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Original Article

Carboxymethyl chitosan perturbs inflammation profile and colonic microbiota balance in mice

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ABSTRACT

Carboxymethyl chitosan (CMC) is widely used in food and medicine as a biodegradable polymer. However, its effects on inflammation profile and colon health are not well investigated. In the present study, CMC was given to mice to evaluate its possible effects on body weight, blood glucose level, inflammation factors, intestinal permeability and colon microbiota. Results showed that blood glucose level of CMC treated mice was relatively higher than control ones. Glucose tolerance test revealed that CMC treated mice presented higher peak glucose level and lower lag level. CMC treatment increased serum LDL-c level, decreased serum HDL-c and IL-10 level in the fat tissue. Moreover, CMC treatment downregulated the expression of tight junction protein, occludin and ZO-1, in colon as evaluated by Western blot. Colon microbiota analysis demonstrated that CMC treatment significantly decreased the OTUs and relative species abundance. The level of *Enterobacteriaceae*, *Lachnospiraceae* and several other bacteria were much higher in the colon content of CMC treated mice. The results collectively suggest that CMC treatment induces disturbance of glucose and fat metabolism, affects the inflammation profile, perturbs colon microbiota balance.

1. Introduction

Chitosan is derived from chitin by deacetylation which enable it dissoluble in acidic solution, but not in natural or alkaline solution. As a derivative of chitin, chitosan is biodegradable and biocompatible that guarantee its applications in food, medicine and many other areas [1]. For instances, chitosan is used to prepare packaging film for food applications [2]. Other researchers used chitosan to develop nano-delivery tools or coating materials to enhance absorption and bio-availability of encapsulated nutraceuticals and drugs [3]. Apart from using as a biopolymer, direct chitosan administration increased the body weight gain, decreased gut lipid content, upregulated immune response and antioxidant-related gene expression in loach [4]. The authors also found chitosan changed the intestine microbiota which showed higher level of *Bacteroidetes* and lower level of *Firmicutes*. However, chitosan is not readily soluble in water and only soluble in acidic

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solution with pH value lower than 6.5. So that further chemical modification was performed to extend its scope of application.

With the aim of higher water solubility, carboxymethyl chitosan (CMC) is developed by carboxymethyl modification of chitosan. Compared with chitosan, CMC is more hydrophilic and readily dissolved in water with broad pH range. Hence, CMC is more widely used as a biopolymer in food, cosmetic and medicine research. Moreover, CMC shows several biological properties like anti-microbial activity, cell function modulation and anti-cancer ability [5–7]. Among them, the strong and broad-spectrum of anti-microbial ability of CMC attracted much attention. In one research, CMC/clay nanocomposite was prepared and exhibited excellent antimicrobial activity against *E. coli* [8]. In another paper, researchers reported that CMC presented strong antibiofilm activity against non-albicans Candida species and growth inhibition ability of planktonic cells [9]. The anti-microbial mechanism of CMC was not well investigated but may related with its higher water solubility and interaction with the negatively charged cell membrane of bacteria [2,10].

CMC based nanoparticles were widely used to increase the bio-availability of nutraceuticals and drugs. It is reported that when exist in intestine, CMC could interact with mucin of the intestinal surface and open the tight junction of intestine epithelia to increase the absorption ratio of encapsulated compounds [11]. Higher water solubility and positive charge of CMC may be responsible for its absorption enhancement effect. One research pointed out that CMC application significant decreased the transepithelial electrical resistance and increased paracellular permeability of heparin based on *in vitro* and *in vivo* tests [12]. Hence, like chitosan, CMC also possesses strong mucoadhesion and tight junction opening ability through ionic interactions [13].

Therefore, CMC is one of the most investigated biopolymers for food and pharmaceutical applications due to strong antimicrobial ability and intestinal absorption promotion function. Nevertheless, when administrated in oral, it is hypothesized that CMC may interfere with the normal function of intestine such as permeability and microbiological balance, but to our best knowledge few studies investigated this problem.

