Probiotics enhance pancreatic glutathione biosynthesis and reduce oxidative stress in experimental acute pancreatitis


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Lutgendorff F, Trulsson LM, van Minnen LP, Rijkers GT, Timmerman HM, Franzén LE, Gooszen HG, Akkermans LM, Söderholm JD, Sandström PA. Probiotics enhance pancreatic glutathione biosynthesis and reduce oxidative stress in experimental acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 295: G1111–G1121, 2008. First published October 2, 2008; doi:10.1152/ajpgi.00603.2007.—Factors determining severity of acute pancreatitis (AP) are poorly understood. Oxidative stress causes acinar cell injury and contributes to the severity, whereas prophylactic probiotics ameliorate experimental pancreatitis. Our objective was to study how probiotics affect oxidative stress, inflammation, and acinar cell injury during the early phase of AP. Fifty-three male Sprague-Dawley rats were randomly allocated into groups: 1) control, 2) sham procedure, 3) AP with no treatment, 4) AP with probiotics, and 5) AP with placebo. AP was induced under general anesthesia by intraductal glycodeoxycholate infusion (15 mM) and intravenous cerulein (5 μg·kg⁻¹·h⁻¹, for 6 h). Daily probiotics or placebo were administered intragastrically, starting 5 days prior to AP. After cerulein infusion, pancreas samples were collected for analysis including lipid peroxidation, glutathione, glutamate-cysteine-ligase activity, histological grading of pancreatic injury, and NF-κB activation. The severity of pancreatic injury correlated to oxidative damage (r = 0.9) and was ameliorated by probiotics (1.5 vs. placebo 5.5; P = 0.014). AP-induced NF-κB activation was reduced by probiotics (0.20 vs. placebo 0.53 OD₅₆₀nm/mg nuclear protein; P < 0.001). Probiotics attenuated AP-induced lipid peroxidation (0.25 vs. placebo 0.51 pmol malondialdehyde/mg protein; P < 0.001). Not only was AP-induced glutathione depletion prevented (8.81 vs. sham 6.18 μmol/mg protein, P < 0.001), probiotic pretreatment increased glutathione compared with sham rats (8.81 vs. sham 6.18 μmol/mg protein, P < 0.001). Biosynthesis of glutathione (glutamate-cysteine-ligase activity) was enhanced in probiotic-pretreated animals. Probiotics enhanced the biosynthesis of glutathione, which may have reduced activation of inflammation and acinar cell injury and ameliorated experimental AP, via a reduction in oxidative stress.

reactive oxygen species; Lactobacillus; Bifidobacterium; glutamate-cysteine ligase; lipid peroxidation

ACUTE PANCREATITIS (AP) is a common condition of which clinical manifestations may range from a mild, self-limiting disease to an inflammatory process with life-threatening complications (13). The severity of AP is difficult to predict at hospitalization, since underlying factors determining severity remain unclear. Although evidence suggests that oxidative stress may be an important determinant of disease severity, little yet is known about the precise mechanisms and the extent of involvement (1, 25, 31).

Oxidative stress can be defined as an imbalance between cellular production of oxidants [reactive oxygen species (ROS)] and the antioxidative capacity and is known to cause acinar injury in the early course of AP (31). Normally, ROS are generated by aerobic cells and are readily removed by endogenous free radical scavenging mechanisms, but antioxidant defense systems of the cells can be overwhelmed by large quantities of ROS, with oxidative stress as a result (19).

The initial stage of AP is characterized by interstitial edema, reducing tissue perfusion and oxygenation, and infiltration of neutrophils, which release large amounts of ROS into the pancreas (25, 43). In a rat model of AP, Rau et al. (31) demonstrated oxidative damage, in the form of lipid peroxidation, and acinar injury within 5 min after induction of AP. Furthermore, oxidative stress activates NF-κB (2, 48), generating an inflammatory response, which attracts more oxidative stress-generating neutrophils, causing a vicious circle in AP (48).

Reduced glutathione (GSH) plays an important role as part of an intracellular defense system to counteract potentially negative effects of ROS and its concentration mainly depends on de novo synthesis by glutamate-cysteine-ligase (GCL) (19). In addition, GSH depletion has been found in early experimental AP to correlate with the extent of pancreatic injury (20).

