Dietary κ-carrageenan facilitates gut microbiota-mediated intestinal inflammation

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Abstract

Background: After nearly half a century, the inflammatory effects of carrageenan (CGN), a ubiquitous food additive, remain controversial. Little is known about its impact on the gut microbiota and intestinal homeostasis.

Results: Mice fed κ-CGN showed no significant inflammatory symptoms, but showed altered colonic microbiota composition, thereby decreasing bacteria-derived short-chain fatty acids (SCFAs) and increased penetrability of the mucus layer. In mice administered the pathogenic bacterium *Citrobacter rodentium*, inflammation and mucosal damage were further aggravated in the presence of κ-CGN. Mucus layer defects and altered SCFA levels could be reproduced by fecal transplantation from κ-CGN-fed mice, but not from germ-free κ-CGN-fed mice. These symptoms could be partially repaired by administering the probiotics *Bifidobacterium longum NCC-2705* and *Faecalibacterium prausnitzii*.

Conclusions: We report a novel evidence that κ-CGN may not be directly inflammatory, but creates an environment that favors inflammation by perturbation of gut microbiota composition and then facilitates expansion of pathogens, which may be partially reversed by the introduction of probiotics.

Introduction

The rise in processed foods during the last half-century has witnessed a steady increase in the consumption of food additives. Many food additives are applied with little or no restriction when they are expanded used [1]. A survey in the Asia-Pacific region found that urbanization is an important correlate of inflammatory bowel disease (IBD) development [2]. In terms of diet, urbanization also leads to an increased adherence to Westernized dietary patterns. Therefore, strict evaluation of food additives in processed foods such as carrageenan (CGN) is appropriate.

CGNs are a family of polysaccharides isolated from red seaweeds. The wide functionality of CGN has led to incorporation of CGN into a myriad of food products. Individuals who consume multiple CGN-containing foods may ingest several grams on a daily basis [3]. The safety and toxicology of CGN has therefore been strictly examined by various agencies and researchers. However, opinions regarding the overall safety and the potential adverse effects that may lead to gut inflammation and even colon cancer remain conflicting. This controversy has been debated for more than half a century, and recent attention by the media has affected the commercial market for CGN [4]. Evidence for the safety of CGN from researches showing that food grade CGN is stable in the gut and shows no harmful effects [5, 6]. On the contrary, a series of papers by Dr. Tobacman's group and others suggest that CGN has potent inflammatory properties [7, 8]. These conflicting findings appear to stem from scientifically rigorous experiments, which suggests that the inflammatory potential of CGN could be conditional. We previously showed that a short course (1−2 weeks) of pre-treatment of mice with CGN led to increased inflammatory bowel symptoms caused by oxazolone, trinitrobenzene sulfonic acid and Citrobacter rodentium [9−11]. Moreover, in the presence of THP-1 macrophages, CGN can destroy a monolayer of Caco-2 cells at very

low concentrations [12]. This leads to the question of whether CGN can affect intestinal epithelial barrier integrity under certain conditions.

The gut microbiota is a crucial environmental factor contributing to host health [13]. The gut microbiota composition is affected by diet [14], and certain microbial can degrade the intestinal mucus layer, contributing to severe colitis in mice [15]. Other studies have found that CGN can alter microbiota composition, including decreasing the abundance of the anti-inflammatory bacterium, *Akkermansia muciniphila* [16]. We therefore hypothesized that CGN may act as a proinflammatory adjuvant, disrupting homeostasis through the alteration of intestinal microbiota, and destruction of mucosal integrity, which may provide a suitable environment for other inflammatory agents or pathogens.

Here, using high molecular weight, non-degraded κ-CGN, we investigate the controversial role of CGN in intestinal inflammation, from the perspective of its impact on intestinal microbiota. We explore the effects of κ-CGN on intestinal microbiota, intestinal mucosal structure, bacterial metabolism and inflammatory changes in mice, and verify these results by fecal microbiota transplantation and germ-free mice.

Results

κ-CGN alters colonic microbiota composition

After 90 days of κ-CGN oral gavage at different concentrations, intestinal microbiota community (based on 16S rRNA analysis) in mice had changed, with a significant increase of species richness (operational taxonomic unit [OTU], Chao1 and abundance-based coverage estimator [ACE] indices). Total bacterial load was increased in the high dose group (CGN-H) by approximately 13% compared with the control group (NC) ($P < 0.01$, Fig. S1a). Shannon and Simpson indices showed no significant differences in microbiota diversity after 90 days of treatment. The changes in the composition of the microbiota led to the changes in the enterotype. Mice in the NC group were clustered into the *Prevotella* (enterotype 2), an intestinal bacteria type are capable of generating short-chain fatty acids (SCFAs) [17]. Mice in the low and medium dose groups (CGN-L and CGN-M) were clustered into *Ruminococcus* (enterotype 3), with dominant bacteria effectively bind mucin and hydrolyze them into monosaccharides. Mice in the CGN-H group was clustered into *Bacteroides* (Enterotype 1), containing bacteria with a large amount of glycosidase capable of degrading polysaccharides (Fig. S1b) [18, 19].

We performed microbiota fecal transplants from NC and κ-CGN-treated mice to germ-free recipients (NC[trans+], CGN[trans+] groups, Fig. 1a). The CGN-L, CGN-M groups and the corresponding fecal transplantation groups had poor separation from the NC mice in Principal coordinates analysis (PCoA) clustering. However, significant separation of microbial composition was found between the CGN-H and NC groups, and the CGN-H[trans+] and NC[trans+] groups. In addition, CGN-H and CGN-H[trans+] mice clustered closely together, indicating high similarity in fecal bacterial composition between the κ-CGN groups and their corresponding transplantation recipients (Fig. 1b, c). Univariate analysis at the phylum level showed

that compared with the NC/NC^trans+ mice, CGN-H/ CGN-H^trans+ mice had a significantly increased abundance of *Bacteroidetes* (54.0% *vs.* 60.3%; 52.3% *vs.* 58.7%, $P < 0.05$), and a significantly reduced abundance of *Proteobacteria* (4.6% *vs.* 2.1%; 4.2% *vs.* 1.5%, $P < 0.01$); *Firmicutes* was decreased in CGN-H/ CGN-H^trans+ mice, but with no significant (37.3% *vs.* 34.1%, 38.1% *vs.* 36.7%; $P > 0.05$;Fig. 1c). *Rhodospirillaceae* was significantly more abundant in CGN-M/CGN-H groups compared with the NC group (CGN-H: 6.4-fold increase, $P < 0.05$; Table S1). At the genus level, the results of fecal bacteria transplantation were consistent with the changes caused by gavaging κ-CGN. Compared with the NC/NC^trans+ groups, the CGN/CGN^trans+ treatments had significantly increased relative abundance of the bacteria *Ruminococcaceae_unclassified* and the intestinal mucus-degrading *Bacteroides* [20] (all increased more than 3-fold; $P < 0.05$). The relative abundance of the intestinal mucosal resident bacteria *Akkermansia*, which inhibits the contact of pathogenic bacteria with the host mucosa [16], was significantly reduced (both reduced by greater than 70%, $P < 0.05$). In addition, the relative abundance of the intestinal mucosal bacterial genera *Anaeroplasma* and *Mucispirillum*, which stimulate host-production of IgG and IgA; the cellulose-degrading bacteria and short-chain fatty acid (SCFA)-producing bacterial genera [20−22], *[Ruminococcus]_torques_group*, *Ruminiclostridium_5, Lachnospiraceae*, and *[Eubacterium]_brachy_group* were also significantly reduced ($P < 0.05$, Fig. 1d, Tables S1 and S2).

