

Low-methoxyl lemon pectin attenuates inflammatory responses and improves intestinal barrier integrity in caerulein-induced experimental acute pancreatitis

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Scope: Acute pancreatitis (AP) is a common clinical acute abdominal disease. The intestinal injury associated with AP will aggravate the condition retroactively. This study investigates whether the low-methoxyl pectin (LMP) isolated from lemon could attenuate AP and associated intestinal injury.

Methods and results: Experimental AP was induced in BALB/c mice by caerulein (CAE) hyperstimulation. Nutritional prophylactic group was pre-fed with 5% LMP supplemented forage 3 days before AP induction. We found that LMP supplementation attenuated the severity of AP as evidenced by reduced serum amylase and lipase levels, pancreatic edema and myeloperoxidase activity. The protective effect was also confirmed by histological examination of pancreatic damage. LMP suppressed the production of pancreatic proinflammatory cytokines including TNF- α , IL-1 β , and IL-6. Moreover, LMP supplementation restored AP-associated disruption of intestinal barrier integrity as evidenced by upregulation of tight junction modulatory proteins occludin, zonula occludens (ZO)-1, antimicrobial peptides β -defensin-1 (DEFB1) and CRAMP as well as increase in SCFAs production. LMP supplemented mice with AP exhibited suppressed intestinal inflammation as shown by decreased ileal and colon cytokine production compared with CAE group.

Conclusion: Our results support dietary LMP supplementation as an effective nutritional intervention for AP and associated intestinal injury.

Keywords:

Cytokines / Inflammation / Intestinal barrier function / Low-methoxyl lemon pectin / Short chain fatty acids / Tight junction



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Abbreviations: AMP, antimicrobial peptides; AP, acute pancreatitis; CAE, caerulein; CRAMP, cathelicidin-related antimicrobial peptide; DEFB1, defensin β -1; H&E, hematoxylin and eosin; HMP, high-methoxyl pectins; LMP, low-methoxyl pectins; MODS, multiple organ dysfunction syndrome; MPO, myeloperoxidase; PBMC, peripheral blood mononuclear cell; QPCR, quantitative real-time PCR; SIRS, systemic inflammatory response syndrome; TJ, tight junction; TNF- α , tumor necrosis factor alpha; ZO-1, zonula occludens-1

1 Introduction

Acute pancreatitis (AP) has become the leading cause of hospital admission for gastrointestinal disorders in many countries and the worldwide incidence is increasing during the past decades [1]. AP starts with autodigestion of the pancreas which leads to local inflammatory responses. The inflammation is accompanied by local production of cytokines such as

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IL-1, IL-6, and tumor necrosis factor- α (TNF- α). The magnitude of release of these cytokines determines the outcome and progress of the disease [2]. Characteristics of AP include pancreatic edema, acinar cell necrosis, immune cell infiltration, hemorrhage, and increased serum levels of pancreatic amylase and lipase. Pancreatic injury is mild in 80% of patients. However, approximately 20–25% of patients with AP develop severe symptoms with systemic inflammatory responses impacting multiple organs including gut, lungs, liver, and kidneys. The mortality rate in this group is as high as 30% [3, 4]. Therefore, design of nutraceutical and/or therapeutic strategies to limit local inflammation and prevent its progression into a severe, systemic form have been critical for the condition.

Accumulating evidence points toward disruption of the intestinal mucosal barrier as an early hallmark of severe AP [5–8]. The mechanisms underlying AP-induced intestinal barrier disruption have not been fully elucidated, although elevated inflammatory cytokines, enhanced oxidative stress and intestinal hypoperfusion are among the contributing factors [7]. Intestinal barrier disruption can lead to translocation of bacteria, endotoxemia, and secondary infection of pancreatic tissue. This causes systemic inflammatory response syndrome (SIRS) or multiple organ dysfunction syndrome (MODS) [5, 8, 9]. Preventing AP-associated intestinal barrier disruption or inflammation is therefore a key target to prevent severe AP-associated mortality.