This study aims to evaluate effects of CMC on the health status of mice focusing on glucose and fat metabolism, inflammation, intestine permeability and colon microbiota. Mice was treated with CMC solution for 8 weeks. Body weight, food intake, water intake, blood glucose level, intraperitoneal glucose tolerance test, organ weight index, serum lipid level, main inflammation factors, tight junction protein level and colon microbiota level were examined.

2. Materials and methods

2.1. Chemicals and reagents

CMC was purchased from Energy Chemical. Co., Ltd. (Shanghai, China). The purity and molecular weight of CMC were measured by gel permeation chromatography (GPC). Test kits for superoxide dismutase (SOD), malondialdehyde (MDA), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), total cholesterol (T-CHO) and triglyceride (TG) were all provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA detection kits for lipopolysaccharide (LPS), IL-1β, IL-6, IL-10, TNF-α and insulin were provided by Shanghai Meilian industrial Co., Ltd. (Shanghai, China). All other chemicals used in this study were of analytical grade without further purification.

2.2. Animals

A total of 10 Kunming male mice, weighing around 20 ± 2 g, were purchased from Laboratory Animal Center of Anhui Medical University (Certificate number: No. 1 license of the Medical Laboratory Animal of Anhui), Hefei, China. All the mice were kept in standard conditions (23 ± 2 °C temperature, 55 ± 5% relative humidity, 12 h light/dark cycle) and fed standard diet for 1 week to acclimatize before experimental process.

2.3. CMC treatment

After acclimatization, mice were randomly allocated into two groups (n = 5), normal control group and CMC treatment group. Mice in normal control group and CMC treatment group could freely access to double distilled water (DDW) and 1% w/v CMC solution prepared with DDW, respectively. Each group of mice was given a commercial mice feed with an initial weight of 250 g. Body weight, food intake and volumes of consumed water and CMC solution were monitored every two days. After 8 weeks of treatment, all the mice were sacrificed by dislocation of the neck. Serum was separated by centrifugation at 1700*g* for 10 min after blood coagulation and stored in fridge at −80 °C for subsequent analysis. Feces in the colon were collected and stored at −80 °C. Livers, kidneys, spleens and fats were collected and weighted, and half the organs were fixed in 10% formalin to prepare paraffin sections and the remaining parts were ground to prepare tissue homogenate and stored at −80 °C for other assays. Organ index was calculated as organ weight (g)/body weight (g) ×100%.

2.4. Intraperitoneal glucose tolerance test (IPGTT)

On the 54th day after the experiment begun, IPGTT was performed according to the previous report [14]. Briefly, prior to IPGTT, all the mice were weighed, marked, fasted for 6 h and injected with 2.0 g/kg body weight of glucose dissolved in normal saline. Blood glucose levels were monitored at 0, 30, 60, 90 and 120 min after the glucose loading using a glucometer (Sinocare, China).

2.5. Biochemical indicators and enzyme-linked immunosorbent measurement

Activity of SOD and concentrations of MDA in liver, HDL-c, LDL-c, TG, T-CHO in serum, were measured using commercial kits. Levels of LPS and insulin in serum, IL-1β, IL-6, IL-10 and TNF-α in adipose tissue, were determined by commercial ELISA kits. All analyses were performed in accordance with the manuals provided by the manufacturers.

2.6. Liver and colon histological staining

Liver and colon tissues were fixed in 10% formalin solution for 24 h. Tissues were then imbedded in paraffin, dehydrated in graded alcohol and cut into slices (5 μm thick). The slices were stained with hematoxylin-eosin (H&E), followed by observation on a light microscope. Finally, the pathological changes were evaluated.

2.7. Colon tight junction protein levels

Western blotting assay was performed according to the procedures reported in literature [15]. Colon tissues from normal control group and CMC treatment group were collected and homogenized in ice-cold RIPA lysis buffer (Beyotime Inc., Nanjing, China). Total protein concentration was quantified using the BCA protein assay kit (Applygen, China). Other procedures were the same with previous reports.

