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Original article

Intestinal bacteria and permeability during experimental acute pancreatitis in rats

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SUMMARY

Background: An increase in intestinal permeability and subsequent bacterial translocation has been demonstrated in critical illness. Cellulose derivatives have in the past been shown to reduce gut leakage following liver resection.

Aims: The aim of the present study was to evaluate changes in microbial counts in experimental acute pancreatitis and the effect of pre-treatment with cellulose derivatives and *N*-acetyl cysteine.

Subjects: 92 male Sprague Dawley rats.

Methods: Acute pancreatitis was induced by intraductal taurodeoxycholic acid infusion. Animals received oral pre-treatment and were randomized to either sham operation or the pancreatitis groups, with or without pre-treatment with cellulose derivatives, the antioxidant or their combinations. Intestinal bacterial populations and permeability were evaluated using bacterial counts and Ussing chamber, respectively.

Results: The number of *E. coli* increased in the luminal content and ileal and colonic mucosa, but levels were restored to almost those seen in controls in all pre-treatment groups except for *N*-acetyl cysteine. When intestinal permeability was measured, none of the treatment groups showed significant differences compared to challenge, except for *N*-acetyl cysteine, which significantly increased permeability.

Conclusion: Pre-treatment with cellulose derivatives was more efficient against disturbances in intestinal permeability and microbial populations than the antioxidant *N*-acetyl cysteine.

Key words: acute pancreatitis, bacterial counts, dietary fibres, intestinal permeability

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INTRODUCTION

Acute pancreatitis (AP) is a rather common disease associated with high morbidity and in its severe form, also a high mortality.[1] The disease progresses from a local auto-digestive reaction confined to the pancreas to a systemic inflammation (systemic inflammatory response syndrome, SIRS). The inflammatory reaction is believed to be elevated by the immunological activity of the gut. The gut barrier consists of several different systems that prevent bacteria and bacterial products to enter the circulation. These barriers include both physical barriers such as the mucosal layer and other mechanisms such as the immunological status and gut flora. Normally, the microbial ecological system is kept in balance but may be altered during disease. Under these circumstances both pathogenic and opportunistic bacteria might increase in number. Overgrowth of *Escherichia coli* (*E. coli*) has experimentally been demonstrated in rats[2,3] and overgrowth of potentially pathogenic bacteria has been suggested to be one of the causes underlying the multiple organ dysfunction syndrome.[4,5] Prevention of these changes in intestinal microbial ecology is of obvious im-

portance, maintaining the functions and integrity of the gut barrier. This is clinically relevant as an increased incidence of sepsis and a worsened outcome in patients with infected pancreatic necrosis have been reported.[6-8]

Ethyl hydroxyethyl cellulose (EHEC) is a water-soluble cellulose derivative. It is reported to be inert to microbial fermentation due to the even distribution of its substituents.[9] It has experimentally been shown to maintain gut barrier integrity and intestinal bacterial populations following surgically induced acute liver failure and *E. coli*growth.[9-11] LM-200 is a water-soluble cathionic ammonium salt of hydroxyl ethyl cellulose (polyquarternium 24). It has a molecular weight of approximately 100 000 and consists of a hydrophilic backbone and lipophilic pendant groups. Because of its hyrdophobicity and also its positively charged groups it adheres to biological surfaces both by hydrophobic interactions and electrostatic association. Intravenously administered *N*-acetyl cysteine (NAC) have previously been shown to reduce systemic and local as well as damage of remote organs in rat subject to taurodeoxycholate induced AP.[12,13]

The present study aimed at studying changes in intestinal enteric bacterial counts and permeability in an experimental model of AP with previously demonstrated increases in gut barrier permeability and translocation.[12,13] Furthermore, the effects of pre-treatment with the cellulose derivatives EHEC and LM-200 as well as the broad-acting antioxidant NAC were evaluated.

Table 1. Experimental design of bacterial measurements. The following groups were used in the experiment: sham operated pre-treated with NaCl (sham), acute pancreatitis pre-treated with NaCl (AP), pre-treatment with LM-200 (LM), pre-treatment with NAC (NAC), pre-treatment with EHEC (EHEC), pre-treatment with combination of NAC and EHEC (NAC +EHEC) and pre-treatment with combination of NAC and LM-200 (NAC+LM-200).