A possible nutraceutical approach to prevent progression of AP into its severe form is by intervening with anti-inflammatory food components such as specific dietary fibers [10]. One family of such dietary fibers is pectins [11, 12]. Pectins have an α (1-4)-linked galacturonic acid backbone and can differ in degrees of methyl esterification (DM) [13, 14]. Earlier, the health-promoting effects of pectins from various sources have been described for its antioxidant, anti-obesity, and anti-cancer functions [15–17]. In addition, dietary pectins of different DMs exhibit anti-inflammatory effects *in vivo* and *in vitro* [18–20]. Compared to high-methoxyl pectins (HMP), low-methoxyl pectins (LMP) with DM values of 50% or less carry negative charge from the free carboxyl groups of the galacturonic acid residues, preventing formation of mucin–pectic aggregates. LMP easily penetrates the mucin layer of the intestine wall [21], which facilitates the interaction of LMP with the intestinal epithelia. It is found that LMP but not HMP stimulate small intestinal mucin secretion by direct interaction with the epithelium [22].

The structure and function of intestinal mucosal barrier, which may be positively regulated by LMP, is essential in limiting the progression of AP into a severe form. We therefore investigated whether a previously undescribed LMP (7 DM) isolated from lemon may have attenuating effects in inflammatory responses in AP via modulating intestinal mucosal barrier dysfunction.

2 Materials and methods

2.1 Animals and treatment

Female BALB/c mice were purchased from Su Pu Si Biotechnology Co., Ltd. (Suzhou, Jiangsu, China) and maintained in specific pathogen-free environment at the Animal Housing Unit of Jiangnan University (Wuxi, Jiangsu, China) under a controlled temperature (23–25°C) and a 12-h light/dark cycle, provided water *ad libitum*, fed standard basal diet or 5% LMP supplemented diet (Supporting Information Table 1, Beijing HFK Bioscience Co., LTD, Beijing, China), and allowed to acclimatize for a minimum of 1 week. The environment was maintained at a relative humidity of 30%–70%. Mice weighing 20 ± 2 g were randomly assigned to three experimental groups ($n = 6$ –8): saline treated control group, caerulein (CAE)-treated CAE group and LMP+CAE group. AP was induced by 8 consecutive hourly intraperitoneal caerulein injections (50 μ g/kg). Nutritional prophylactic group was pre-fed with 5% LMP supplemented forage three days before AP induction. Humane killing was 1 h after the last caerulein injection. All animal-related experimental protocols were approved by the Institutional Animal Ethics Committee of Jiangnan University (JN.No20150301-0229) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals. The investigational compounds, lemon pectins with a degree of methyl esterification of 7% (7 DM) were acquired from CP Kelco, Lille Skensved, Denmark. Endotoxin levels in pectin samples were confirmed with endotoxin detection kit (Thermo Scientific, Sunnyvale, CA, USA) to be below the detection level of 0.1 ng/mL.

2.2 Serum amylase measurements

Serum was collected by allowing the blood to coagulate at ambient temperature for 25 min and subsequently centrifuged at $3000 \times g$ at 4 °C for 10 min. The supernatant was then collected and stored at –80°C until analysis. An iodine-starch colorimetric method was used to measure serum amylase levels. Briefly, serum samples were incubated with a prewarmed substrate buffer for 7.5 min at 37°C. After adding iodine and ddH₂O to the mixture, absorbance was measured at 660 nm using UV-2450 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

2.3 Pancreatic edema

A portion of freshly harvested pancreatic tissue was trimmed of fat and weighed. Pancreatic water content was evaluated by the ratio of initial weight (wet weight) of the pancreas to its weight after incubation at 80°C for 48 h (dry weight).

Table 1. Primers used for qPCR

Gene	Forward	Reverse
ZO-1	5'-CTTCTCTTGCTGGCCCTAAAC-3'	5'-TGGCTTCACTTGAGGTTTCTG-3'
Occludin	5'-CACACTTGCTTGGGACAGAG-3'	5'-TAGCCATAGCCTCCATAGCC-3'
DEFB1	5'-GCACAAGAAGGTCACACGGA-3'	5'-CTAAGTTGCAGATGGGGTGT-3'
CRAMP	5'-CTTCAAGGAACAGGGGGTGG-3'	5'-CTTGAACCGAAAGGGCTGTG-3'
β -actin	5'-CCCAGGCATTGCTGACAGG-3'	5'-TGGAAGGTGGACAGTGAGGC-3'

2.4 Myeloperoxidase (MPO) activity

MPO activity was determined by an MPO assay kit according to the manufacturer's protocol (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.5 RNA isolation and quantitative PCR

Total RNA was extracted from fresh tissues using TRIzol (Thermo Scientific, Szeged, Hungary) and quantitated by spectrophotometry (NanoDrop 2.0; Thermo Fisher Scientific, Rockland, DE, USA). Complementary DNA was prepared by reverse transcription of 2 μ g total RNA using the PrimeScript RT Master Mix (Takara BIO INC., Shiga, Japan) in S1000 Thermal Cycler machine (MJ Research, Waltham, MA, USA). SYBR Green PCR reagents (BIO-RAD) were used to determine the mRNA levels. Calculations were made based on the comparative cycle threshold method ($2^{-\Delta\Delta C_t}$). Relative expression ratios were calculated as normalized ratios to β -actin internal control gene. Detailed primer sequences are shown in Table 1.