The approach to modify the intestinal microbiota by oral intake of live bacteria to prevent intestinal disorders has been of growing interest (15). It has been suggested that probiotics have antibacterial and immunomodulatory effects (33, 41, 44). In addition, probiotics have been shown to increase GSH levels (23, 26) and to reduce intestinal oxidative stress in several experimental models (26, 27, 47). Furthermore, there is evidence showing that probiotics ameliorate morphological severity (22) and pancreatic DNA damage (34) in experimental AP. On the contrary, opposed to any expectations, our group recently found that probiotics, administered as treatment, more than doubled the relative risk of mortality in severe AP patients, which was probably related to an increased incidence of intestinal ischemia and necrosis (4), suggesting that probiotics may have been causative of an extra oxidative assault rather than ameliorative of oxidative damage. These results unfortunately demonstrate that detailed mechanism-based investiga-
tions of the effects of prophylactic multispecies probiotics on oxidative stress are largely lacking. This study therefore investigated the effects of pretreatment with probiotics on oxidative stress, acinar cell injury, and proinflammatory markers during the early phase of AP. Since the current concept of the pathophysiology of severe AP resembles that of major abdominal trauma, sepsis, and other critical illnesses, the results of this experiment may shed further light on mechanisms of action behind positive effects of probiotics in preventive applications, such as elective abdominal surgery (39).

**MATERIALS AND METHODS**

**Rats.** Male specific pathogen-free Sprague-Dawley rats (250–350 g, B&K, Sollentuna, Sweden) were maintained under constant housing conditions [temperature (22°C), relative humidity (60%), and a 12-h light-dark cycle] and had free access to water and standard rat chow throughout the experiment. The experimental design (Fig. 1) was approved by the local committee of animal ethics. Fifty-three rats were randomly allocated into five groups: 1) nonoperated control animals (n = 5), 2) sham procedure (n = 12), 3) AP (n = 12), 4) AP and probiotics (n = 12), and 5) placebo (n = 12).

**Surgical procedures.** At the start of the experiment, under general anesthesia using a combination of 2% isoflurane gas (flow: 0.5 l/min O2, 1.5 l/min air), a permanent gastric cannula was fitted in all rats as described previously (44), except for nonoperated control rats. Animals in the probiotics and placebo groups were allowed to recover for 4 days, prior to the start of daily probiotics or placebo administrations.

The study product (Ecologic 641, Winclwo Bio Industries, Amsterdam, the Netherlands) consisted of six viable and freeze-dried strains: *Lactobacillus acidophilus* (W70), *L. casei* (W56), *L. salivarius* (W24), *Lactococcus lactis* (W58), *Bifidobacterium bifidum* (W23), and *B. lactis* (W52) (previously classified as *B. infantis*), with cornstarch and maltodextrin as carrier substances. Placebo (the same substance without bacteria) was packed in identical sachets and coded.

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AP was induced as originally described by Schmidt et al. (36) and previously performed by our group (44). Pressure-controlled retrograde infusion of 0.5 ml sterilized glycodelyoxicholic acid (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) into the common bile duct was followed by intravenous infusion of cerulein (5 μg·kg⁻¹·h⁻¹, 1 ml/h, for 6 h, Sigma-Aldrich). No animals needed to be excluded for infusion pressures exceeding 35 mmHg. During the sham procedure, the papilla of Vater was cannulated, but no glycodelyoxicholic acid was infused, followed by 6 h of intravenous saline infusion (1 ml/h).

**Collection and preservation of samples.** After infusion of cerulein or saline, rats were anesthetized by isoflurane inhalation. Whole blood was collected via cardiac puncture into EDTA-coated tubes. A portion of the pancreatic tail was frozen in situ using a prefrozen forceps, freeze dried, and stored at −80°C for analysis of ATP levels. Samples from the central part of the pancreas were fixed in neutral 4% buffered formalin, then washed in PBS containing 6.8% sucrose, dehydrated in acetone, embedded in Technovit 8100-plastic (Hereus-Kultzer, Wehrheim, Germany), and sectioned for histological analysis. For terminal-deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL), formalin-fixed pancreas samples were embedded in optimum cutting temperature compound (Histolab, Västra Frölunda, Sweden) and frozen at −80°C until sectioned. Remaining part of the pancreas was snap frozen in liquid nitrogen, stored at −80°C, and freeze dried within 1 wk. Biochemical analyses, run in duplicate, were performed in freeze-dried tissues.

**Histological grading.** Pancreatic tissue sections (2 μm) were hematoxylin-eosin stained, coded, and examined by a pathologist blinded to the experimental design. Severity of pancreatitis was graded according to the previously described (35), modified Spormann et al.’s (38) scoring criteria (Table 1). Infiltrating neutrophils were counted in four random fields per slide. Necrosis was defined as loss of the acinar cell structure including zymogen degradation, loss of the basal basophilic/apical acidophilic staining of the cytoplasm, a pyknotic nucleus, and rupture of the cell membrane. At ×100 magnification, a minimum of 1,000 random acinar cells per histological section were counted. The amount of acinar cell necrosis was related to the total area of pancreatic parenchyma and presented as a percentage of the total.