Group	Challenge	Pre-treatment
Sham	Sham OP	NaCl
Chall	AP	NaCl
LM	AP	LM-200
NAC	AP	NAC
EHEC	AP	EHEC
NAC+EHEC	AP	NAC+EHEC
NAC+LM	AP	NAC+LM-200

Table 2. Experimental design of permeability measurements. The following groups were used in the experiment: sham operated pre-treated with NaCl (sham), acute pancreatitis pre-treated with NaCl (AP), pre-treatment with LM-200 (LM), pre-treatment with NAC (NAC) and pre-treatment with EHEC (EHEC).

Group	Challenge	Pre-treatment
Sham	Sham OP	NaCl
Chall	AP	NaCl
LM	AP	LM-200
NAC EHEC	AP	NAC EHEC AP

MATERIALS AND METHODS

Animals, pre-treatment and experimental design

A total of 42 male Sprague-Dawley rats (SD, Scanbur BK AB, Sollentuna, Sweden) rats, weighing approximately 180 g, were used in the ecology experiments, allocated into seven groups, one AP group and five AP pre-treatment groups were used (Table 1). An additional 50 animals were used for the permeability experiments and were evenly allocated into five groups, ten animals in each. The groups were; sham, AP and three AP pre-treatment groups (Table 2). All animals were kept under standard conditions (12 hours dark/light cycle, 22°C) for five days prior to the experiment. The rats had free access to water and rodent chow (R34, Lactamin AB, Kimstad, Sweden).

The animals were kept in standard laboratory cages, with three animals in each cage. The study was approved by the local Ethical Animal Research Committee (Malmö/Lunds Djurförsöksetiska Nämnd).

Twenty-four and one hour prior to the induction of pancreatitis, all animals received 1 ml of saline (NaCl, 0.9%), *N*-acetyl cysteine (NAC, 50 mg/kg rat), ethyl hydroxyethyl cellulose (EHEC, 16.8 mg/ml), LM-200 (LM, 16.8 mg/ml) or combinations of EHEC or LM-200 together with NAC as pre-treatment. All pre-treatment substances were dissolved in saline. The administrated volume in all groups was 1 ml and it was given orally through a soft feeding tube.

Induction of AP

The animals were anaesthetized using a mixture of ketamine hydrochloride (Ketalar, Parke-Davis, Warner Lambert Nordic AB, Solna, Sweden; 50 mg/ml) and xylazine hydrochloride (Rompun vet., Bayer AB, Gothenburg, Sweden; 20 mg/ml) administered intramuscularly at least 20 min prior to the induction of AP. The abdomen was shaved, disinfected and opened along the midline. AP was induced by clamping the proximal end of the common bile duct and cannulating the biliary-pancreatic duct. 0.2 ml of 5% sodium taurodeoxycholate (in

glycyl-glycin-NaOH buffer 0.025 mol/l, pH 8.0) was administered by means of an infusion pump at a flow rate of 0.04 ml/min into the duct.[14] The sham operation was performed as above but omitting the canulation and infusion. The abdomen was closed in two layers with continuous 4/0 polypropylene sutures. After surgery, buprenorphine hydrochloride (Temgesic®, Schering-Plough AB, Stockholm, Sweden; 0,3 mg/ml) was administered intramuscularly.

Sham operation was performed similarly but without infusion of the sodium taurodeoxycholate solution.

The animals were killed by exsanguination and organs were collected for bacterial analysis nine hours after pancreatitis induction and six hours after AP induction when performing the permeability studies.

Microbiological measurements

Viable counts were performed for total aerobic counts, total anaerobic counts and *E. coli*. A midline incision was made using sterile technique. A sample of 0.2mL of blood was collected from the femoral artery for detection of bacterial presence in blood. Organ samples from mesenteric lymph nodes (MLN), pancreas, liver, lung, ileal, caecal and colonic mucosa as well as luminal content from the ileum, caecum and colon were collected and weighed. The luminal content was separated from the intestine. Next the intestine was rinsed with saline to remove any luminal content from the mucosa. The mucosa was separated from adjacent tissue by sharp dissection. The blood samples were inoculated in test tubes containing 3.0 mL of Brain – Heart Infusion (BHI) broth (Difco laboratories, Detroit, IL, USA). Aliquots of 0.2mL of the supernatant were cultured for aerobic, microaerophillic and anaerobic bacteria. All media for aerobic culture were incubated at 37˚C for at least 3 – 5 days, while organ samples for anaerobic bacteria were cultured at 37˚C for 7 days before discarded as negative. Enteric Gram–negative bacteria were identified using the API 20 system (Biomιrieux, Marcy-lιtoile, France) and *Lactobacillus spp.* by API 50 CH (Analytab Products, Plainview. NY, USA). All other aerobic microaerophylic and anaerobic bacteria isolated were identified by standard procedures.[16] The number of living bacteria were calculated and expressed as the number of living organisms per gram organ tissue. The results are presented as colony forming units (CFU) per gram.

