probiotics and their products keep gastrointestinal tract homeostasis and regulate immune responses. For example, probiotics can regulate IgE production level and maturation of T cell in the gut.

Innovations and breakthroughs

To study the benefits of probiotics for sepsis, the authors observed the survival of cecal ligation and puncture (CLP) rats using different amounts mixed probiotics. The mixture of probiotics included *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. They also detected

bacterial populations in ascites and blood of CLP rats using cultivation and real-time polymerase chain reaction. The data suggested that *Escherichia coli* and *Staphylococcus aureus* may be primary pathogens in the septic model. Probiotics improve survival in the septic model by suppressing the conditioned pathogens.

Applications

The results of this study suggest that probiotics improve survival in the septic model by suppressing the conditioned pathogens. This study helps to know whether probiotics can improve the clinical course of sepsis.

Terminology

CLP of rats produces cecal ischemia and polymicrobial infection. The bacteria of fecal contents will spill into the abdomen, and produce severe peritonitis and bacteremia. So CLP has been used as a classic animal model of sepsis. There are complex microbial populations in intestinal tract. Some of them are probiotics. When administered in adequate amounts, probiotics confer a health benefit to the host. The products of probiotics include mucin, organic acids, branched chain fatty acids, H₂, CO₂, ammonia, amines and vitamins. These products regulate host health through different pathways such as regulating energy, gene expression and cell differentiation, producing anti-inflammatory agents and keeping gut homeostasis.

Peer review

This study investigated the potential benefit of probiotic supplement in preventing septic death by studying the survival rates in rats treated with different doses of a probiotic mixture and then further investigated the effects of probiotics administration on the bacteria proliferation in blood and ascites in a cecal ligation and puncture sepsis model. This study shows the importance of knowing whether probiotics can improve the survival of experimental sepsis.

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