**Amylase activity.** Amylase activity in plasma (U/l) was measured by routine methods in our clinical chemistry laboratory, via a two-step specific pancreas amylase assay (Diasys Diagnostic Systems International, Holzheim, Germany). After inhibition of salivary amylase the substrate 4-ethylidene-(G7)-1-4-nitrophenyl-(G1)-α-d-maltoheptaoiside was cleaved by pancreas associated amylase in the sample, releasing 4-nitrophenylenedoligosaccharides. A second substrate was added (ε-glucosidase), resulting in release of p-nitrophenol, which was measured as an increase in absorbance at 410 nm by use of a Advia 1800 autoanalyzer (Siemens, Munich, Germany).

**Pancreatic water content.** To estimate pancreatic edema, frozen tissue was weighed on a balance with readability of 0.01 mg (Mettler AT 250, American Instrument Exchange, Haverhill, MA) before and after freeze drying. Results are expressed as percentages water of total tissue weight.

**Apoptosis.** Apoptotic cells were detected by use of the in situ cell death detection kit (Roche Diagnostics, Bromma, Sweden). Pancreas sections (5 μm) were incubated in TUNEL reaction mixture for 1 h at 37°C and counterstained with 0.5 μM 4′,6-diamidine-2-phenylindole (DAPI). Slides were mounted in antifading Fluorescent Mounting Medium (DakoCytomation) and examined by confocal laser

![Fig. 1. Experimental design. Nine days prior to induction of pancreatitis, a permanent gastric cannula was fitted into all animals, except for the nonoperated control animals. Probiotics or placebo was administered intragastrically once daily to the animals allocated into the probiotics and placebo group, starting 5 days prior to induction of acute pancreatitis. Nine days after the start of the experiment, acute pancreatitis (AP) was induced or a sham procedure was performed. After cerulein infusion, all animals were anesthetized for euthanasia (†) and removal of organ samples.](http://ajpgi.physiology.org/Downloadedfromhttp://ajpgi.physiology.org/)
the number of TUNEL-positive cells per 100 cells in 20 random controls were included. Apoptotic rate was determined by counting scanning microscopy (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA; peripheral necrosis

IPF, intermediate-power field.

scanning microscopy (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA; ×60 oil immersion objective). For each test, negative
controls were included. Apoptotic rate was determined by counting the number of TUNEL-positive cells per 100 cells in 20 random photomicrographs in four tissue sections of four rats in each group.

Further quantification of apoptosis was performed by histone-associated DNA-fragmentation analysis in 50 μg pancreatic tissue, using the Cell Death Detection ELISA Plus Kit (Boehringer Mannheim, Mannheim, Germany) as previously described (42). Results were normalized to protein content as measured according to Bradford et al. (7) in the original homogenates.

Caspase-3 activity was assessed in pancreatic tissue extracts, corresponding with 100 μg protein content. Freeze-dried tissue (5 μg) was homogenized at 4°C in 500 μl Reporter Gene Assay Lysis Buffer (Roche Diagnostics) as performed previously by our group (42). In short, the homogenate was centrifuged at 15,000 g for 30 min. The supernatant was used for the caspase activity assay and protein content determination. Equal amounts of protein were assayed in duplicates for each sample. Cleavage of the fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, Pharmingen, Becton-Dickinson, Stockholm, Sweden) by active caspase-3 was measured at an excitation wavelength of 380 nm and emission wavelength of 435 nm, by use of a fluorescence spectrophotometer (Varian Cary Eclipse Instrument, Palo Alto, CA). Results are expressed as fluorescence units per milligram protein content.

ATP levels. For maximal stability, cellular ATP was extracted in 1 M perchloric acid containing 1 mM EDTA for 30 min on ice. After centrifugation, 200 μl supernatant was neutralized by adding 100 μl 2.2 M KHCO₃. Samples were repeatedly mixed for 20 min on ice and subsequently centrifuged for 2 min at 6,000 g at 4°C. Cellular ATP levels were determined by using a commercially available assay (ATP Determination Kit, A22066, Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Light formed from ATP and luciferin in the presence of luciferase was measured at 560 nm (Multilabel Counter Victor 2™, V, PerkinElmer, Växjö, Sweden). ATP concentrations, expressed in micromolar per milligram protein, were calculated by using the included standard, diluted to the range of 0.5–10.0 μM.

Proinflammatory mediators. To investigate the proinflammatory reaction, the activity of IL-1β converting enzyme (ICE), which converts IL-1β into its active form (17), and NF-κB activation were quantified.

ICE activity was assessed in tissue containing with 100 μg protein content. Preparation of the tissue was similar to the previously described method for caspase-3 activity analysis (42). ICE activity was measured by use of the ICE-specific substrate Ac-Tyr-Val-Ala-Asp-AMC (AcYVAD-AMC, Calbiochem, Darmstadt, Germany). Fluorescence of enzymatically cleaved AMC was measured at an excitation wavelength of 380 nm and emission wavelength of 435 nm. Results are given in fluorescence units per milligram protein content.