2.6 ELISA assays

Serum was obtained by allowing the blood to coagulate at ambient temperature for 25 min and subsequently centrifuging at $3000 \times g$ for 10 min. Pancreatic, ileal, colon homogenates, and serum were assayed for inflammatory mediators using a sandwich ELISA kit for pancreatic lipase, TNF- α , IL-1 β , and IL-6 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm within 30 min, using an automated microplate reader (Multiskan GO; Thermo Fisher Scientific Oy, Vantaa, Finland). Data are expressed as pg/mL.

2.7 Histology

Freshly harvested pancreatic and colon tissues were fixed in 4% phosphate-buffered formaldehyde overnight, dehydrated with gradient ethanol solutions, and embedded in paraffin blocks. Tissue sections (5 μ m) were dewaxed in xylene, hydrated through upgraded ethanol solutions, and stained with hematoxylin and eosin (H&E). Morphological changes were

examined under a DM2000 light microscope at $400\times$ magnification.

2.8 Quantification of SCFAs

Concentrations of acetate, propionate, and butyrate were analyzed in pooled feces by gas chromatography coupled mass spectrometer (GC-MS) as previously described [23]. Briefly, feces (50 mg) were homogenized in 500 μ L of saturated NaCl solution. Thereafter, we acidified samples with 40 μ L 10% sulfuric acid and extracted SCFAs by 800 μ L diethyl. Samples were centrifuged at $14\,000 \times g$ for 15 min at 4°C . 1 μ L of supernatants were used for analysis with the GC-MS-QP2010 (Shimadzu, Japan). Real time analysis software GC-MS Postrun (GC-MS solution Version 2.72) was employed to calculate the concentrations of the acids. An external standard method was employed to determine concentration of each SCFA.

2.9 Statistical analysis

Data are expressed as mean \pm SEM. The parametric distribution of data was confirmed using Kolmogorov-Smirnov test. Statistical analysis between two groups was performed by independent *t*-test, or when multiple comparisons were made, by one-way analysis of variance followed by Tukey's post-hoc test using GraphPad Prism (v5; GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered as a statistically significant difference.

3 Results

3.1 LMP supplementation alleviates the severity of AP

The effect of LMP supplementation was tested in the most widely used mouse model of AP induced by caerulein hyperstimulation [24]. LMP in a 5% concentration through the feed was given 3 days before caerulein hyperstimulation. No difference in food intake or body weight between basal diet-fed and LMP supplemented mouse groups was observed (data not shown). LMP supplementation significantly reduced AP symptoms. LMP-pre-fed mice exhibited reduced pancreatic edema (Fig. 1A, $p = 0.0431$) and significantly