κ-CGN aggravates the inflammatory outbreak of pathogenic bacteria by influencing colonic microbiota

κ-CGN did not induce overt colitis in conventional C57BL/6 or germ-free mice, with no significant increase in level of lipocalin-2 (Lcn-2, a sensitive marker of intestinal inflammation in mice) ($P > 0.05$; Fig. 2a, b), CGN-H^trans+mice did not exhibit changes in fecal Lcn-2 levels, either ($P > 0.05$). Administration of *C. rodentium* led to significantly increased levels of Lcn-2 compared to NC group, with a 2.3-fold increase ($P < 0.01$). Interestingly, in mice treated with CGN-H, followed by *C. rodentium* (CGN^C. rodentium), fecal Lcn-2 was significantly increased (~ 1.8 fold) relative to mice administered *C. rodentium* alone ($P < 0.01$).

Similar to Lcn-2, after 90 days of κ-CGN treatment, six pro-inflammatory cytokines were unchanged compared to NC group ($P > 0.05$, Fig. 2c). *C. rodentium* treatment significantly increased the expression of TNF-α and interleukin-6 (IL-6), by 2.8 and 2.4-fold, respectively ($P < 0.01$) relative to the NC group. Importantly, TNF-α and IL-6 were significantly elevated in CGN^C. rodentium compared with the *C. rodentium*-only treatment group by 3.6 and 1.4-fold, respectively ($P < 0.01$), as well as the chemokine MCP-1, which was increased by 1.6-fold ($P < 0.01$, Fig. 2c).

H&E staining of the colon tissues showed that the intestinal tissue structure of mice in the κ-CGN, CGN-H^trans+, and CGN-H^free+ (CGN-H treated germ-free mice) groups was normal, with tightly arranged goblet cells and no inflammation and infiltration in the crypts (Fig. 2d). The CGN/CGN^trans+ treatment groups had no sign of hyperemia, edema or ulceration (Fig. 2d, Fig. S2). *C. rodentium* induced colon tissue damage in conventional mice, which was further aggravated in CGN^C. rodentium mice; both groups had submucosal edema in colon tissues, and a significantly increase of inflammatory cells in the basal layer.

The colonic mucosa of CGN$^{C. rodentium}$ mice was clearly hyperemic, with edema and hemorrhagic ulcers (Fig. 2d, Fig. S2), demonstrating a state of heightened mucosal inflammation in CGN$^{C. rodentium}$ mice.

κ-CGN alters microbiota composition, leading to changes in mucus degradation genes and SCFA

Metagenomics analysis was consistent with the results from 16S rRNA analysis, Chao1 and ACE indices increased significantly after 90 days with CGN-H treatment (P< 0.01, Fig. S3a), while Shannon and Simpson indices did not. In addition, the relative abundances of SCFA-producing genera, including *Ruminiclostridium_5*, *Lachnospiraceae* and *Eubacterium brachy_group* [23] were reduced in CGN-H-treated mice. The relative abundance of *Bacteroides* was increased in CGN-H-treated mice (Fig. S3b, Table S3), and excessive proliferation of *Bacteroides* may lead to destruction of polysaccharides in the intestinal mucus layer [24]. We therefore sought to determine the abundance of carbohydrate utilization genes in relation to intestinal mucus composition. A total of 28,687 genes encoding carbohydrate active enzymes (CAZymes) were identified in mouse fecal samples, with the largest number of genes belonging to the family of glycoside hydrolases (Fig. 3a). CGN-H treatment led to a significant increase in genes encoding mucosal polysaccharide binding proteins and mucin degrading enzymes. For example, CBM32 and CBM40, which encode mucosal glycan binding proteins, were increased 12.5 and 2.1-fold, respectively, compared with the NC group (P< 0.05); the genes encoding N-acetyl galactosidase, CE9 and CE11 were increased by 9.8 and 36.1-fold, respectively (P< 0.01); genes encoding mucosal polysaccharide and glycosyltransferase, GH106 and GT4, were increased 29.5 and 26.7-fold, respectively (P< 0.01, Fig. 3b). By performing correlation analysis between CAzymes genes changes and microbial abundance (Fig. 3c, Table S4), we found that the abundance of *Bacteroides ovatus* (*Bacteroides* genus) containing CBM32, CE11, GH106, GH109, GH84, and GH85 genes increased 25.35-fold (P< 0.01). The abundances of *Bacteroides nordii* and *Bacteroides thetaiotaomicron* containing GH33 were 17.89 and 21.35 folds higher, respectively than that of the NC group (P< 0.01). The abundance of *Bacteroides intestinihominis* containing GT4 gene was increased 3.45-fold (P< 0.05).

As shown in Fig. 3b, the abundance of genes involved in the use of cellulose, starch and mannan were significantly reduced in CGN-H treated mice. For example, compared with the NC group, GH5 and GH151 encoding β-1, 4-xylan hydrolase were reduced 68.3 and 51.6-fold, respectively (P< 0.01); GH36 and GH77 encoding α-glucosidase were reduced 29.3 and 28.4-fold (P< 0.01), respectively; GH30 encoding β-mannosidase was decreased by 23.2-fold (P< 0.01). In Fig. 3c, correlation analysis showed that the abundances of *Lachnospiraceae_ bacterium_10* and *Ruminococcus torques* containing cellulose degrading genes (GH5, GH42, GH15 and GH151) and starch and mannan degrading genes (GH36, GH77 and GH30) were reduced 13.25 and 6.75-fold, respectively after CGN-H treatment (P< 0.01).

These bacterial can produce large amounts of SCFAs [19, 23]. We therefore quantified SCFAs and, as expected, fecal SCFA contents were significantly altered by κ-CGN treatment. OPLS-DA demonstrated clear separation between CGN-M/CGN-H and NC group, but with limited separation between CGN-L and NC groups (Fig. S4). Among several typical SCFAs, butyric acid, isobutyric acid, valeric acid, and

isovaleric acid were all reduced in CGN-M and CGN-H groups, especially butyric and valeric acid, which were significantly reduced by 67.4% and 60.6%, respectively in the CGN-H group ($P < 0.01$). Similar changes in SCFA contents were seen in mice receiving fecal transplants from CGN-H mice. Compared with the NC^trans+ group, the CGN-H^trans+ group had a 65.3% reduction in butyric and a 69.7% reduction in valeric acid ($P < 0.01$). In germ-free mice, SCFA contents were very low in general, even after κ-CGN transplantation (Fig. 3d), which indicates that the change in SCFA contents in feces of CGN-treated mice was related to changes in microbiota composition.

Correlation analysis between SCFA abundance and intestinal microbiota (Fig. 3e, Table S1) showed that bacteria affected by κ-CGN treatment, including cellulose degrading bacteria, and the SCFA-producing bacterial genera *Ruminiclostridium_5*, *Lachnospiraceae*, and *[Eubacterium]_brachy_group* [23] were positively correlated with changes in isovaleric acid, caproic acid, and acetic acid ($P < 0.05$). In addition, positive correlations were also found between *Ruminiclostridium_5* and isobutyric acid and butyric acid; and between *Lachnospiraceae* and butyric acid and valeric acid ($P < 0.05$); whereas a negative correlation was found between *Ruminococcaceae* and valeric acid and butyric acid ($P < 0.05$).