To determine NF-κB activation, subunit p65 of activated NF-κB/Rel was quantified in nuclear protein by the ELISA kit TransAM NF-κB Family Kits (Active Motif North America) as previously described by our group (42). Activity of NF-κB is expressed as the absorbance (OD450nm) of subunit p65 per milligram of nuclear protein.

Lipid peroxidation. To assess oxidative-induced cellular damage to lipid membranes, lipid peroxidation was analyzed in pancreatic tissue. Pancreatic samples were homogenized in 20 mM PBS containing 5 mM butylated hydroxytoluene to prevent sample oxidation. Malondialdehyde (MDA) concentration was determined in supernatants via a lipid peroxidation assay kit (LPO-586; Byoixtech, OXIS International, Portland, OR) according to manufacturer’s instructions. MDA levels were normalized to protein content of the original homogenate and are expressed as picomoles per milligram protein.

Glutathione assay. To estimate the antioxidative capacity, GSH and oxidized glutathione (GSSG) contents were determined in pancreatic tissue and plasma by using a commercially available assay (Glutathione Assay Kit II, Merck Chemicals, Hull, UK) according to the protocol provided by the manufacturer. Briefly, pancreatic tissue (1 mg) was homogenized in 150 μl ice-cold lysis buffer and centrifuged at 10,000 g for 15 min at 4°C. After protein determination, supernatants and plasma aliquots were deproteinized with 5% metaphosphoric acid (Sigma-Aldrich Chemie) and 4 M triethanolamine (Sigma-Aldrich Chemie) and plasma samples were lyophilized. Samples were then mixed with the Assay Cocktail reagents. After incubation at 25°C for 25 min, absorbance was measured at 405 nm. GSH levels were calculated by using a standard curve generated by standards provided by the manufacturer. GSH content was determined by subtracting the amount of GSSG from the total GSH content and GSH-to-GSSG ratios were calculated. Results are expressed in micromoles per milligram protein.

Cysteine. In pancreas and plasma, total cysteine, the rate-limiting precursor of GSH, was determined by the spectrophotometric method developed by Gaitonde (14) and expressed as nanomoles per milligram protein and as nanomoles per milliliter plasma, respectively. Briefly, samples were reduced in acidic medium with 10 mM dithiothreitol, and conjugates of ninyhdrin were assayed spectrophotometrically at 560 nm.

Glutamate-cysteine-ligase. De novo synthesis of GSH in red blood cells and pancreas was analyzed by quantification of GCL enzyme activity as previously described (45). To yield hemolysates, erythrocytes were obtained from separate EDTA blood samples by centrifugation at 900 g for 3 min after washing three times with five volumes of cold isotonic NaCl solution. Erythrocytes were lysed by the

Table 1. Histological scoring of pancreas injury

<table>
<thead>
<tr>
<th>Edema</th>
<th>None</th>
<th>Minimal edema, expanded interlobular septa</th>
<th>Moderate edema, expanded intralobular septa</th>
<th>Severe edema, separated individual acini</th>
</tr>
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<tbody>
<tr>
<td>Fat inflammation</td>
<td>None</td>
<td>Minimal (&lt;20 inflammatory cells per IPF) at ×200</td>
<td>Moderate (20–50 inflammatory cells per IPF)</td>
<td>Severe (&gt;50 inflammatory cells per IPF)</td>
</tr>
<tr>
<td>Parenchymal inflammation</td>
<td>None</td>
<td>Minimal (&lt;20 inflammatory cells per IPF) at ×200</td>
<td>Moderate (20–50 inflammatory cells per IPF)</td>
<td>Severe (&gt;50 inflammatory cells per IPF)</td>
</tr>
<tr>
<td>Peripheral necrosis</td>
<td>None</td>
<td>Moderate</td>
<td>Severe</td>
<td></td>
</tr>
<tr>
<td>Fat necrosis</td>
<td>None</td>
<td>Focal necrosis, &lt;5% of the total area of fat</td>
<td>and/or sublobular necrosis, &lt;20% of the total area of fat</td>
<td>and/or lobular necrosis, &gt;20% of the total area of fat</td>
</tr>
<tr>
<td>Parenchymal necrosis</td>
<td>None</td>
<td>Focal necrosis, &lt;5% of the total area of parenchyma</td>
<td>and/or sublobular necrosis, &lt;20% of the total area of parenchyma</td>
<td>and/or lobular necrosis, &gt;20% of the total area of parenchyma</td>
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addition of 1 volume of 50 mM Tris·HCl buffer (pH 7.4), containing 1 mM EDTA, and by sonication for 2 × 20 s. The erythrocyte membranes were removed by centrifugation at 18,000 g for 40 min. Tissues were homogenized in 250 mM sucrose containing 20 mM Tris, 1 mM EDTA, 20 mM boric acid, 2 mM serine, pH 7.4. The cytosolic protein fraction from crude homogenates was obtained by centrifugation and was subsequently filtered through microcon-10 (Millipore) tubes to remove endogenous inhibitors and substrates for GCL. GCL activity was determined as the difference between GSH synthesis in unblocked and GSH synthesis in samples blocked with 200 mM 5-sulfosalicylic acid dehydrate and expressed as millimoles GSH per minute per milligram protein.