2.8. 16S rDNA amplicon pyrosequencing and high-throughput sequencing analysis

Total bacterial genomic DNA was extracted using the PowerMax DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. PCR amplification of the bacterial 16S rRNA genes V4 region was performed using the forward primer 515F (5′-GTGCCAGCMG CCGCGGTAA-3′) and the reverse primer 806R (5′- GGACTACHVGGGTWTCTAAT-3′) [16]. PCR reactions and bioinformatic analysis were carried out according to previous publications [17].

3. Results

3.1. Impact on food intake, solution intake, body weight, blood glucose level and organ weight

In the present study, the molecular weight and purity of CMC were first analyzed by gel permeation chromatography (GPC). Result was showed in Table S1 and Fig. S1. The weight average molecular weight (Mw) of CMC used was determined as $1.254x10^5$ Da and the number average molecular weight (Mn) is $1.051x10^5$ Da. CMC used in the present study was a mixture with low relative molecular weight distribution index about 1.193, indicating that CMC could be considered as a homogeneous polymer.

As shown in Figs. S2a and b, CMC treated mice showed higher body weight than the control group despite the intake of food was less. The higher body weight suggests higher absorption and storage of energy from food. One previous study reported that chitosan supplement lowered the body weight of mice feed with high fat diet [18]. However, the effect of CMC treatment on the body weight of mice has not been reported. In addition, solution intake was recorded for each cage and depicted in Fig. S2c.

From the third week after the treatment, blood glucose level was measured weekly and was depicted in Fig. S2d. It is observed that CMC treatment exerted no significant impact on the glucose level. Before the end of the animal test, IPGTT was conducted which was showed in Fig. S2e. After the injection of glucose solution, blood glucose level increased to the peak value and then decreased gradually. CMC treated group presented higher peak value and lower lag value. It is assumed that CMC treatment may influence glucose metabolism which need further investigations.

Weight percentage of the major organs were calculated to the body weight of each mouse (Figs. S2f, g, h, i). Data revealed that CMC treated mice showed slightly heavier liver and body fat, and lower kidney weight. Nevertheless, the difference is not significant. Meanwhile, liver, spleen, kidney and colon histomorphology properties were displayed in Fig. S3. No obvious pathosis change was observed between control and CMC treated ones.

3.2. Serum lipid profile, insulin and LPS concentration

Chitosan was reported to decrease the lipid absorption and serum lipid level [19]. As to CMC, scarce information is available. As shown in Fig. 1, CMC treated mice have higher TC level, and higher LDL-c level with no significant difference. TG of CMC treated group is lower than that of control. As to the HDL-c, CMC treated ones presented significant lower value than that of control.

As mentioned before, there is a paucity of literature on the physiological effects of CMC. The result presented in our study indicated that mice treated with CMC solution exhibited disturbed serum lipid profile as lower TG and HDL-c concentration which may be caused by the unnormal intestinal health or lipid metabolism. It was reported that lipid reducing and antioxidant ability of CMC in rabbits [20]. Along with chitosan, CMC at the concentration of 1.5% reduced TG, TC and LDL-c but increased HDL-c concentration. Different results obtained in our study may be caused by different materials used (such as different molecular weight) or the impact of different animal model. But further reports about CMC on lipid lowering effects is lacking in the literature.

3.3. Inflammation, redox status and colon tight junction protein expression

To evaluate the inflammation profile, four inflammation cytokines including IL-1β, IL-6, TNF-α and IL-10 were measured. As showed in the Fig. 2a, b, c, the concentration of IL-1β, IL-6 and TNF-α in both serum and fat tissue presented no significant difference among the two mice groups. On the other hand, IL-10 (in Fig. 2d), which is considered as cytokine to repress the inflammation reaction, is lower both in serum and fat tissue of CMC treated group. Particularly, in fat tissue, IL-10 concentration of CMC treated mice was significantly lower than that of control group.