Shifts in microbial composition contribute to intestinal barrier dysfunction

Fluorescent in situ hybridization (FISH) examination revealed both κ-CGN treatment of conventional mice and the corresponding fecal bacteria transplantation caused thinning of the intestinal mucus layer, by reducing 61% and 58%, respectively (Fig. 4a, b; $P < 0.01$), but not correlated with the expression of the major intestinal mucin Muc2, Krüppel-like factor 4 (Klf4) or goblet cell protein (Tiff3). The expression of these three proteins were not altered by κ-CGN treatment ($P > 0.05$, Fig. 4c). However, after 90 d of κ-CGN treatment in germ-free mice, the thickness of the mucus layer was almost unchanged. No bacteria were observed in the intestinal mucus layer within 25 μm from the epithelial cells of NC group (Fig. 4d, e). In contrast, bacteria penetrated the mucus layer more frequently in mucosal biopsies obtained from κ-CGN-H treated and fecal transplant groups, compared with NC group, with the average distance reduced by greater than 87% in the CGN-H group and 83% in CGN-H^trans+ group ($P < 0.01$). Such microbiota encroachment correlated with reduced mucus thickness. A classical permeability marker FITC-dextran test revealed a significant increase in mucus penetrability in CGN-M, CGN-H and CGN^trans+ groups with higher FITC-dextran levels than in NC mice ($P < 0.05$, Fig. 4f). To determine whether the observed defective mucus layer was caused directly by κ-CGN treatment, we next studied mucus properties in germ-free mice. Compared with NC^free+ group (germ-free control mice), no significant difference in penetrability was detected in CGN-H^free+ mice: mucus thickness, penetrability and expression of Muc2, Klf4, Tiff3 were not affected (Fig. 4a, b, f).

Probiotics attenuate pathogen-induced inflammatory mucosal damage

We supplemented mice with two probiotics *Bifidobacterium longum* NCC-2705 and *Faecalibacterium prausnitzii* after 90 d of oral gavage of κ-CGN (CGN^{Pro+}, Fig. 5a). PCoA analysis of 16S rRNA sequencing at genus level showed two distinct clusters between NC or CGN-H mice; after 30 d of subsequent probiotic intervention, the bacterial composition of CGN-H^{pro+} group was closer to that of the NC group (Fig. 5b). Univariate analysis at phylum level showed decreased relative abundance of *Bacteroides* in CGN-H^{pro+} mice compared with CGN-H mice (45.1% vs. 55.7%, $P < 0.01$), and increased relative abundances of *Firmicutes* (50.4% vs. 43.0%, $P < 0.01$) and *Proteobacteria* (3.2% vs 1.1%, $P < 0.01$; Fig. 5c). At genus level, the increases in relative abundance of CGN-H-induced proinflammatory bacterial genera *Comamonas* [25], *Alistipes, Escherichia-Shigella* [26] and *Clostridium* were ameliorated by probiotic intervention as was the CGN-H-induced decrease in the relative abundance of *Akkermansia*. While the abundance of *Bifidobacterium and Faecalibacterium*, two gavaged probiotics, increased significantly ($P < 0.01$, Fig. 5d, Table S5). *Anaeroplasma* and *Mucispirillum* bacteria, and the genera *Ruminiclostridium_5*, *Lachnospiraceae*, and *[Eubacterium]_brachy_group* were significantly increased in CGN-H^{pro+} vs. CGN-H mice ($P < 0.05$). In addition, the polysaccharide-degrading bacteria *Enterorhabdus* were significantly reduced in CGN-H^{pro+} vs. CGN-H mice ($P < 0.05$, Fig. 5d, Table S5).

Probiotic intervention significantly increased butyric acid, isobutyric acid, and valeric acid ($P < 0.05$, Fig. 5e), which were originally reduced by CGN-H. Spearman's correlation analysis the different SCFAs between CGN-H^{pro+} and CGN-H groups and the intestinal microbiota after probiotic intervention showed that several SCFAs, including butyric acid and valeric acid were significantly and positively correlated with *Lachnospiraceae_bacterium_615* and *Lachnospiraceae_bacterium_DW8/17/22* and with *F. prausnitzii* ($P < 0.05$, Fig. 5f). In addition, *Mucispirillum_schaedleri* was positively correlated with changes in caproic acid ($P < 0.05$); and *Alistipes_sp._CHKC1003, Alistipes_finegoldii, Clostridium_sp._Culture-27, Enterorhabdus_mucosicola* and *Desulfovibrio_sp._ABHU2SB* were negatively correlated with changes in butyric acid, isobutyric acid, acetic acid, and propionic acid ($P < 0.05$).

In addition, the phenomenon of thinning of the mucus layer and increased intestinal permeability caused by exposure to CGN-H was reversed after probiotic intervention. The thickness of the mucus layer recovered by 55.6% ($P < 0.01$, Fig. 5g, h), and intestinal permeability was dampened by probiotics, approaching levels similar to that of the NC group ($P < 0.05$, Fig. 5i). Colonic tissue and inflammatory factors were not significantly changed by probiotic intervention (Fig. S5).

Then, *C. rodentium* was administered by oral gavage after 90 d of CGN-H or after 90 d of CGN-H plus 30 d of probiotic intervention (CGN$^{C. rodentium+Pro+}$). *C. rodentium* treatment significantly reduced the content of SCFAs in feces of conventional mice, except for caproic acid ($P < 0.05$, Fig. 6a); compared with the *C. rodentium* group, SCFA contents were further reduced in the CGN$^{C. rodentium}$ group ($P < 0.05$, Fig. 6a). Probiotic intervention significantly reversed this reduction in intestinal SCFAs ($P < 0.05$), with the content of propionic acid and butyric acid higher than that of the *C. rodentium* group ($P < 0.05$). Even acetic acid, isobutyric acid, and isovaleric acid were basically restored to normal levels. H&E histochemical analysis produced similar findings: Probiotic intervention significantly reduced intestinal inflammation, eliminated

submucosal edema, and significantly reduced the infiltration of inflammatory cells in the basal layer ($P <$ 0.05, Fig. 6b, Fig. S6). Similarly, probiotic intervention significantly improved *C. rodentium*-induced increase in inflammatory cytokines, which was further promoted by CGN-H. Compared with the CGN$^{C. rodentium}$ group, TNF-α, IL-6 and MCP-1 were reduced by 26%, 20%, and 18%, respectively in the CGN$^{C. rodentium+pro+}$ group ($P <$ 0.05, Fig. 6c), suggesting the ability of probiotics to dampen the pro-inflammatory effect of *C. rodentium*.

Discussion

Some researchers have suggested that CGN may exert chronic low-grade inflammation, defined by elevated systemic expression of proinflammatory cytokines in the absence of the classical aggregates of immune cell infiltrates [27]. In our data, without increased inflammatory cytokines, this definition for κ-CGN may be slightly inappropriate. These are consistent with most official reports [1, 3, 4]. However, in the present study, 90 days of κ-CGN gavage promoted colitis-associated gut inflammatory symptoms in *C. rodentium*-treated mice, which also had been observed in our short-term tests [11]. We therefore hypothesized that κ-CGN provides a favorable environment for inflammation.

Mucosal compartmentalization functions to minimize direct contact between intestinal bacteria and the epithelial cell surface (stratification). Bhattacharyya et al. reported that 14 weeks of CGN administration led to disruption of the mucosal surface in mice [28]. We previously found that κ-CGN can lead to destruction of the differentiated colonic layer via immune system effects [12], which suggest that the intestinal mucus layer may be a target of κ-CGN. Administration of κ-CGN to germ-free mice did not result in low-grade inflammation, as assessed by fecal Lcn-2 and proinflammatory factors, or any of the parameters related to mucus thinning and altered localization of bacteria, even the alteration of SCFA contents. However, in normal C57BL/6 mice, κ-CGN caused mucosal thinning, increased gut permeability and decreased SCFA levels. These findings oppose the notion that κ-CGN directly impacts the mucus layer and, rather, suggests that microbial dysbiosis may be a key driver of κ-CGN-dependent inflammatory susceptibility.