Statistical analysis. All data were assessed for normal distribution by Shapiro-Wilk's test. Parametric values are presented as means (SE). Statistical analysis was performed by ANOVA followed by Tukey's honestly significant difference test for multiple comparisons (SPSS 13.0, Chicago, IL). Nonparametric values are given as median (25–75th interquartile range). Comparisons were done by Kruskal-Wallis when more than two groups were compared. Mann-Whitney U-test was used to compare two groups. Spearman's rank correlation coefficients were computed for correlation analyses. Considering Bonferroni's correction, P values <0.01 were considered significant.

RESULTS

Safety of administration of viable probiotics. No rats receiving probiotics showed signs of diarrhea, weight loss or loss of appetite during probiotic treatment. During the 5 day pretreatment period, the increase in animal weight was equal in all groups [sham 22.3 (3.8) vs. AP 21.9 (3.9) vs. placebo 20.6 (2.8) vs. probiotics 20.5 (2.1) gr/5 days]. Mortality due to AP did not occur.

Probiotics ameliorated AP-associated pancreatic damage. Pancreatitis-induced injury was characterized by extensive edema, hemorrhage, and infiltration of neutrophils (Fig. 2A). Pretreatment with probiotics markedly reduced the severity of pancreatic injury [1.5 (0.88–3.25) points vs. placebo 5.5 (3–6.1); P < 0.01, Fig. 2B]. In the pancreatitis animals, light microscopy revealed areas of complete structural damage by necrosis involving 12% of the pancreatic parenchyma and the presence of infiltrating neutrophils, both of which were reduced after probiotic pretreatment (Fig. 2, C and D). The histopathological score for edema was reduced by pretreatment with probiotics [1 (0.5–1.1) points vs. placebo 1.5 (1.3–2); P < 0.01]. This corroborated the reduction in water content in the probiotic-treated animals (Fig. 2E). Interestingly, pretreatment with probiotics did not affect the increase in plasma amylase despite the significant attenuation in the severity of pancreatitis (Fig. 2F).

Probiotics reduced AP-induced apoptotic cell death. Laser confocal microscopy of fluorescent TUNEL-stained slides revealed that AP-induced apoptosis was confined to small peripheral areas of pancreatic parenchyma (Fig. 3A). Pretreatment with probiotics attenuated the AP-induced apoptosis, showing a 70% reduction of the apoptotic rate in pancreas (P < 0.01) (Fig. 3B). To further quantify apoptosis, DNA fragmentation and caspase-3 activity were determined. Elevation in histone-associated DNA-fragmentation in the AP group was prevented by pretreatment with probiotics (Fig. 3C). This was in keeping with the reduction in caspase-3 activity in probiotic-pretreated animals (Fig. 3D).

Probiotics reduced AP-induced ATP depletion in pancreatic tissue. Apoptosis is a highly regulated form of cell death, involving many ATP-dependent steps. Depletion of cellular ATP is known to cause switching of form of cell death from apoptosis to necrosis (18). AP reduced ATP levels by 37% (sham 0.93 vs. AP 0.59 μM/mg protein; P < 0.001) 6 h after induction of pancreatitis (Fig. 4A). Pretreatment with probiotics normalized the ATP levels. ATP levels correlated inversely with the area of necrosis (r = −0.76) (Fig. 4B), which supports the hypothesis that ATP depletion is the major determinant for a necrotic form of cell death (18).

Probiotics reduced AP-induced inflammatory markers in pancreatic tissue. To further investigate the effects of probiotics on AP-associated elevation of proinflammatory mediators we assessed activation of NF-κB by analysis of translocated p65 into the nucleus and ICE, which converts pro-IL 1β into its active form (17).

The induction of AP resulted in an approximately threefold elevation of activated ICE (Fig. 5A, P < 0.001). Pretreatment with probiotics attenuated the AP-associated elevation of ICE activity in pancreas. In addition, the AP-induced increase in activated NF-κB was normalized by probiotics (Fig. 5B).