Permeability measurements

Six hours after induction of AP the animals were anesthetised and a midline lapratomy was performed. Permeability measurements were performed similarly to what has been described earlier.[17] Ten cm sections of proximal ileum, distal ileum and colon were removed and immediately placed in room tempered modified Krebs solution (composed of 0.11 M NaCl, 3.0 mM $CaCl_2$, 5.5 mM KCl, 1.4 mM KH_2PO_4, 29.0 mM $NaHCO_3$, 5.7 mM sodium pyruvate, 7.0 mM sodium fumarate, 5.7 mM sodium glutamate, 13.4 mM glucose) equilibrated with physiological gas by bubbling the solution with carbogen (95% O2 and 5% CO2). The intestine was rinsed in Krebs solution, cut along the mesenterial border and mounted in the Ussing chamber (Precision Instrument Design, Los Altos, CA, USA). The time between organ harvest and mounting in the Ussing chamber never exceeded 30 minutes. The serosal side was filled with modified Krebs solution containing 0.001% fetal calf serum and the mucosal side was filled with modified Krebs solution containing mannitol (2.44 mg/ml) and the two marker substances ^{14}C-mannitol and ovalbumin. The Ussing setup was kept at 37˚C and carbogen was bubbled through the solutions during the whole experiment. The permeability was measured every 20 minutes during two hours by pipetting 1 ml from the serosal side and the volume was compensated with the same amount of modified Krebs solution. The ^{14}C-mannitol was measured in a gamma counter (Beckman, LS 6500) and the ovalbumin was measured by rocket electrophoresis and the concentrations were plotted versus time. The permeability values were presented as coefficients (k-values) from these plots.

Statistics

The statistical calculations were performed using the non-parametric Mann-Whitney U-test. The results are presented as box plots and in the figures a p value of <0.05 is labelled * and <0.05 is shown as **. In the case of comparisons between sham to challenge + was used and in the case of treatment versus challenge * was used. Outliers were marked as such in the tables (o) if the value was greater than three standards from the mean in either direction. All statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Bacterial ecology in colon and ileum

During AP, intestinal bacterial counts were altered in both the colonic and ileal mucosa and corresponding luminal contents. This was most pronounced for *E. coli* (Figure 1), while less pronounced for the total concentration of anaerobes (Figure 2) and less, but still significant for total aerobic counts (Figure 3). Higher levels of *E. coli* were found in luminal content as compared to mucosal

counts in the ileum and colon (Figure 1). Anaerobes demonstrated a similar pattern in the luminal content as well as in the mucosa (Figure 2). Aerobic bacterial counts of the luminal content were lower than in the corresponding mucosa in both the ileum and the colon (Figure 3).

Translocation data was inconclusive. Differences in bacterial counts in blood, mesenteric lymph, liver and lung nodes between neither groups reached statistical significance (data not shown).

E. coli

In both colonic and ileal mucosa and luminal content, concentrations of *E. coli* were significantly increased in SAP versus the sham group ($p<0.05$). Pretreatment with LM-200 normalized the levels in both the colonic and ileal mucosa as well as in the luminal content (Figure 1). Combination treatment with NAC did not demonstrate any additive effects. EHEC pretreatment reduced *E. coli* levels in the colonic mucosa and luminal content but not in the ileum. Ileal luminal content of the EHEC and NAC groups, when these three agents were administered individually, did not significantly differ as compared to untreated pancreatitis animals, but in combination, a synergistic effect could be demonstrated. NAC pre-treatment alone did not result in any significant effect in neither the colon nor ileum (figure 1).