Here, high intake of κ-CGN induced changes in gut microbiota composition in mice, with increased relative abundance in several taxa belonging to the phylum *Bacteroidetes*, and decreased relative abundance of *Proteobacteria* and *Firmicutes* taxa. Shang et al. found that CGN profoundly decreased the relative abundance of the anti-inflammatory bacterium *A. muciniphila* in the gut [16]. Our study supports these findings where *Akkermansia* was significantly decreased in response to κ-CGN treatment. Surprisingly, we found that κ-CGN exposure did not decrease microbiota diversity, but increased total bacterial abundance. In general, individuals with chronic inflammation have lower bacterial diversity than their healthy counterparts. This may explain why although it caused mucus defection, but did not translate to colitis development compared to other food additives, such as carboxymethylcellulose and polysorbate-80. In their results, gut microbiota diversity was decreased [16]. Thus, κ-CGN's preservation of bacterial diversity may maintain a delicate balance in the intestinal microbiota and its environment. For example, some bacteria associated with inflammation and immunity showed no negative changes. We

even observed the decrease of the relative abundance of gut *Proteobacteria*, which has positive correlation to colitis [29], and the increase of the abundance of Rhodospirillaceae, which can enhance the cellular and humoral immunity of the body [30].

The analysis of the gut metagenomes revealed altered microbial gene profiles in κ-CGN-treated animals. It confirmed the significant increase in relative abundance of CAZymes belonging to the genus *Bacteroides* in response to κ-CGN. A variety of CAZymes encoded by this taxon were regulated, including genes encoding glycoside hydrolases and polysaccharide lyases. For example, increased expression of CBM32 and CBM40, which encode mucous membrane-binding proteins; CE9 and CE11 degradation of mucin O-glycans, and the mucosal polysaccharide lyase and glycosyltransferase genes GH106 and GT4, which may allow switching to utilize host glycans of the intestinal mucus layer [20].

SCFAs contribute to the maintenance of immune homeostasis in the intestine and enhance intestinal epithelial barrier by assembly of tight junctions [22]. We observed a significant up-regulation of the genus *Ruminococcaceae_unclassified*, which is capable of inhibiting SCFA synthesis, and decreased abundance of *[Ruminococcus]_torques_group*, *Lachnospiraceae*, *Ruminiclostridium_5*, and *E. brachy_group*, which can synthesize SCFAs [23]. Among these, *R. torques* and *L. bacterium_10* possess enzymes necessary for the hydrolysis of cellulose, starch and mannan, and contribute to the production of high amounts of SCFAs [31, 32], such as GH5, GH42, GH15, GH151, GH36, GH77, GH30, for which the gene contents were down-regulated. Furthermore, the decreased fecal levels of SCFAs, especially butyric acid and valeric acid, was confirmed. However, the expression of Muc2, a building block of colonic mucus; Klf4, a transcription factor involved in barrier function, and Tiff3, a mucosal repairing protein [31], were not affected by κ-CGN treatment. Therefore, based on changes in microbial composition and CAZymes and SCFA synthesis gene contents, we speculate that κ-CGN treatment led to increased bacterial consumption of mucin-derived nutrients, which exceeded production of these compounds, hence destroying the integrity of the mucus layer, which led to increased penetrability. We previously reported that once κ-CGN has a conversation with the immune system, mucosal damage is intensified [12]; our latest findings may indicate that κ-CGN increases crosstalk between gut microbiota and the immune system by increased contact with the gut epithelium following mucosal damage.

The observation that germ-free mice receiving fecal transplants from κ-CGN-treated mice also experienced mucosal thinning, decreased production of SCFAs and similar levels of inflammatory factors to conventional mice, suggests relatively stable colonization by transplant microbiota with subsequent inheritance of similar phenotypes.

Various probiotics have demonstrated clinical efficacy in patients with ulcerative colitis. *Bifidobacterium* strains have several beneficial effects, including strengthening epithelial barrier function in human ulcerative colitis patients [31]. Indeed, administration of the probiotics *B. longum NCC-2705* and *F. prausnitzii* led to a partial recovery of gut microbiota and mucus barrier destruction by ameliorating SCFA deficiency, reinforcing intestinal barrier and dampening intestinal permeability. Probiotic administration further limited the *C. rodentium*-induced immune reaction, metabolic impairment and inflammation.

Conclusion

Overall, this approach revealed that the consumption of κ-CGN, a ubiquitous food additive, does not induce histopathologically evident inflammation in mice, but does alter gut microbiota in a manner that leads to colonic mucus layer destruction, and disruption of intestinal homeostasis, thereby creating a weakly pro-inflammatory environment (scheme 1). These phenomena can be reproduced by fecal transplantation from κ-CGN-treated mice to germ-free mice. It worth noting that when animals are exposed to pathogens or other inflammatory agents, κ-CGN may exacerbate pathogen-induced intestinal inflammation, as seen here in *C. rodentium*-treated mice. Most of these κ-CGN-associated alterations were partly reversed by supplementation with probiotics, which therefore has potential therapeutic implications.

Methodology

Experimental materials

Male C57BL/6 mice (6 weeks old, SCXK 2018-0004) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Mice were housed in the animal center of Ningbo University Medicine College (Ningbo, China), with four mice per cage, under a 12-h light/12-h dark cycle (lights on at 08:00 AM) in temperature- and humidity-controlled environments (21 ± 2 °C and approximately 60%, respectively) with free access to food and water. Male germ-free mice (genetic background of C57BL/6, 6 weeks old) from the National Laboratory Animal Seed Center (Shanghai, China) were housed with one mouse/Combibloc aseptic package isolator under germ-free conditions. All mice were habituated to their housing environment for 5 days prior to oral gavage. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and animal-related experiments were approved by the Ethical Committee of Animal Use and Protection of Ningbo University Health Science Center.

Experimental design

Mice were treated with different concentrations of κ-CGN (1.7 mg/kg, CGN-L; 8.3 mg/kg, CGN-M; or 41.7 mg/kg, CGN-H; n = 32) for 90 days [9–11]. For the treatment, 8 mice were treated with κ-CGN only; 8 mice with κ-CGN and *C. rodentium* (CGN$^{C.\ rodentium}$, gavaged daily with 10^9 CFU/mouse of *C. rodentium* for 3 consecutive days); 8 with κ-CGN and probiotics (CGN^{Pro+}, gavaged with 10^9 CFU/mouse suspension of 2 probiotics for 23 days); and 8 with κ-CGN, *C. rodentium* and probiotic (CGN$^{C.\ rodentium+Pro+}$, treated with 2 probiotics for 23 days, stopped treatment for a week, followed by gavage with *C. rodentium* for 3 days). Mice (*C. rodentium* group) were given an equivalent volume of saline for 90 (n = 8) or 120 days (n = 8), followed by administration of *C. rodentium* for 3 days. The control group (NC) was given only saline (n = 8).

Fresh feces were collected at days 0, 45, and 90. Blood was collected by retrobulbar intraorbital capillary plexus at day 90. Serum was obtained by centrifugation of blood. Mice were then euthanized, and colons

were collected.

For germ-free experiments, 16 male germ-free mice were sorted into two groups (n = 8), including a control group (NC^{free+}) and CGN-H-treated group (41.7 mg/kg for 90 days, CGN-H^{free+}). For the microbiota transplantation experiment, fecal samples were suspended in 30% glycerol. Male germ-free mice were divided into 4 groups (NC^{trans+}, CGN-L^{trans+}, CGN-M^{trans+}, and CGN-H^{trans+}; n = 8). They were orally administered 200 μL of fecal suspension (from the feces of mice of CGN or NC group) with a concentration of 400 mg/mL for 3 consecutive days. After 90 days, each group was sampled as described above (Fig. S7).

16S rRNA gene sequence analysis

Genomic DNA was extracted from the cecal content samples of mice [32]. The hypervariable regions V3-V4 of 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA) ([33]). For 16S rRNA gene sequence analysis, refer to the supplementary material.