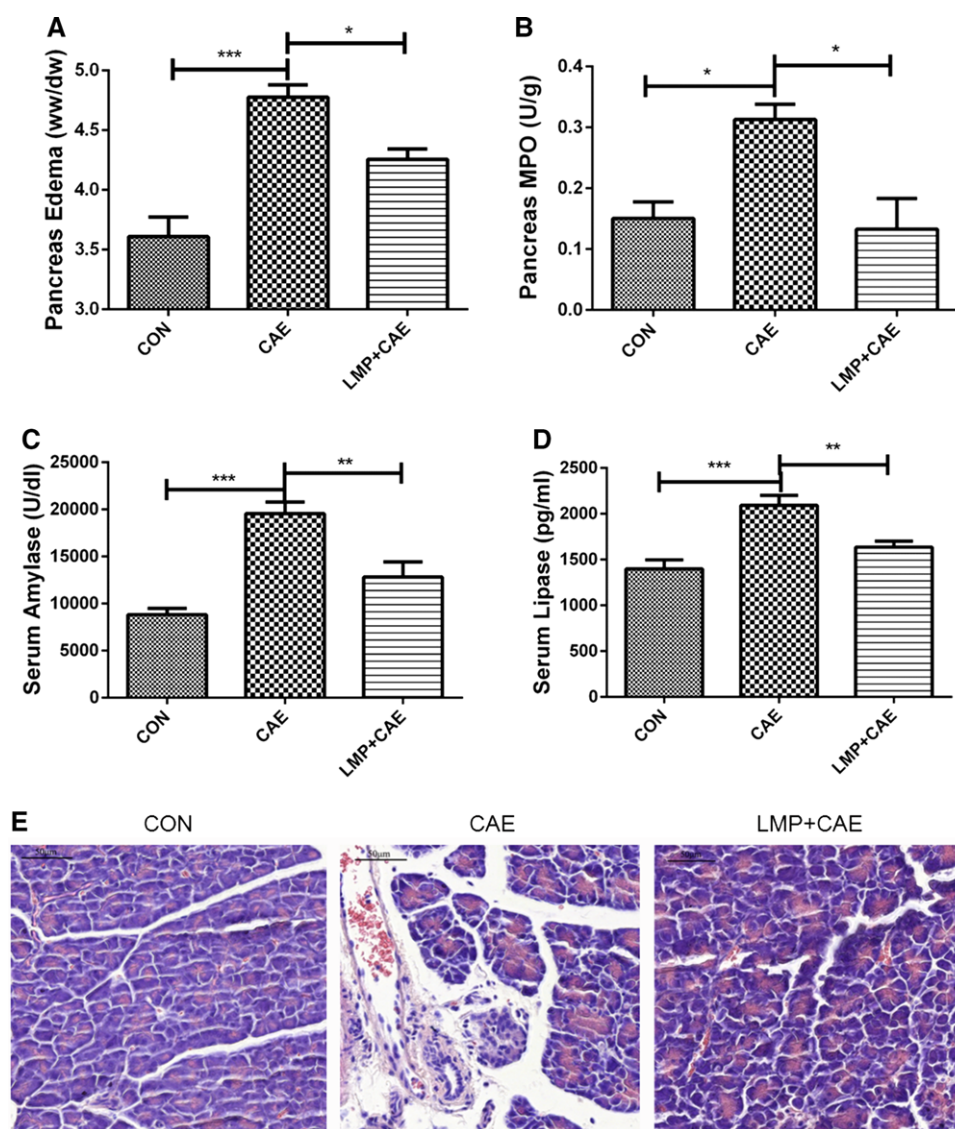


Figure 1. LMP supplementation alleviates the severity of AP. Pancreatic edema (A) pancreatic MPO (B), serum amylase (C), serum lipase levels and pancreatic histology (E) in CON (saline-treated), CAE (caerulein-treated) and LMP+CAE (LMP prophylactically supplemented before AP induction) groups of BALB/c mice. Data are means \pm SEM from at least three independent experiments of at least five independent mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Representative histological sections from at least three independent experiments of five independent mice were shown. Original magnifications: $\times 400$.

reduced caerulein-induced increases in pancreatic MPO levels ($p = 0.0287$), serum amylase ($p = 0.0059$), and lipase ($p = 0.0068$) (Fig. 1B and D). Histological examination of pancreatic injuries showed remarkable reductions of all measures of pancreatic AP histopathology including edema, inflammatory infiltrates, vacuolization, and necrosis in 5% LMP pre-fed group, compared with CAE group (Fig. 1E).

3.2 LMP supplementation promotes a modulatory cytokine profile during AP

The development of AP is accompanied by increased pancreatic and systemic pro-inflammatory cytokine production, which amplifies the condition and promotes systemic inflammatory responses [25, 26]. To study the effects of LMP intake on inflammatory responses during AP, we examined TNF-

α , IL-1 β , and IL-6, in the pancreas and serum. We found that feeding 5% LMP significantly reduced pancreatic TNF- α ($p = 0.0003$), IL-1 β ($p = 0.0022$), and IL-6 ($p = 0.0387$) compared to CAE group (Fig. 2A–C). This effect of LMP was not restricted to the pancreas but was also found in serum TNF- α ($p = 0.0011$), IL-1 β ($p = 0.0116$), and IL-6 ($p = 0.0385$) (Fig. 2D–F).