Anaerobes

The total anaerobic count was significantly ($p<0.05$) raised following SAP in both colonic and ileal content but

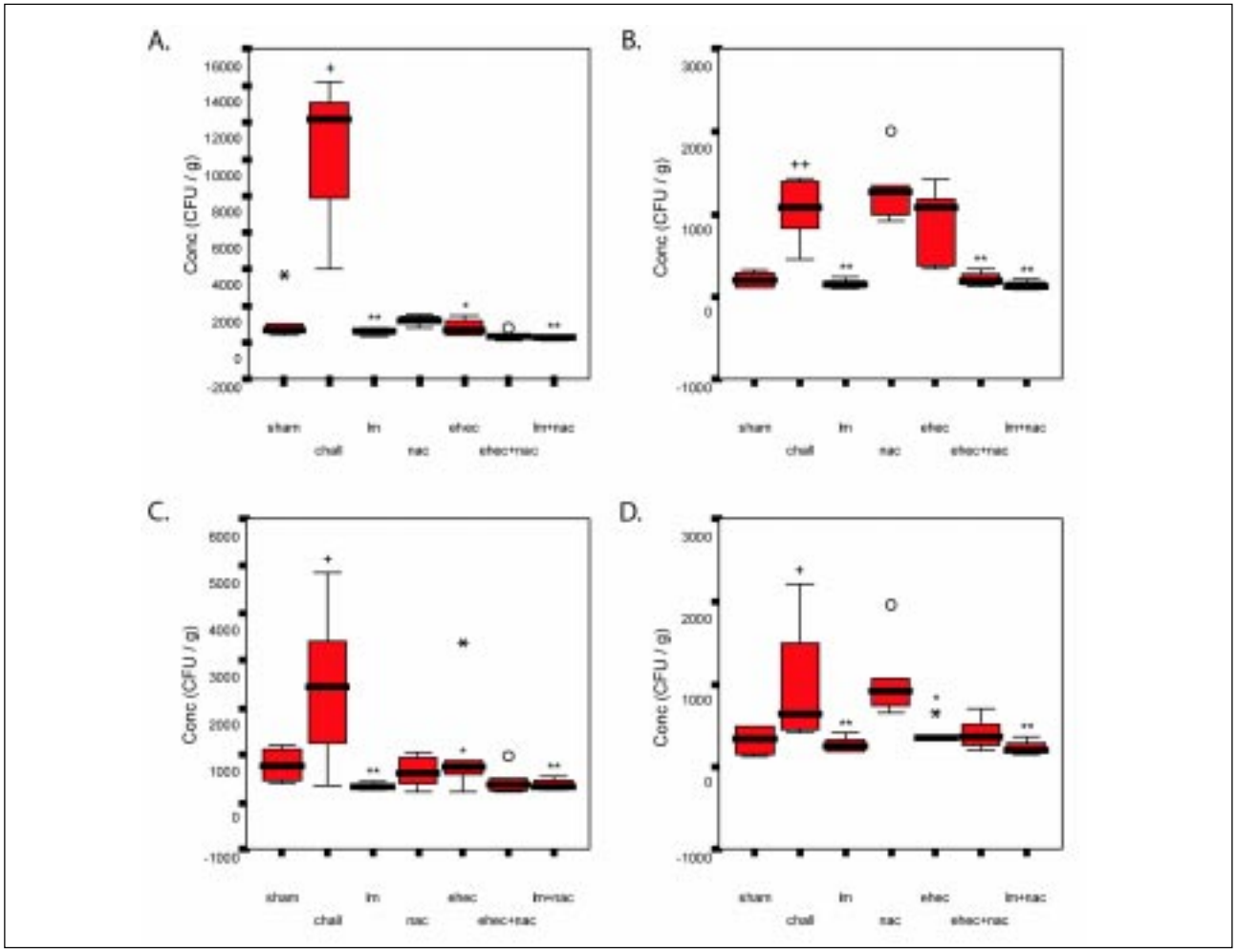

Fig. 1. Intestinal ecology of *E. coli* in the A) colonic luminal content, B) ileal luminal content, C) colonic mucosa and D) ileal mucosa. In all groups, the levels were increased in all groups during SAP. Treatment effects were most pronounced in the colon where all treatment groups normalized the disturbance otherwise caused by SAP. In the ileum, LM-200 and the combination groups were most effective.