Macroscopic evaluation and histological analysis

Clinical severity of gut tissues was graded as outlined in Table S2, mice were weighed daily, checked for stool consistency, rectal bleeding and presence of blood in the stool [34].

Colonic tissues were treated for H&E staining. The histological grade and level of inflammation were determined by blinded veterinary pathologists. Colitis severity was quantified using the histological activity index (HAI) [35, 36], as detailed in Table S8.

Analysis of Lcn-2 and cytokine levels

Lcn-2 levels in fecal samples were determined using the DuoSet murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, Minnesota, USA).The levels of six cell cytokines, IL-6, TNF-α, interferon (IFN)-γ, IL-10, IL-12p70, and MCP-1, were determined using the BD Cytometric Bead Array Mouse Inflammatory Cytokine Kit (BD BioSciences, Franklin Lakes, NJ, USA). Samples were analyzed on a BD FACSCalibur flow cytometer (BD BioSciences).

Fecal microbiota analysis by metagenome sequencing

DNA was extracted from mice fecal samples using standard methods [37]. Paired-end (PE) library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA) and subjected to Illumina PE 150-bp PE150A sequencing (Illumina, Inc., San Diego, CA, US). For metagenome assembly and construction and analysis of the gene catalog, refer to the supplementary materials. Representative sequences of non-redundant gene catalog were aligned to NCBI NR database with e-value cutoff of $1e^{-5}$ using BLASTP (Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) for taxonomic annotations.

SCFA composition analysis

Lyophilized samples were extracted and analyzed via GC/MS according to Zhao et al [23]. SCFA levels were analyzed by hierarchical clustering with the pheatmap package in R (version 3.6.1). OPLS-DA was used to evaluate the effect of each treatment group. The correlation between SCFA levels and intestinal microbial abundance was analyzed by calculating Spearman's rank correlation coefficient.

Mucin immunostaining and localization of bacteria by FISH

Fixed colonic tissues containing fecal material were washed and paraffin-embed; 5 μm sections were dewaxed. Hybridization was performed at 50 °C overnight with EUB338 probe (5'-GCTGCCTCCCGTAGGAGT-3', with 5' labeled Alexa 647, Gene Pharma Co., Ltd, Shanghai, China), followed by mucin-2 primary antibody (1:1500, Cell Signaling Technology Inc., Shanghai, China) overnight at 4 °C, and anti-rabbit Alexa 488 secondary antibody (1:1500) at room temperature for 1 h, then washed thrice with TBST. Then, 10 μg/mL DAPI (Sigma-Aldrich, St. Louis, Missouri, US) was applied for 2 h, washed, and observed with a Zeiss LSM 700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). The distance between bacteria and epithelial cell monolayer, as well as the thickness of the mucus layer, were analyzed by ZEN software 2011.

In vivo epithelial barrier permeability

An *in vivo* assay of intestinal barrier function was performed using a FITC-labeled dextran method, where the serum concentration of FITC-labeled dextran was used as a proxy for paracellular permeability according to the method of Chassaing et al [13].

Statistical analysis

Analyses were performed using the SPSS software, version 16.0 (IBM Corp., Armonk, New York, USA). The results were expressed as means ± SEM. Differences among groups were analyzed using a post-hoc Fisher's LSD test after a one-way ANOVA. $P < 0.05$ was considered statistically significant. The Levene test was used to verify the homogeneity of variance.

Abbreviations

ACE: abundance-based coverage estimator, CGN: carrageenan, FISH: fluorescent in situ hybridization, Klf4: Krüppel-like factor 4, HAI: histological activity index, IBD: inflammatory bowel disease, IFN: interferon, IL: interleukin, OUT: operational taxonomic unit, SCFAs: short-chain fatty acids.

Declarations

Availability of data and materials

16S rRNA gene sequence data in the Annoroad database (http://crftp.annoroad.com); the account number and password are ANCZJ180308.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interests, financial or otherwise.

Authors' contributions

HC and WW conceived and designed the study. WW, JZ and HH performed the experiments. WW, JZ, FW, YZ, JL analyzed data. WW prepared figures. HC and WW primarily wrote and edited the manuscript. WW assisted with mucus measurements. FW conducted blinded histology scoring. All authors discussed the results and provided comments on the manuscript.

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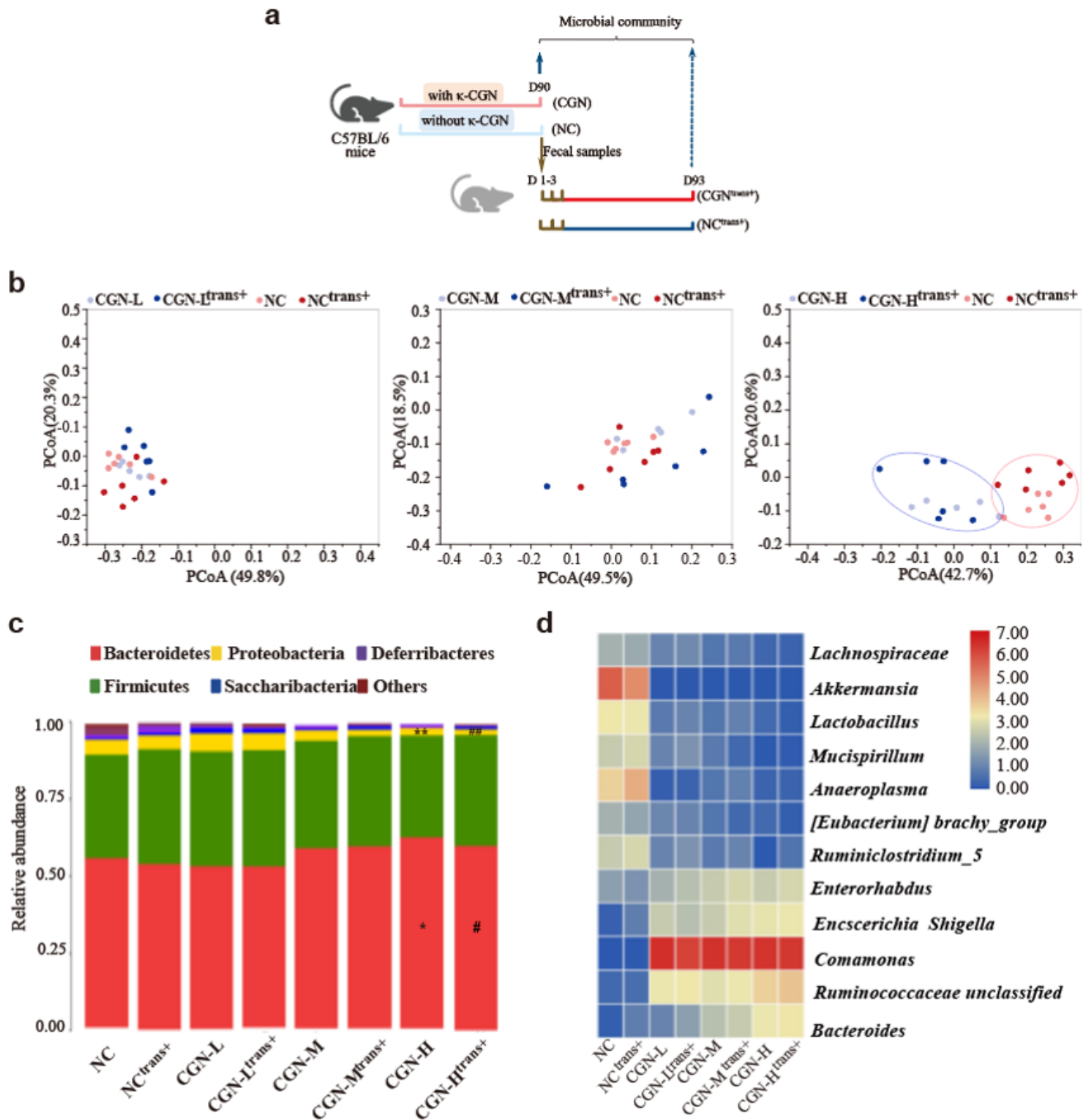
Figures