3.3 LMP supplementation normalizes intestinal barrier function

Intestinal injury and disruption of intestinal mucosal barrier integrity are early hallmarks of severe AP and marked by dysregulation of TJ modulatory proteins, barrier reinforcing antimicrobial peptides (AMPs) and health-promoting SCFAs. To investigate the effect of LMP on intestinal barrier function,

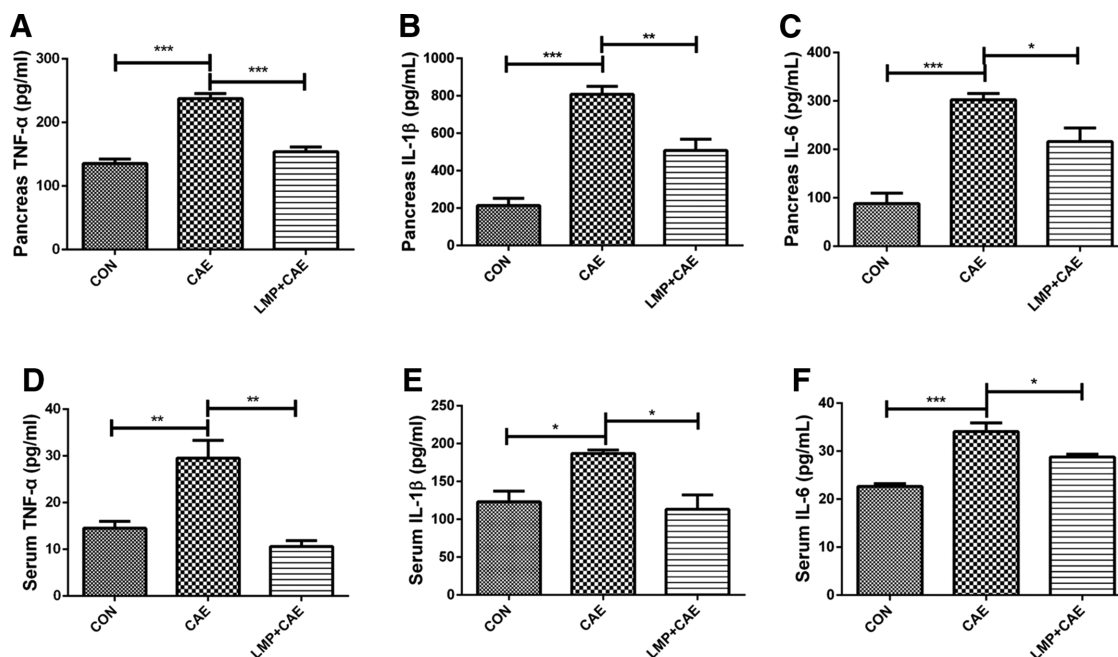


Figure 2. LMP supplementation promotes a modulatory cytokine profile during AP. Quantitative analysis of pancreatic (A–C) and systemic (D–F) TNF- α (A, D), IL-1 β (B, E), and IL-6 (C, F) in CON, CAE, and LMP+CAE groups of mice. Data are means \pm SEM from at least three independent experiments of at least five independent mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

we measured the mRNA expression of major TJ proteins, ZO-1, and occludin, as well as AMPs defensin- β -1 (DEFB1) and CRAMP in the colon and ileum. As shown in Fig. 3A and B, LMP prevented CAE-induced decreases in ZO-1 ($p = 0.0121$ colonic and $p = 0.0011$ ileal) and occludin ($p = 0.0137$ colonic and $p = 0.0348$ ileal) expression. Similarly, LMP significantly upregulated colon and ileal DEFB1 ($p = 0.0021$ and 0.0348 , respectively) and CRAMP ($p = 0.0004$ and 0.0416 , respectively) which were downregulated by CAE treatment (Fig. 3C, D). In histology we found that LMP prevented a reduction in the mucous layer and crypt length in the colon (Fig. 3E, F), a sign of prevention of barrier dysfunction.

Next, we determined the concentrations of acetic acid, propanoic acid, and butyric acid in feces by GC-MS. After feeding mice with LMP, we found that the concentrations of individual and total SCFAs in feces of 5% LMP fed mice were significantly increased compared with mice fed with normal chew (Fig. 3G, H).

3.4 LMP supplementation protects gut inflammation during AP

Having found clear effects on the intestinal integrity, we next questioned whether LMP lowered inflammatory events in the intestine already. To determine this we examined ileal and colonic TNF- α , IL-1 β , and IL-6 levels. LMP treated mice had statistical significant lower TNF- α ($p = 0.0087$ colonic, $p = 0.0120$ ileal), IL-1 β ($p = 0.0409$ colonic and $p = 0.0044$

ileal), and IL-6 ($p = 0.0040$ colonic and $p = 0.0108$ ileal) levels (Fig. 4A–F) when compared with the control group.