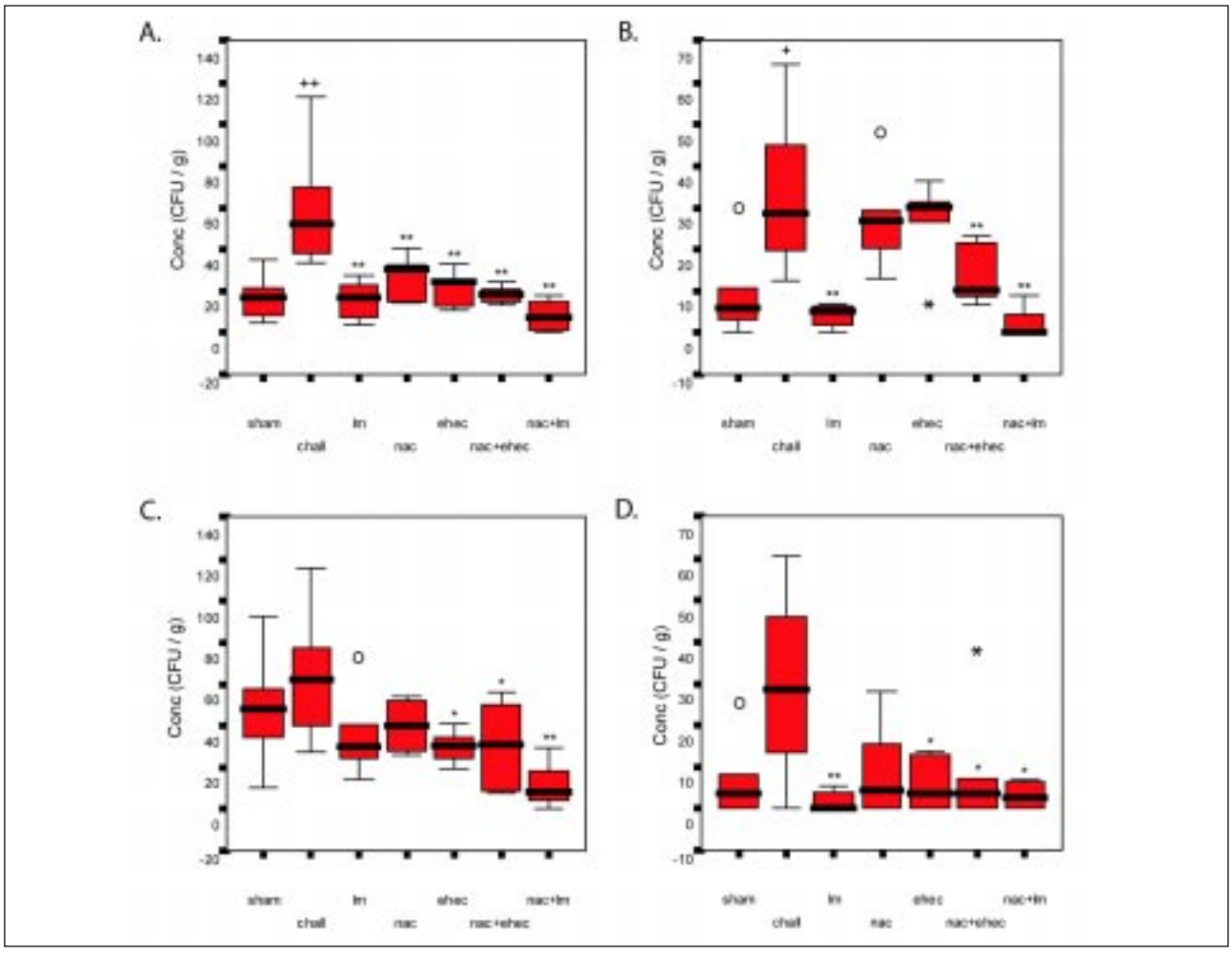

Fig. 2. Intestinal ecology of anaerobic bacteria in the A) colonic luminal content, B) ileal luminal content, C) colonic mucosa and D) ileal mucosa. Also the total number of anaerobic bacteria showed similar patterns as for *E. coli*.

not in the mucosa (figure 2). In colonic contents, anaerobic bacterial levels in all pre-treatment groups were significantly ($p<0.01$) reduced. In ileal luminal content LM-200 pre-treatment resulted in a significant decrease in the number of anaerobic bacteria versus saline pre-treatment ($p<0.05$), while EHEC and NAC were not effective. In figure 2, unlike other samples, NAC and EHEC exhibit significant additive effects on ileal content (figure 2b). The concentrations in the ileum were lower than in the colon but were at both sites elevated during SAP. LM-200 reduced anaerobic bacterial levels ($p<0.05$), as did the combination of LM-200 and NAC ($p<0.01$) as compared to saline pre-treatment. Combining NAC and EHEC did not result in any significant additive effects (figure 2).