Redox status of liver was evaluated by examining MDA and SOD level (in Fig. 2e and f). SOD level between CMC and control group was almost the same. But MDA level of CMC treated group was higher than that of control even though the difference was not significant. Our result was not in line with other from the previous literature in which CMC treatment reduced MDA level in rabbit [20]. We are not sure if the results are contradictory due to the different materials used and animal models. CMC at the tested concentration had no

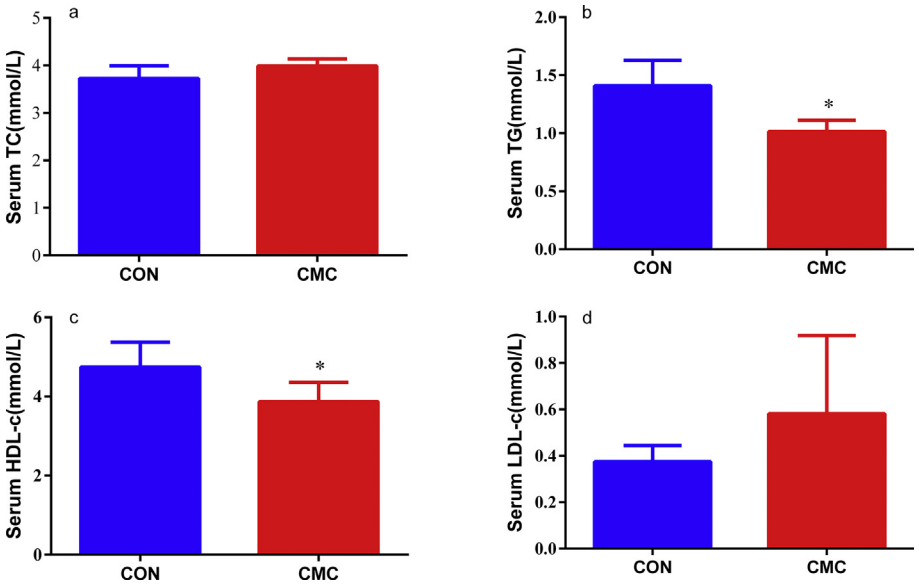

Fig. 1 – Total cholesterol concentration (a), total triglyceride concentration (b), high density lipoprotein cholesterol concentration (c) and low density lipoprotein cholesterol concentration of control (CON) and CMC treated mice (CMC).