4 Discussion

In the present study, prophylactic anti-inflammatory effects of an LMP (7 DM) were tested on caerulein hyperstimulation induced AP and associated intestinal barrier dysfunction. Notably, dietary LMP supplementation mitigates the severity of AP by suppressing pro-inflammatory cytokine production and several characteristics of the disease. Intriguingly, LMP protects AP-associated intestinal mucosal barrier dysfunction by upregulating barrier reinforcing TJs and AMPs as well as by enhancing SCFA production. Our data suggest that dietary LMP intake prevents the development of AP by three-graded actions: (i) reducing pancreatic inflammation and damages, (ii) reducing AP associated intestinal barrier dysfunction and intestinal inflammation and (iii) preventing its progression into a systemic inflammatory response as the consequence of the two aforementioned effects.

Earlier studies on dietary pectins have been functionally described for their nutritional, antioxidant, antiapoptotic, anticancer, and antidiabetic properties [19, 27–29]. Supplementation of pectins varies from 1 day up to weeks in vivo [18]. Here, we observed significant prophylactic effects of pectins on common abdominal inflammation including preceding intestinal dysfunctions associated with severe AP after 3-day feeding prior to AP induction. Earlier, it has been suggested

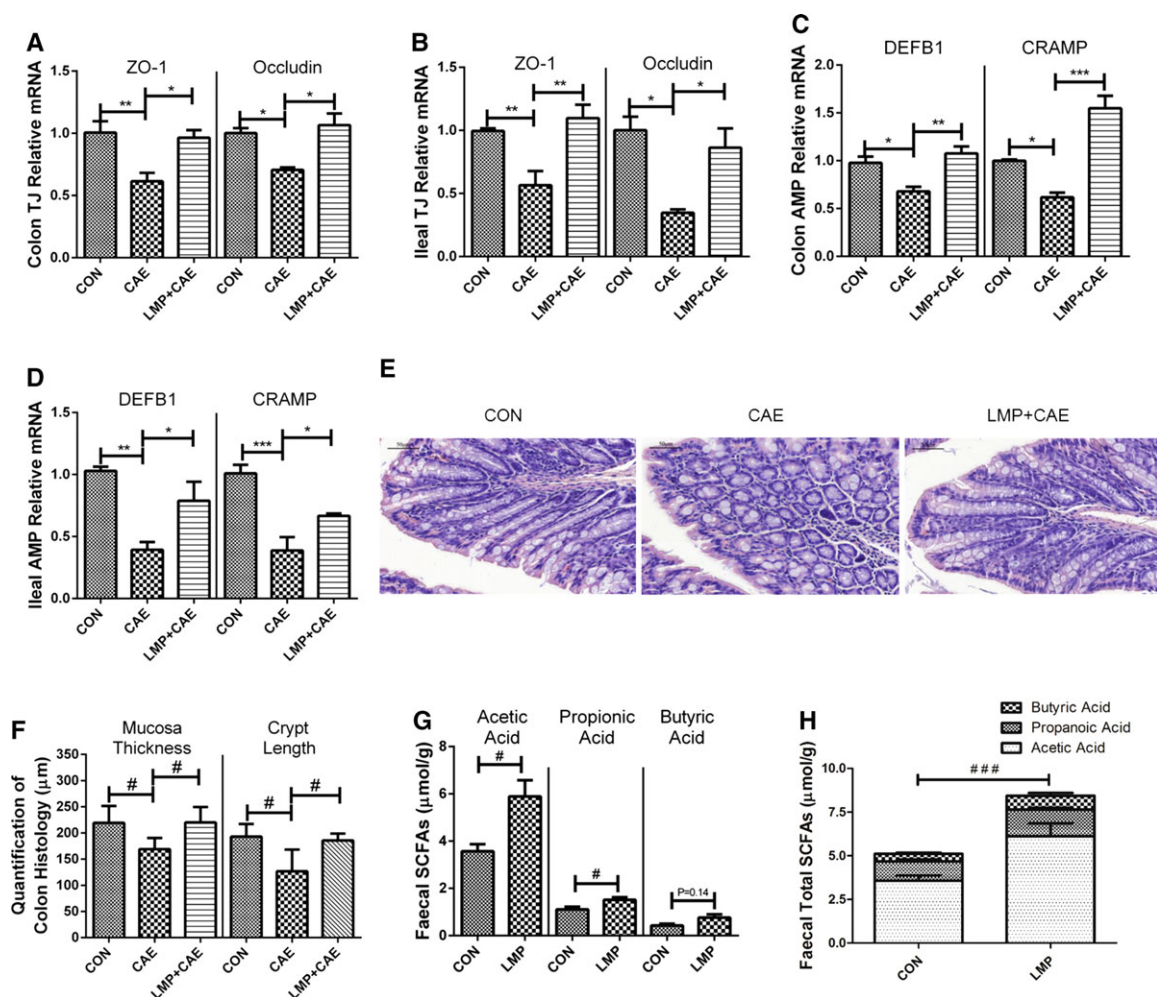


Figure 3. LMP supplementation protects AP-associated intestinal barrier dysfunction. Relative mRNA levels of colon (A, C) and ileal (B, D) TJ proteins ZO-1, occludin (A, B), and AMPs DEFB1, CRAMP (C, D) of each group. E: Histological examination. Representative histological sections from at least three independent experiments of five independent mice were shown. Original magnifications: $\times 400$. F: Quantification of mucosa thickness and crypt length. G, H: Faecal concentrations of individual (G) and total SCFAs (H) in control and 5% LMP-fed groups. Data are means \pm SEM from at least three independent experiments of at least five independent mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. # $p < 0.05$, ### $p < 0.001$ by independent *t*-test.