Total aerobes

In the colonic content, the concentration of aerobic bacteria increased ($p<0.01$) during SAP compared to saline pre-treatment. Levels of aerobic bacteria were otherwise not significantly altered in colonic mucosa or ileal mucosa or content (Figure 3). LM-200 reduced these levels significantly ($p<0.05$) as compared to saline in colonic content, as did the two types of combination treatment ($p<0.01$) (Figure 3).

Permeability

In the NAC group the permeability significantly ($p<0.01$) increased as compared to challenge in all three intestinal segments as noted both for the smallest marker mannitol and the larger ovalbumin.

Pre-treatment of EHEC significantly ($p<0.01$) decreased the permeability to mannitol in all segments, while the permeability to ovalbumin only decreased ($p<0.01$) in distal ileum (Figure 4).

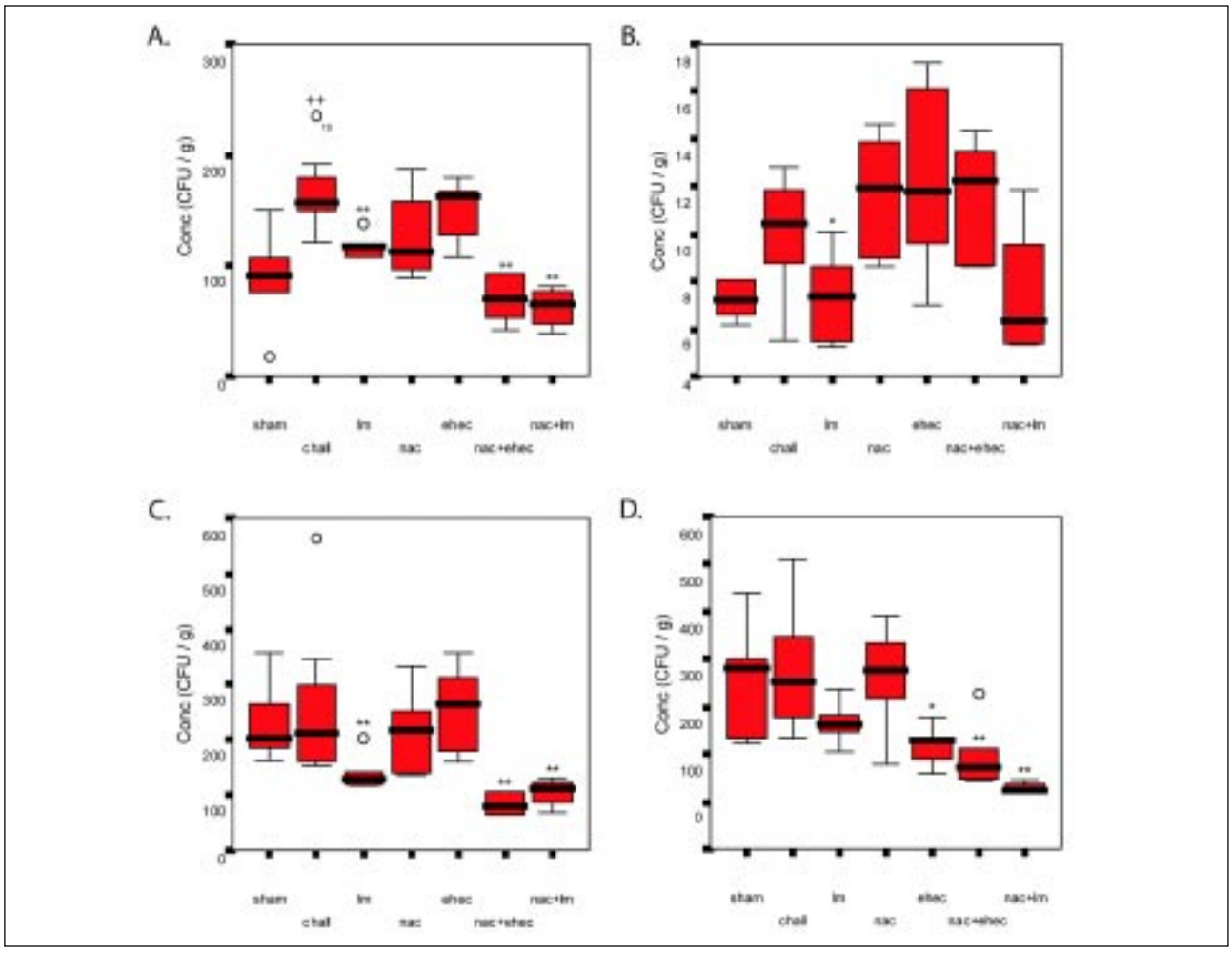

Fig. 3. Intestinal ecology of aerobic bacteria in the A) colonic luminal content, B) ileal luminal content, C) colonic mucosa and D) ileal mucosa.