Figure 1

κ-CGN significant alters microbiota composition. (a). Experimental scheme. (b). PCoA plots of microbial community dissimilarity (Bray-Curtis) in fecal samples at genus level by 16S rRNA-based sequencing. (c). Relative abundance of microbial taxa determined by 16S rRNA analysis of fecal bacteria at phylum level, for which abundances > 1% are shown; *P < 0.05, compared with the NC group. #P < 0.05, compared with the NCtrans+ group. D. Heatmap of the relative abundance of individual genera after κ-CGN or microbiota transplantation treatment.
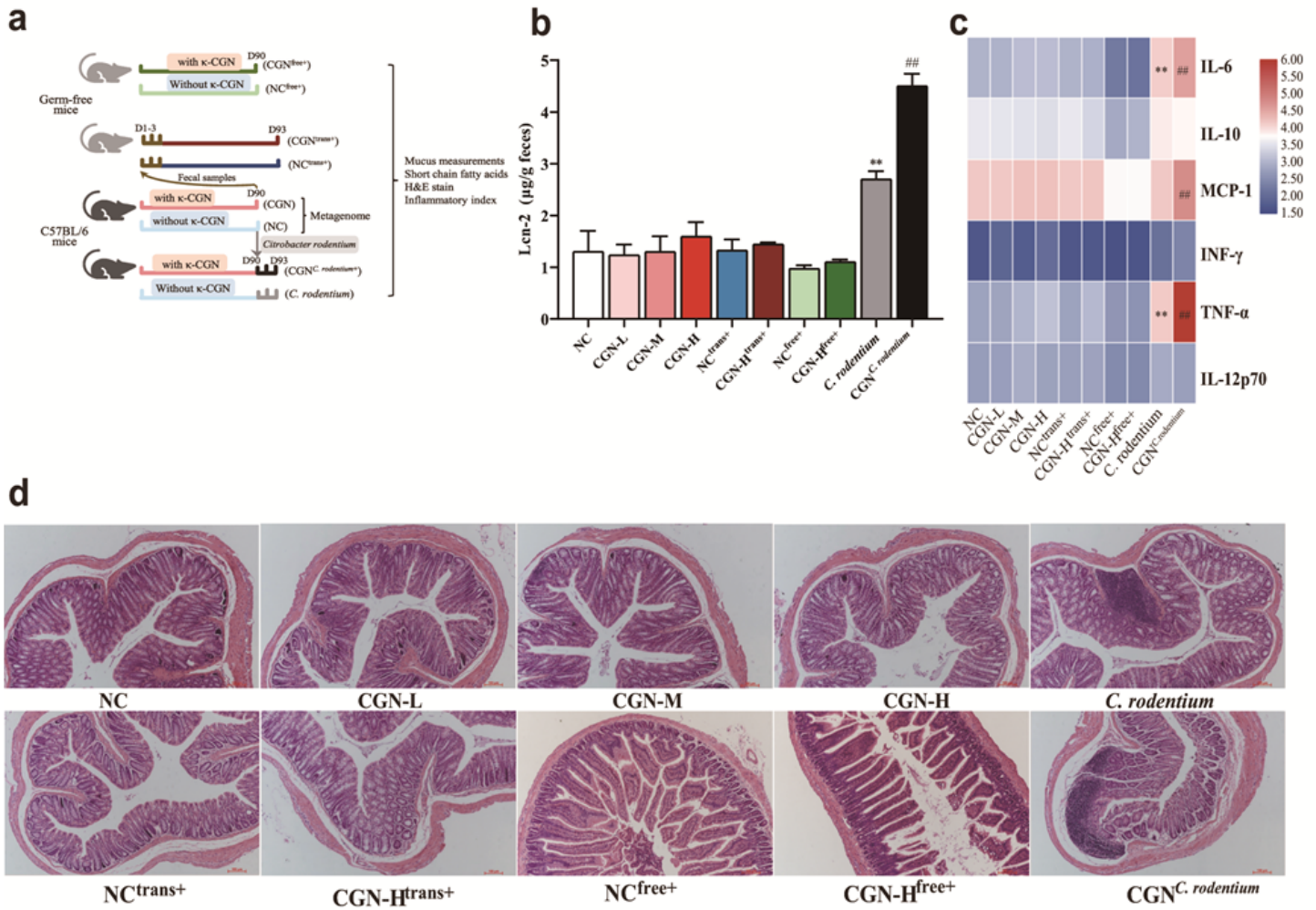
Figure 2

κ-CGN enhances Citrobacter rodentium−induced inflammation. (a). Experimental scheme. (b). Fecal levels of the inflammatory marker Lcn-2. (c). Heatmap of mean concentrations (pg/mL) of inflammatory cytokines in serum of mice (n = 8 mice). **$P < 0.01$, compared with the NC group. ##$P < 0.01$, compared with the C. rodentium group. (d). The impact of κ-CGN and C. rodentium on colonic histological changes. Colon was H&E stained. Bar = 100 μm.
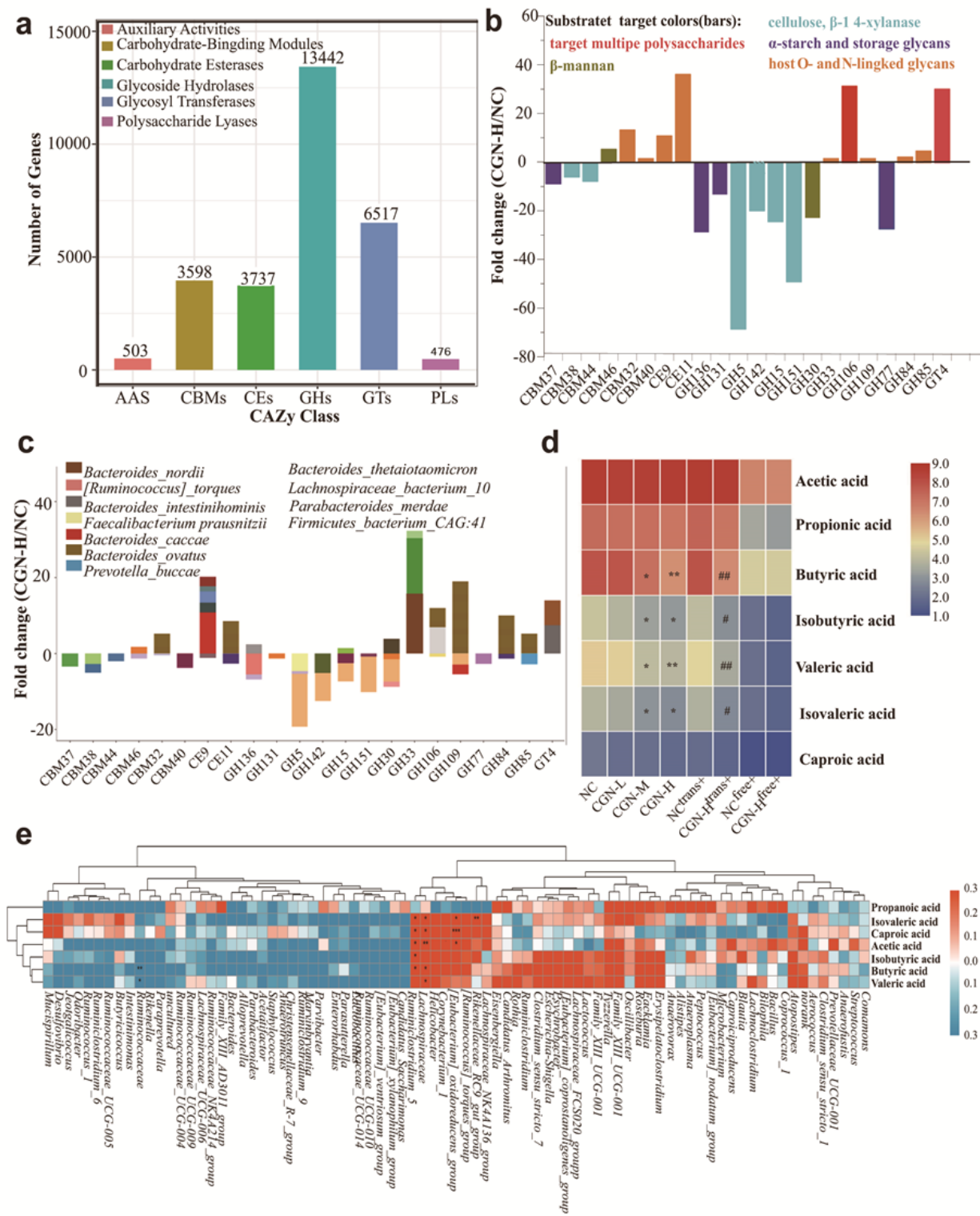
Figure 3