that dietary fibers including pectins are inversely related with pancreatic enzyme activity and that pectins inhibit pancreatic digestive enzyme activities in vivo [30]. Premature activation of pancreatic zymogens is a triggering event of AP, which supports the potential role of pectins in protecting AP. As AP disease progresses, TNF- α induces IL-1 β and IL-6, which act on macrophages primarily and other immune cells to induce more TNF- α , forming a positive feedback loop. By the “trigger like” effect, the inflammatory mediator cascade is triggered and causes damage to the pancreas and the other vital organs [31]. The immunomodulatory effects of pectins are dependent on its source and chemistry. Pectins isolated from alfalfa, citrus, celery stalks, and passiflora edulis have been reported to have anti-inflammatory effects [29, 32, 33]. Apple pectin downregulates the inflammatory responses by modulating proinflammatory cytokines and immunoglobulins in

colitis [18]. Pectins at different degrees of esterification (DE) are found to have differential effects. Capsicum, a 25% methyl esterified pectin from the sweet pepper *Capsicum annuum*, was found to decrease TNF- α release and to increase production of IL-10 in lipopolysaccharide-stimulated whole blood cells [34]. Citrus and apple pectins with 34–38% methyl esterification suppress endotoxin-induced pro-inflammatory responses by blocking LPS signaling pathways, whereas similar pectins with a high degree of methyl esterification (68–76%) failed to show this effect [35–37]. Moreover, the ability of pectin to inhibit intracellular mediators of inflammation in vitro is inversely related with its degree of methyl esterification [19]. Here, we observed less severe AP in mice fed with 5% LMP, and reduced pancreatic acinar injury, as compared with mice fed with normal diet. LMP-fed mice had a lower level of TNF- α , IL-1 β , and IL-6. The differential