DISCUSSION

Disturbances in intestinal microbial population densities occur during critical illness and *E. coli* may, apart from being a naturally occurring species in the gastrointestinal tract, also act as a pathogen. An increased *E. coli* overgrowth may have harmful effects and have been observed in several critically ill patients including severe acute pancreatitis.[5] In line with previous results[2,3] using the same pancreatitis model, an increased overgrowth of the facultative anaerobic and also potentially pathogenic *E. coli* was observed in the pancreatitis group as compared to sham-operated animals. This increase was reduced in the treatment groups and was effectively normalized in the group receiving LM-200, both in colonic and ileal counts. Similar results were shown for total anaerobic bacteria. This effect could be explained by several different mechanisms. One explanation may be that LM-200 acts as a prebiotic, i.e. a substrate for beneficial aerobic species, which in turn may have a suppressive effect on potentially pathogenic anaerobic species. It has previously been stated that EHEC is an inert substance,[9-11,17] but the results in the present study suggests that this may not entirely be true and this fact may strengthen the hypothesis of its action as a prebiotic substrate. The finding of a significantly increased reduction of anaerobic bacteria in the colon as compared to the ileum may support this hypothesis.

The finding that also aerobic bacterial levels were elevated during pancreatitis is somewhat difficult to explain. The fact that they also were significantly decreased during LM-200 pre-treatment may though suggest that the substance binds and inactivates bacteria in a non-specific manner.