Probiotics reduced AP-induced oxidative stress and increased GSH content in the pancreas. Because the amount of generated ROS exceeds the cellular antioxidative capacity, ROS cause cellular damage in the form of oxidative degradation of lipid membranes, which can be measured as lipid peroxidation (19). Therefore, to assess oxidative stress-induced damage in the early phase of AP, lipid peroxidation was quantified. AP caused a marked increase in lipid peroxidation in the pancreas, which was normalized by pretreatment with probiotics (Fig. 6A). Since this reduction in lipid peroxidation after probiotic pretreatment may have resulted from either reduced amounts of ROS or an enhanced antioxidative capacity, we quantified local GSSG and GSH. In all pancreatitis groups GSSG increased twofold compared with sham or control animals (Fig. 6B) and administration of probiotics did not prevent this increase, suggesting that both probiotic and placebo-pretreated animals encountered a similar amount of ROS during the early phase of AP. Interestingly, probiotic pretreatment did not only prevent AP-induced GSH depletion, it also increased pancreatic GSH levels, even compared with control and sham-operated rats (Fig. 6C), suggesting an increase in antioxidative capacity after pretreatment with probiotics. As expected, there was an inverse correlation between pancreatic GSH content and lipid peroxidation (r = −0.70) (Fig. 6D). Furthermore, there was a strong positive correlation between lipid peroxidation and pancreatic injury (r = 0.90) (Fig. 6E) and an inverse correlation between GSH content and histological pancreatic damage (r = −0.67) (Fig. 6F), which supports the hypothesis that intracellular GSH protects against oxidative stress-induced pancreatic injury. In addition, pancreatic ATP levels showed an inverse correlation with lipid peroxidation (r = −0.78) (Fig. 7A), as well as a positive correlation between ATP levels and pancreatic GSH content (r = 0.70) (Fig. 7B).

Probiotics induce enhanced systemic glutathione levels. To determine whether the local increase in GSH found after probiotic pretreatment was associated with a change in systemic GSH, we next analyzed plasma GSH levels. Systemically, probiotic pretreatment demonstrated an even more pronounced effect on GSH levels resulting in an...
almost twofold increase in plasma GSH compared with control animals (Fig. 8).

De novo synthesis of glutathione. In search of the source of increased levels of GSH the possibility of enhanced GSH biosynthesis was explored. De novo synthesis of GSH is regulated by availability of cysteine and GCL enzyme activity (19). Firstly, plasma cysteine levels were equal in all groups (Fig. 9A), in contrast to local levels in the pancreas which were reduced during AP, regardless of pretreatment (Fig. 9B), suggesting that cysteine availability did not play a major role in the enhanced levels of GSH. Secondly, GCL activity in red blood cells (Fig. 9C) and in pancreas (Fig. 9D) was most abundant in the probiotics group, indicating enhanced GSH biosynthesis in probiotic-pretreated animals.

DISCUSSION

The present study is the first to demonstrate that multispecies probiotics enhance de novo synthesis of GSH and increase GSH content locally in the pancreas as well as systemically. After pretreatment with probiotics, oxidative stress, subsequent acinar cell injury, and the local inflammatory response were ameliorated in a well-established model of severe AP. Oxidative stress is defined as a lack of sufficient antioxidative reserve during times of increased ROS production. There has been increasing awareness of the important role that oxidative stress plays in many inflammatory illnesses, including AP. In experimental as well as in clinical AP there is evidence of depletion of circulating antioxidative levels (6, 31) with the degree of depletion corresponding to disease severity...
When produced under physiological conditions, ROS are captured by sufficient amounts of antioxidants. However, during the development of AP the increase in ROS exceeds the antioxidant capacity of endogenous defense systems (20) resulting in oxidative stress-induced damage (31, 43). Indeed, our data show that AP induced increased oxidative damage to lipid membranes, which was prevented by pretreatment with probiotics.

Besides lipid membranes, ROS are also capable of attacking other cellular targets resulting in cellular injury. Indeed, antioxidant therapy has been shown to ameliorate pancreatic injury in experimental AP (10, 24). In keeping with those findings our results show that pretreatment with probiotics attenuates the morphological severity and edema formation in experimental AP. Moreover, the correlation between lipid peroxidation and pancreatic injury suggests that oxidative stress mediates the progression of AP.

In response to (oxidative) injury, acinar cells produce and release TNF-α, a proinflammatory cytokine that has shown to stimulate apoptosis in acinar cells (16, 48). In addition, ROS oxidant therapy has been shown to ameliorate pancreatic injury in experimental AP (10, 24). In keeping with those findings our results show that pretreatment with probiotics attenuates the morphological severity and edema formation in experimental AP. Moreover, the correlation between lipid peroxidation and pancreatic injury suggests that oxidative stress mediates the progression of AP.