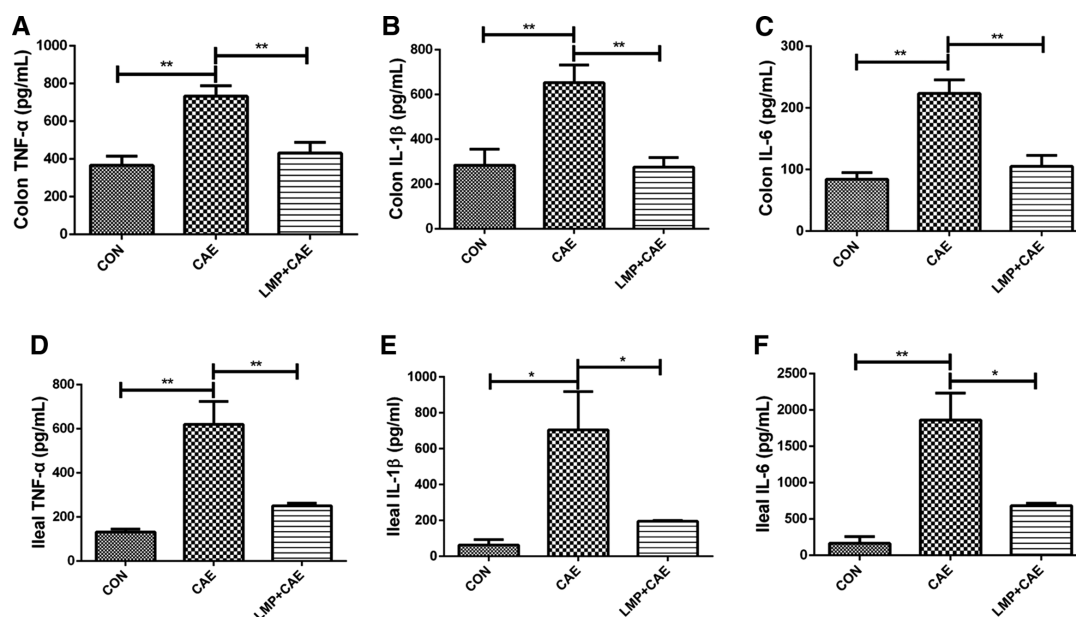


Figure 4. LMP supplementation protects gut inflammation during AP. Quantitative analysis of colon (A–C) and ileal (D–F) TNF- α (A, D), IL-1 β (B, E), and IL-6 (C, F) in each group. Data are means \pm SEM from at least three independent experiments of at least five independent mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

modulation may be explained by in vitro versus in vivo and physiological versus pathophysiological conditions of the models studied. Our data point to clear modulatory effects of low-methoxyl (7 DM) lemon pectin on local inflammatory responses during AP and the disease severity.

The mucous layer covering the intestinal mucosal surface acts as a major defensive barrier between the luminal contents and the epithelial cells, protecting them from potential luminal insults. As our results demonstrate, feeding with LMP protects the mice from AP-associated intestinal injury. Tight junction is the main connection between the intestinal epithelial cells, critical in maintaining mechanical integrity and intestinal mucosa barrier function [38]. It has been shown that loss of ZO-1 and occludin after development of significant intestinal inflammation increases the permeability of the epithelial barrier [39]. We found that during AP, ZO-1 and occludin are downregulated by caerulein administration which can be prevented by dietary LMP supplementation. In addition to TJ proteins, AMPs with antimicrobial and immunomodulatory properties reinforce the barrier integrity and have been shown to promote the healing of colon lesions during colitis [40]. A similar trend of modulation to TJs was observed for DEFB1 and AMPs with LMP feeding, suggesting that the low esterified pectin protects gut barrier by upregulating TJs and AMPs.

In addition to effects on barrier integrity, soluble dietary fibers are known to impact gastrointestinal functions by stimulating proliferation of intestinal cells [41]. Among the dietary soluble fibers, pectin has the most rapid fermentation rate [42] and comparing pectins of different DMs, LMP is more efficiently fermented by the microbiota in the ileum than HMP

present which is mainly fermented in the proximal colon [43]. The anaerobic fermentation of pectin in the large bowel results in production of SCFAs, which stimulate intestinal mucosal growth and have their positive effects on intestinal function [44]. It has been shown that pectin increased the concentration of SCFAs in rat caecum and colon samples [45]. In accordance with these findings, our results show that LMP increased the concentration of faecal SCFAs in treated mice. In fact, SCFAs could be the effector metabolites of LMP with health-promoting and barrier-reinforcing effects on gut, including inhibition of inflammation [46] and modulation of oxidative stress [47] which are key pathological events underlying AP. Furthermore, SCFAs has been reported to augment barrier function [48] and by crosstalk with intestinal epithelial hypoxia-inducible factor (HIF) [49] or by activation of AMP-activated protein kinase [50].

Last, we observed that dietary LMP lowered the secretion of intestinal inflammatory cytokines. As AP progresses, the inflammatory cytokines, such as TNF- α and IL-1, of local pancreas reach gut by microcirculation, activate NF- κ B in affected intestinal cells, and induce more of inflammatory mediators that contribute to intestinal barrier dysfunction and mucosal injury [51, 52]. Gut barrier dysfunction and worsened barrier permeability in AP in turn contributes to the second attack to the pancreas as well as systemically, causing excessive activation of leukocytes, release of multiple cytokines and other inflammatory mediators and development of multiple-organ complications [51, 53]. Thus, gut barrier and immune homeostasis which is the key to prevent the severe form of AP, could be positively modulated by dietary LMP intervention.

Collectively, our data suggest that LMP supplementation remains a promising dietary intervention to control local inflammation and protect intestinal barrier injury, thereby preventing the development of severe AP.

Y.S. and Y.H. performed experiments and analyzed data. P.D.V. purified the low-methoxyl pectin. F.W., H.Z., P.D.V. provided intellectual inputs and critically reviewed the manuscript. J.S. designed and interpreted experiments. Y.S. and J.S. wrote the paper.

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The authors have declared no conflict of interest.

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