In conditions with a systemic inflammatory response

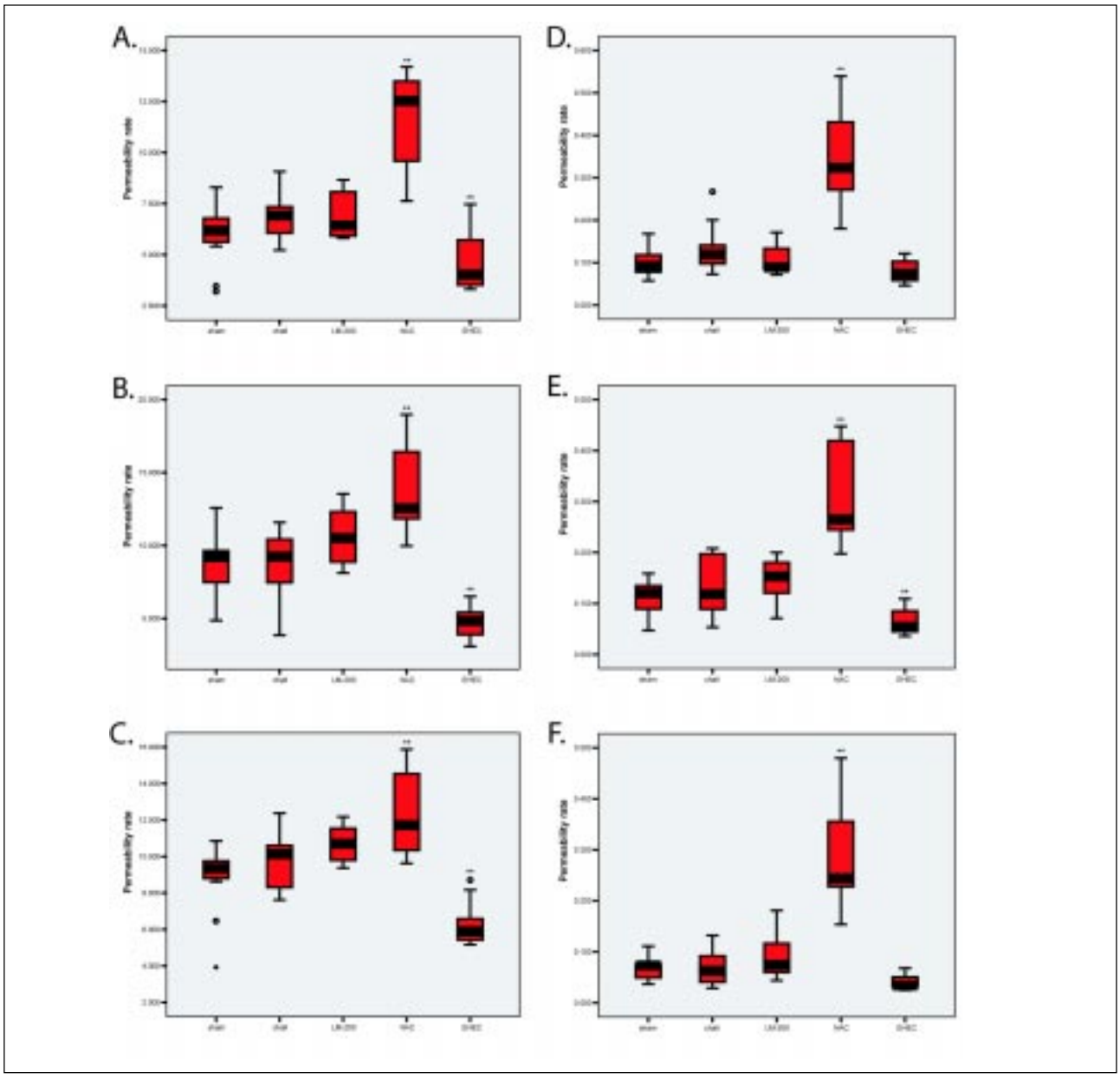

Fig. 4. Permeability of mannitol in the A) proximal ileum B) distal ileum C) colon and permeability of ovalbumin in the D) proximal ileum E) distal ileum F) colon.

or fulminant critical illness, e.g. severe acute pancreatitis, oxidative stress plays an important role. In addition to possessing probable probiotic effects, LM-200 may have antioxidative properties. This could be implied by the absence of synergistic or additive effects when administered in combination with NAC, a well-known antioxidant.

The impaired intestinal barrier integrity and increase in permeability, caused by oxidative damage and other processes, may play a central role in the subsequent reactions during the course of SAP. The increase in intestinal permeability together with alterations in enteric bacterial composition with overgrowth of potentially pathogenic and invasive enteric bacteria are probably a prerequisite for concomitant bacterial translocation and sepsis, as has been shown experimentally in taurodeoxycholate-induced acute pancreatitis.[12] Also clinically, an increase in intestinal permeability in patients with acute pancreatitis has been demonstrated.[18-21] Frequently, though not uniformly, bacterial translocation has been detected in severe acute pancreatitis.[22-26] Translocation

of bacteria primarily derived from the gut may both increase circulating levels of lipopolysaccharides but also may cause a secondary infection of an otherwise sterile pancreatic necrosis and thereby worsen the outcome.[27]

The permeability changes are not coupled with changes in the intestinal ecology. EHEC seem to restore the hyperpermeability during the AP insult and even decrease the permeability to levels below those of the sham group. The fact that it does not by itself affect the microbial populations suggests that it may only act as a mechanical barrier.

NAC increases the permeability in all groups. This is probably caused by its mucolytic properties exercised locally when administered per orally. Its antioxidative properties can be utilized if given intravenously, an administration route where the effect on permeability is not as pronounced.[28,29]

Maintenance of gut barrier integrity including restoring changes in enteric bacterial levels thus seems essential in severe acute pancreatitis. One such component could be orally administered cellulose derivatives. Water-soluble derivatives like ethyl hydroxyethyl cellulose have been reported to alter surface characteristics of enteric bacteria and inhibiting bacterial attachment onto the intestinal surface.[9-11] However, if these aspects also are applicable in the present model on severe acute pancreatitis is to be demonstrated, as are the mechanisms explaining the potential synergistic effects with antioxidants and potential effects on intestinal motility.

In conclusion, maintaining intestinal microbial ecology is important for a preserved gut barrier function in critical illness including acute pancreatitis. Findings in the present study imply that cellulose derivatives like EHEC and LM-200 might be of potential value, where LM-200 seem to possess somewhat more potent effects. The combination with e.g. broad-acting antioxidants, as done in the present study, or together with other potential agents could render cellulose derivatives as a potentially interesting component in a multimodal, orally administered, management regime in critical illness.

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