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Fig. 3. Probiotics reduced acute pancreatitis-associated apoptosis in the pancreas. After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis or a sham procedure or were not operated on (control n = 5; sham n = 12; AP n = 12; AP pla n = 12; AP pro n = 12). Sections of pancreas, obtained after completion of cerulein infusion or sham procedure, were stained with fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL, purple) and counterstained with 0.5 μM 4',6-diamidino-2-phenylindole (DAPI, blue). A: compared with sham-operated animals, AP caused apoptosis confined to small peripheral areas of pancreatic parenchyma. Placebo-treated animals also showed focal apoptosis in peripheral areas. Probiotic animals showed a reduction in apoptotic rate. The results shown are typical of those obtained from at least 4 rats per group. Bar = 100 μm. B: average (± SE) number of TUNEL-positive cells per 100 cells in 20 random photomicrographs of 4 sections per sample of at least 4 independently acquired samples from each group. Apoptosis was further quantified by determination of DNA fragmentation (C) and caspase-3 activation (D). Graphs show the average (± SE). All analyses were run in duplicate. Comparisons were performed by ANOVA followed by Tukey’s HSD.

Fig. 4. Probiotics attenuated acute pancreatitis-associated ATP depletion. After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis or a sham procedure or were not operated on (control n = 5; sham n = 12; AP n = 12; AP pla n = 12; AP pro n = 12). A: after completion of cerulein infusion or sham procedure, pancreas tissue samples were frozen in situ and freeze dried, and ATP levels were determined. The graph shows the median (± range). Comparisons were performed using Kruskal-Wallis followed by Mann-Whitney U-test. B: correlation between the extent of acinar cell necrosis and pancreatic ATP levels was computed by using Spearman’s rank correlation coefficients.
also directly induce apoptosis by damaging DNA. It is worth mentioning that an inverse correlation between acinar cell apoptosis and severity of AP has been reported (5). Indeed, extensive apoptotic acinar cell death was associated with a mild course of pancreatitis whereas severe AP is characterized by extensive necrosis (5). This, however, is in apparent contrast with our present study, which demonstrates a concurrent reduction in severity and apoptosis in the probiotic-pretreated group. Although our findings show that probiotics reduce DNA fragmentation and caspase-3 activity in the pancreas, the maximum apoptotic rate of 1.1% suggests that apoptosis most likely plays a less significant role in the early phase of this model of AP. Similarly, Rau and colleagues (30), studying the effects of ICE inhibition on cell death during severe AP, showed that apoptosis is not a relevant factor in the determination of severity during the early phase of experimental severe AP.

The development of pancreatic necrosis results in a dramatic increase of mortality in AP patients (13). In AP, ROS cause severe mitochondrial dysfunction, resulting in ATP depletion.

Fig. 5. Probiotics reduced levels of acute pancreatitis-associated early inflammatory markers. After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis or a sham procedure or were not operated on (control n = 5; sham n = 12; AP n = 12; AP pla n = 12; AP pro n = 12). After completion of cerulein infusion or sham procedure, inflammatory markers were assessed in pancreas tissue by quantification of IL-1β converting enzyme (ICE; A) and NF-kB levels (B). Graphs show the average (± SE). All analyses were run in duplicate. Comparisons were performed by ANOVA followed by Tukey’s HSD.

Fig. 6. Probiotics reduced acute pancreatitis-induced oxidative stress in the pancreas. After 5 days of pretreatment with placebo or probiotics, rats were subjected to acute pancreatitis or a sham procedure or were not operated on (control n = 5; sham n = 12; AP n = 12; AP pla n = 12; AP pro n = 12). Antioxidant status was investigated in pancreas tissue, after cerulein infusion or sham procedure, by determination of lipid peroxidation [malondialdehyde (MDA) levels; A], oxidized glutathione levels (GSSG; B), and reduced glutathione levels (GSH; C). Graphs show the average (± SE). All analyses were run in duplicate. Comparisons were performed by ANOVA followed by Tukey’s HSD. Correlations between lipid peroxidation and pancreatic GSH content (D), pancreatic injury and lipid peroxidation (E), and pancreatic injury and GSH content (F) were computed by use of Spearman’s rank correlation coefficients.
Apoptosis is an ATP-dependent process, which makes it impossible for the injured and ATP-depleted acinar cell to complete the apoptotic process. Under these conditions necrosis would be the only form of cell death possible (12, 18). In accordance with this hypothesis, the present study shows a concurrent amelioration of ATP depletion and necrosis after pretreatment with probiotics. In addition, the hypothesis that oxidative stress causes mitochondrial damage and subsequent loss of ATP (18) is in keeping with the inverse correlation between pancreatic ATP levels and lipid peroxidation, found in our study.

Acinar cells respond to oxidative stress-induced injury with activation of NF-κB, causing a proinflammatory response (2, 48). Activation of cytokines such as TNF-α and IL-1β has been shown to enhance local tissue destruction and to cause distant organ complications (21). Our data show that pretreatment with probiotics, possibly mediated by a reduction of pancreatic oxidative stress, attenuates AP-induced NF-κB and ICE activity. ICE converts pro-IL-1β into its active form (17), which in turn has been shown to play a pivotal role in local and systemic complications in severe AP (9, 29). Moreover, both clinical and experimental studies have shown that ICE activity is an important determinant of severity of AP (29, 30).