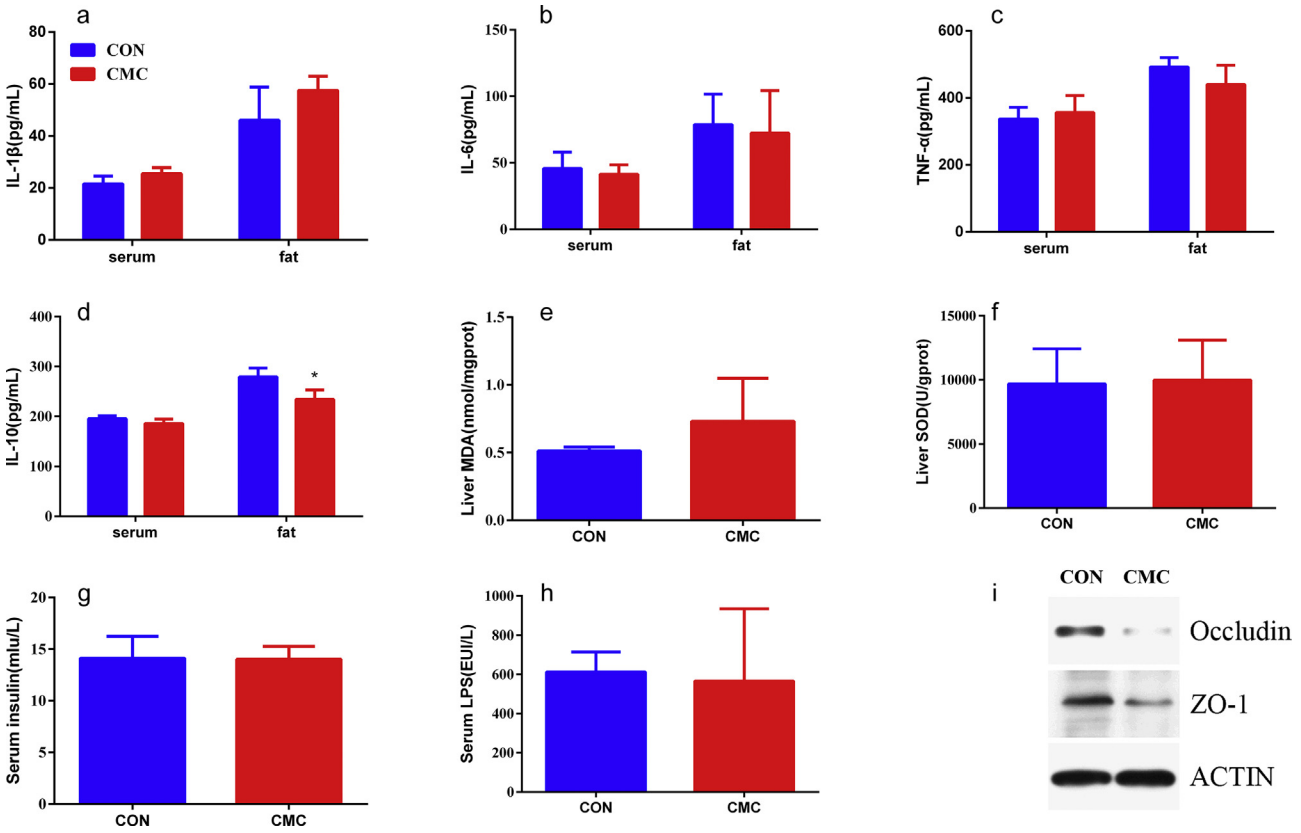

Fig. 2 – Inflammation factors, IL-6 (a), IL-1β (b), TNF-α (c) and IL-10 (d), concentration in serum and fat tissue of control (CON) and CMC treated mice (CMC); Liver MDA (e) and SOD (f); Serum insulin (g) and serum LPS (h); Colon occludin and ZO-1 protein expression level (i).

significant impact on the insulin concentration level (Fig. 2g) and serum LPS concentration (Fig. 2h) in mice.

Moreover, the expression level of occludin and ZO-1 was measured by WB which indicates that CMC treatment could down-regulate the concentration of both occludin and ZO-1 compared with that of control group (Fig. 2i). The decreased tight junction protein level may be caused directly by CMC treatment or indirectly associated with changed colon

microbiota profile as suggested by previous publication [21]. Hence, in the following experiment, the microbiota profile was analyzed.

3.4. *Imbalance on OTUs and microbiota abundance*

Colon microbiota profile was analyzed by sequencing the V4 hypervariable region of the 16S rRNA gene. After quality trimming, assembly and quality filtering, sequences were classified into OTUs at the similarity cutoff of 97%. Then taxonomic assignment, alpha diversity, beta diversity and related analyses were carried out according to previous reports. Table S2 listed the number of clean tags of each colon content which could be used for OTUs assignment.

In the control group, 1057 OTUs were assigned while for the CMC treated group 705 OTUs were delineated (Fig. 3a). Venn results also indicated that between CMC treated group and control group, they share 576 same OTUs. Lower OTUs number suggests decreased microbiota abundance which is similar to the result of high fat diet-induced obesity or intestinal microbiota disorder. In Fig. 3b and Fig. S4, the rank abundance curve presented both the abundance and evenness of colon microbiota. Longer line projected on x-coordinate means more OTUs assigned for the sample. And the degree of the slope indicates the evenness of OTUs distribution in the microbiota and gradual slope means more evenness of distribution. It is evident that most (4 out of 5 mice) control ones has more OTUs as well as uniform distribution which is the characteristic of health colon microbiota profile (Fig. S5).