κ-CGN-induced changes in microbiota composition results in altered levels of carbohydrate active enzyme (CAZyme) genes and short-chain fatty acids (SCFAs). (a). Classification of whole-community carbohydrate active enzymes (CAZymes) based on microbial community metagenomic data. (b−c). Positive and negative regulation of encoding CAZymes between CGN-H/NC comparisons. CAZyme families (x-axis) in which > 2-fold changes (P < 0.05, Student's t test) were observed for all genes in that

family in RPKM-normalized fecal community metagenomes (n = 3 mice/group). (d). Heatmap of mean values of SCFA contents in mice feces (µg/g). *P < 0.05, **P < 0.01, compared with the NC group. #P < 0.05, ##P < 0.01, compared with the NCtrans+ group. (e). Spearman's correlation between changes in fecal SCFAs and genus abundances of individual genera after CGN-H treatment. The intensity of the colors represents the degree of association between changes in fecal SCFA concentrations and the relative abundances of individual genera. Scale represents correlation coefficients. *P < 0.05, **P < 0.01.
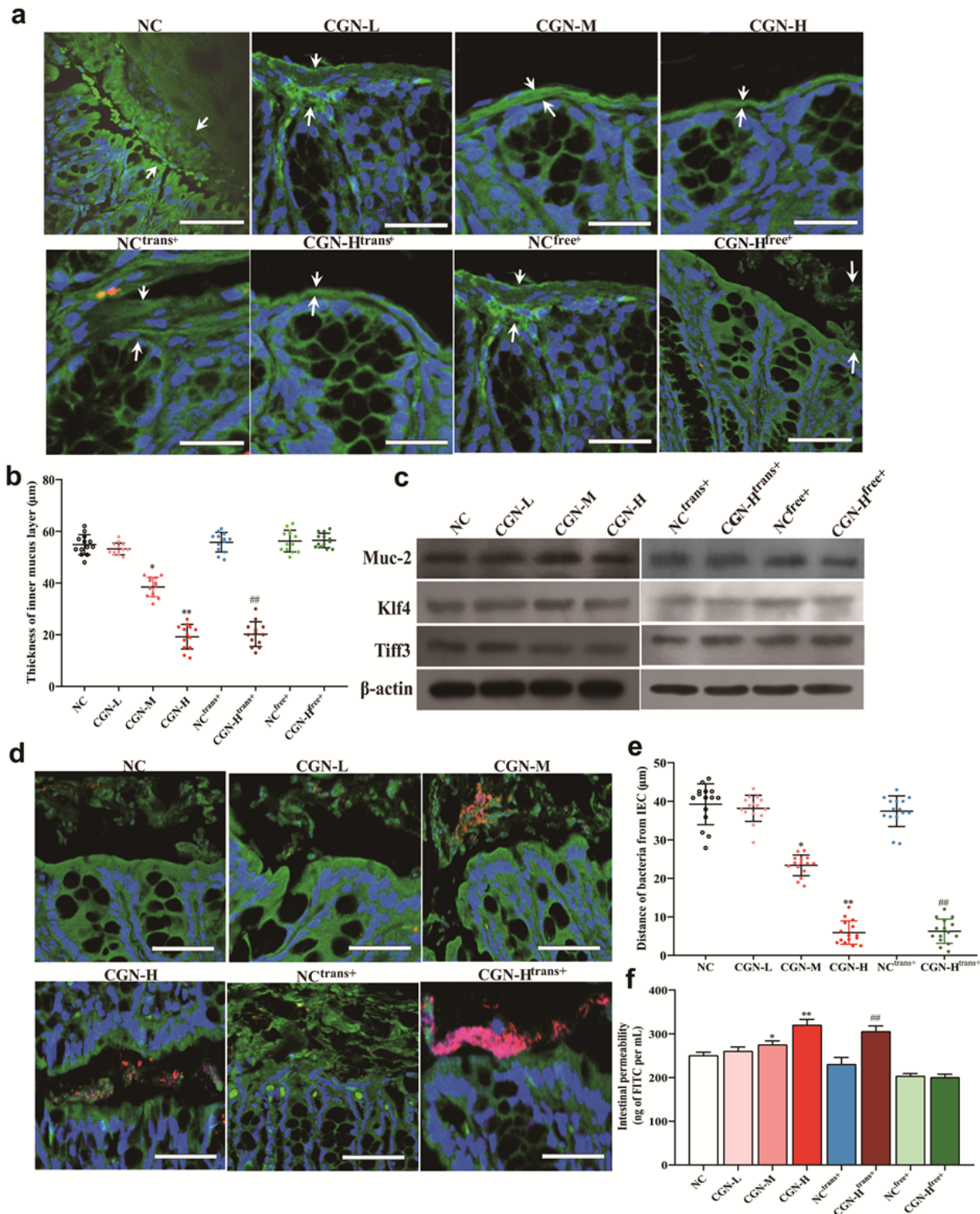