Of note, no difference in plasma amylase levels was observed, despite a marked amelioration of the severity of the AP after probiotic pretreatment. Although a direct role of GSH cannot be excluded, these results suggest that enhanced pancreatic GSH levels are associated with attenuated severity, without changing pathways of regulation of pancreatic protein secretion. Our findings are in accordance with data from Ethridge et al. (11) demonstrating that despite an attenuation of severity of AP that was sorted by inhibition of cyclooxygenase-2, no differences in serum amylase were demonstrated. In addition, also in AP patients, only a weak association between amylase levels and severity of the pancreatitis can be demonstrated (46).

Taking together the above, our study demonstrates that pretreatment with probiotics putatively ameliorates the severity of AP via a reduction in oxidative stress-induced injury. This probiotic effect may have been due either to a reduction of ROS in the pancreas or to an enhanced defense mechanism against oxidative stress. GSH represents the major endogenous defense system against oxidative stress (19) and has been shown to be inversely correlated with the APACHE II score in patients with AP (28). While scavenging ROS, GSH is oxidized to GSSG, being an indirect measure of the total amount of ROS neutralized by GSH. In our study, probiotics showed no effect on pancreatic GSSG levels, indicating an equal cellular ROS production in placebo and probiotic-pretreated rats. AP-induced GSH depletion, however, was not only prevented in probiotic-pretreated animals; probiotics increased pancreatic as well as systemic GSH levels even compared with control animals. Our data concur with recent data (26, 27, 47) showing that probiotics enhance GSH levels. Peran et al. (26) showed in a model of rat colitis that the preventive effect of *L. fermentum* was mediated by bacterial release of GSH, attenuating oxidative stress. However, this is the first time that probiotics have been shown to increase GSH in pancreatic tissue. Moreover, in the probiotic-pretreated group, enhanced pancreatic GSH content may have maintained normal lipid peroxidation levels by counteracting the large amount of pan-
creatic ROS (19). The inverse correlation between pancreatic GSH content and lipid peroxidation, indicating oxidative stress-induced cellular injury and histological scoring of pancreatic injury, emphasizes the importance of this endogenous defense mechanism.

Our results describe an increase in GSH biosynthesis after pretreatment with probiotics, demonstrated by an increase in GCL activity and increased local and systemic GSH levels. GCL activity is upregulated under conditions in which increased cellular defense is necessary, i.e., low-dose proinflammatory stimuli as TNF-α or NF-κB or sublethal oxidative stress, resulting in increased cell resistance against subsequent and potential lethal insults (19).

In contrast to the results described herein, our group recently conducted a double-blind, randomized trial in nearly 300 severe AP patients that demonstrated, contrary to any expectations, that administration of probiotics early after the onset of AP more than doubled the relative risk of mortality (4). Taking together the above, a hypothetical model can be envisioned in which administration of probiotics in healthy subjects causes minor stress, inducing upregulation of antioxidative enzymes and consequently preconditioning for a large oxidative assault, whereas in critically ill AP patients the same minor stressor may only further aggravate the critical condition. Consequently, probiotics may not be a treatment option in critically ill patients.

In addition, even though antioxidant therapy has been shown to ameliorate the severity of experimental AP (2, 48), it should be noticed that pancreatic oxidative stress and excessive ROS generation are early features in AP and consequently not a good target for clinical therapy. This has recently been shown in a randomized controlled trial in which the results found in human subjects were not that encouraging (37). Probiotics, however, possess a whole array of mechanisms of action (15) and to fully benefit from the antioxidative action of probiotics preventive applications need to be thought of. For instance, in elective major abdominal surgery, in which intestinal oxidative stress is to be expected (40), pretreatment with probiotics has already shown promising results in clinical trials (32, 39). In addition, post-endoscopic retrograde cholangiopancreatography pancreatitis is one of the major complications that has plagued this endoscopic procedure and occurs at a relatively constant rate of 10% (3). The elective nature of the procedure gives a unique opportunity for early modification of the disease and perhaps prophylaxis.

Since the product used is a multispecies combination of probiotic strains, it is worth noting that the found effects depend on the specific combination of the applied bacteria. Additional studies will be necessary to elucidate the effects of the separate strains as well as a possible synergistic effect of this specific combination of probiotics. In addition, probiotic effects are mostly strain specific and cannot be generalized for other probiotic combinations.

Overall, our findings show that administration of multispecies probiotics enhances GSH biosynthesis both systemically and locally and consequently increases GSH content in the pancreas and reduces oxidative stress in acinar cells, resulting in prevention of pancreatic peroxidative damage and amelioration of the local inflammatory response, finally reducing the severity of experimental AP. Our data not only demonstrate the ability of probiotics to augment the antioxidative defense but also underscore the importance of oxidative stress and the endogenous antioxidant defense mechanisms during the early phase of AP.
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