Taxonomic assignment results were showed in Table S3 and Fig. 3c. It is arranged into phylum, class, order, family, genus and species. It is shown that CMC treated mice possess much less count in phylum, class, order and family compared with control ones. Since the method employed in this research detected very few microbes to the species level, the difference at this level is not significant.

Rarefaction analysis was carried out to determine whether the sequencing per sample is enough to fully reflect the microbiota diversity. Fig. S5 showed that under the sequence deep, most of the OTUs were revealed. Shannon–Winner curve was depicted using Shannon number as the indicator to reveal the diversity (Fig. S6). So, the current analysis condition is enough to reflect the microbiota profile in samples. For alpha diversity analysis, 3 indicators including Shannon, Simpson and Chao1 were used. All the 3 indicators of CMC treated mice were similar or lower than that of control group as showed in Fig. S7. Shannon and Simpson incline to reflect the evenness degree of microbiota with Shannon emphasizes richness and rare OTUs while Simpson emphasizes evenness and dominant OTUs. Chao1 inclines to reflect the richness degree of microbiota with emphasize on rare OTUs. Usually, lower alpha diversity is associated with high fat diet or high cholesterol and disturbed gut microbiota profile. Hence, CMC treatment

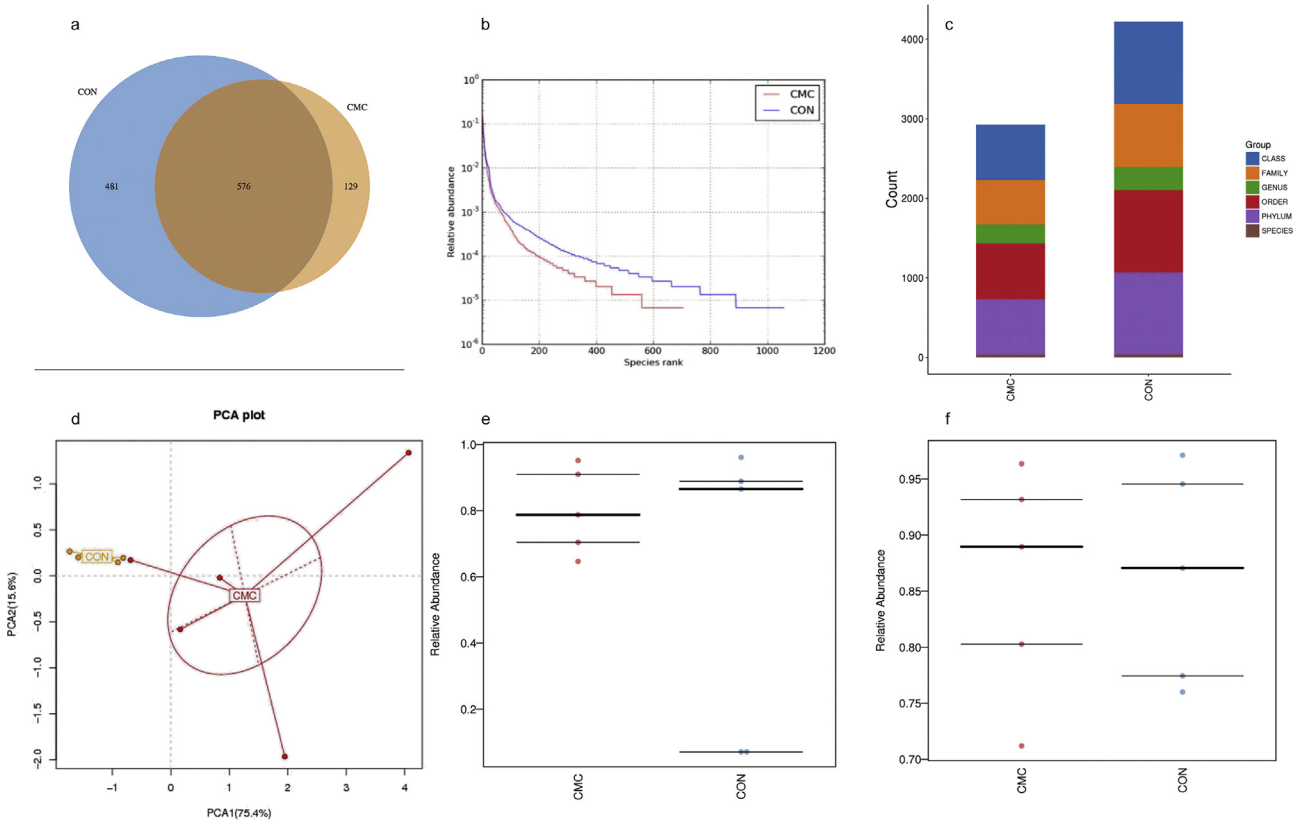

Fig. 3 – Colon microbiota analysis. a, Wayne diagram of OTUs comparison; b, relative species abundance; c, Taxonomic assignment; d, PCA analysis result; e, relative tolerant ability of colon microbiota to oxygen stress; f, relative abundance of Gram-negative bacteria.

induced similar impact on the gut microbiota as decrease the diversity and OTUs number as the same time.

PCA analysis was employed to describe the microbiota difference on the genus level. It is obvious that control group clustered together on the PCA plot while CMC treated ones showed much variance (Fig. 3d). Shorter distance between spots means similarity between different sample. Results indicated that control group share similar microbiota profile, but CMC treated ones were significant different from control group. Moreover, the CMC treated ones also showed significant variance