Figure 4

κ-CGN alters erosion of the mucus barrier in conventional C57BL/6 mice, but not germ-free mice. (a). Confocal microscopy analysis of colonic sections showing the mucus layer (arrows). Muc2 (stained with Muc2 antibody, green) and DNA (DAPI, Blue), bar = 100 μm. (b). The effect of κ-CGN on the thickness of the inner mucus layer (n = 8). (c). Protein expression of Muc2, Tiff3 and Klf4 in the colon (n = 8). (d). Confocal microscopy analysis of microbiota localization (n = 10). Muc2 (stained with Muc2 antibody, green), bacteria (stained with EUB338 probe, red), and DNA (DAPI, Blue), bar = 100 μm. (e). The effect of κ-CGN on the distance of closest bacteria to intestinal epithelial cells. (f). Intestinal permeability measured by levels of serum FITC-dextran following oral gavage (n = 8). *P < 0.05, **P < 0.01, compared with the NC group. ##P < 0.01, compared with the NCtrans+ group.
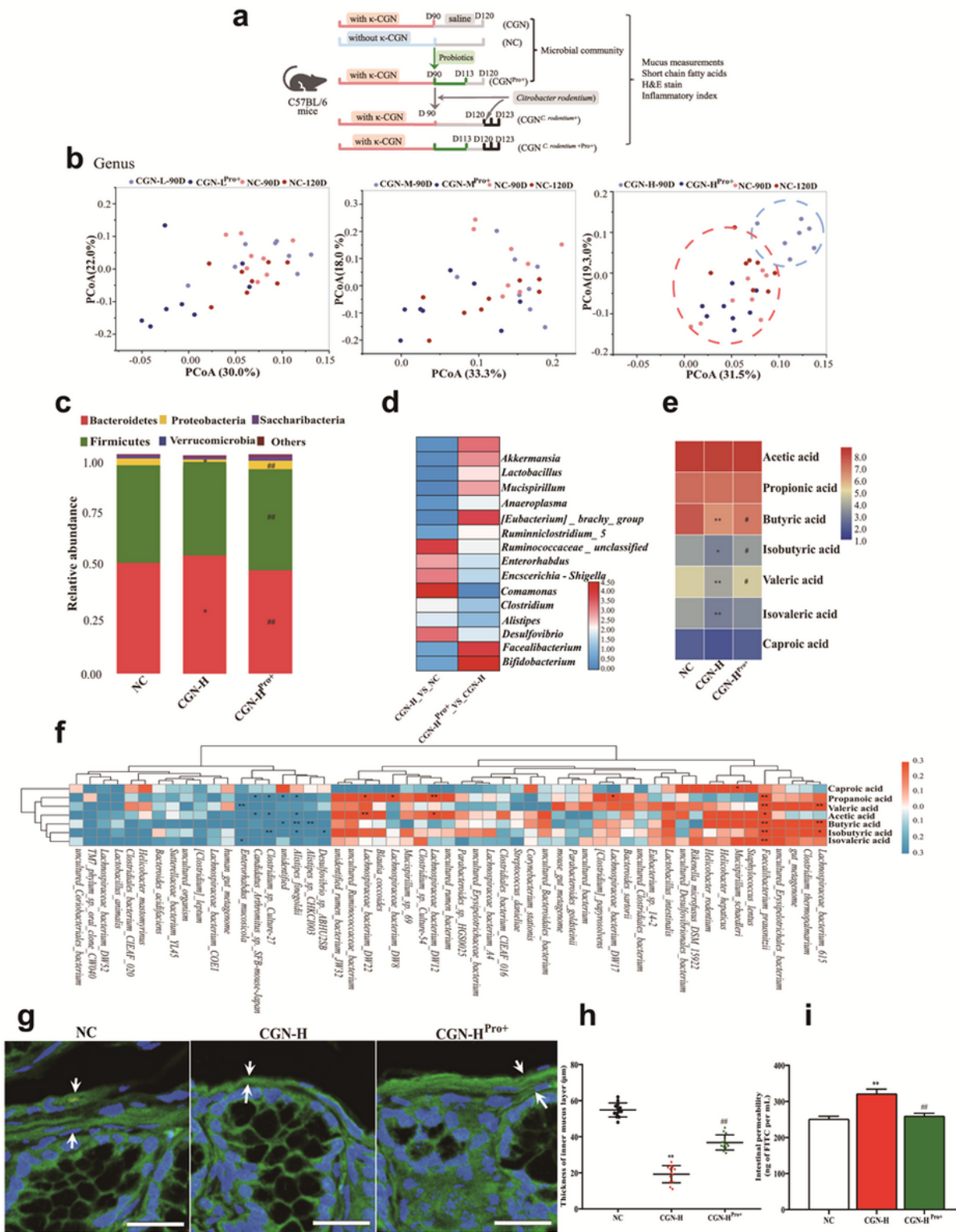
Figure 5

Probiotics reversed the effects of κ-CGN on gut microbiota, short-chain fatty acids, and mucus destruction. (a). Experimental scheme. (b). Genus-level PCoA plots of microbial community dissimilarity (Bray-Curtis) of 16S rRNA-based sequencing results in fecal samples before and after probiotic intervention. (c). Phylum-level relative abundances of microbial taxa of fecal bacteria, for which abundances > 1% are shown. (d). Heatmap of the relative abundances of individual genera after

treatment with κ-CGN or probiotics. (e). Heatmap of fecal SCFA contents (μg/g). (f). Spearman's correlation between changes in fecal SCFAs and in genus abundances of individual genera after probiotic intervention. The intensity of the colors represents the degree of association between changes in the concentration of fecal SCFAs and the relative abundances of individual genera. (g). Confocal microscopy analysis of colonic sections showing the mucus layer (arrows). Muc2 (stained with Muc2 antibody, green) and DNA (DAPI, Blue), bar = 100 μm. (h). The impact of κ-CGN or probiotics on the thickness of the mucus layer (n = 8). (I). Intestinal permeability measured by serum FITC-dextran following oral gavage (n = 8). *P < 0.05, **P < 0.01, compared with the NC group. #P < 0.05, ##P < 0.01, compared with the CGN-H group.
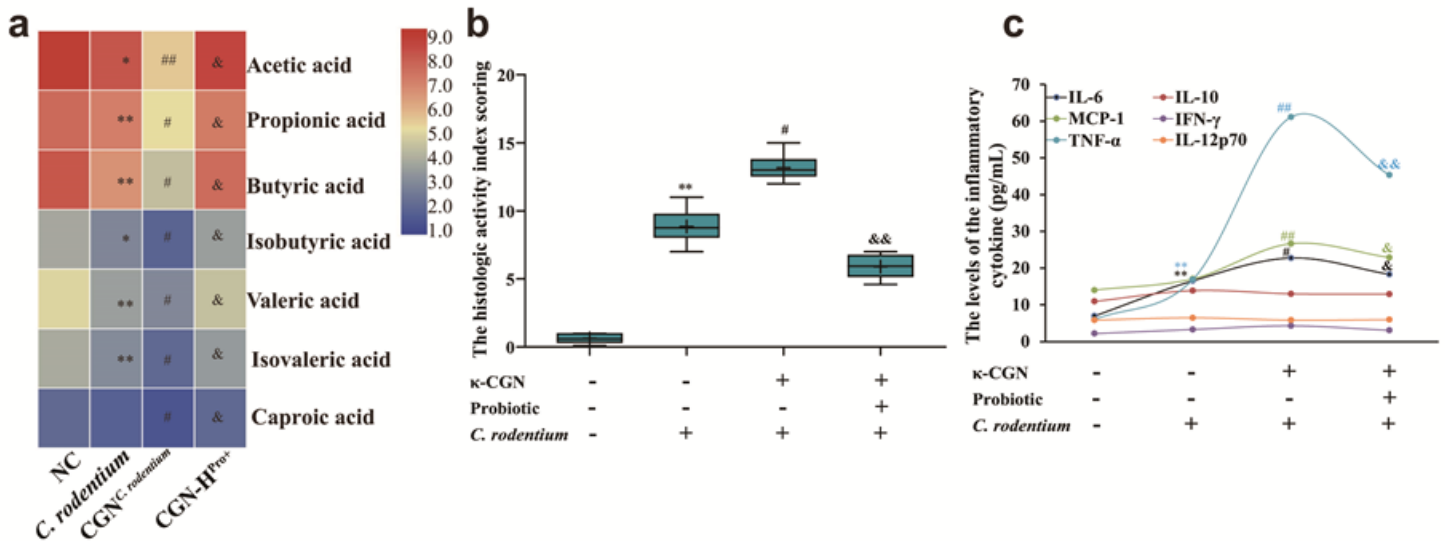

Figure 6

Probiotics suppress the pathogen-induced inflammation. (a). Heatmap of fecal SCFA contents (μg/g). (b). The histologic activity index scores of colons in Citrobacter rodentium-stimulated mice, reduced by probiotics. (c). Probiotics improve C. rodentium−induced increase in pro-inflammatory cytokines (pg/ml, mean ± SEM). *P < 0.05, **P < 0.01, compared with the negative group, #P < 0.05, ##P < 0.01, compared with the C. rodentium group, &P < 0.05, &&P < 0.01, compared with the CGNC. rodentium group.

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