between themselves which is in line with OTUs and other experiment results. PCA analysis suggest that CMC treatment changed the microbiota from normal to disturbed one. CMC treatment also decreased microbiota stress tolerant ability to oxygen as showed in Fig. 3e which indicates decreased microbiota stability. At the same time, CMC treatment increased gram-negative bacteria level (Fig. 3f) and decreased gram-positive bacteria level (Fig. S8).

3.5. *CMC treatment changed microbiota profile and microbiota metabolism functions*

CMC treatment not only decreased OTUs number of colon content compared with control group but also disturbed abundance of several bacterial species (Fig. 4). It is observed that CMC treatment increased *Enterobacteriaceae* (denovo 38, 231, 658, 288 and 657), *Bacteroidaceae* (denovo 40, 66, 89) *Lachnospiraceae* (denovo 93, 121, 542), decreased *Lachnospiraceae* (denovo 95, 155, 193), *Ruminococcaceae* (denovo 159, 162 145) etc.

From OTUs group count, CMC and control mice presented significant different microbiota profile. CMC treatment increased OTUs belongs to *Pseudomonadaceae*, *Lachnospiraceae*, *Citrobacter*, *Clostridiales*, *Oligella*, *Parabacteroides*, *Prevotella* copri, *Clostridiales* while decreased OTUs belongs to *Prevotella* sp, *Bacteroidales*, and *Ruminococcaceae*. It should be noted some microbes were not identified to the species level, so it is quite common that CMC treatment increased and decreased microbe level belong to the same genus or family. For instances, CMC treatment increased the level of *Prevotella* copri but at the same time also decrease certain *Prevotella* sp. So, the effect of CMC is complex and should be interpreted case by case.

As it is widely accepted that microbiota undertake several important metabolism functions in intestinal tract, changed microbiota profile would also impact on the metabolism pathways pertained to microbiota. Functions significantly changed by the CMC treatment was analyzed using KEGG and results were showed in Fig. S9. It is obvious that several genes or enzymes expression level in the colon microbiota of CMC treated mice were upregulated including several nitrogen metabolism related enzymes. Decreased genes including lipoprotein metabolism related enzymes as correlated with the result that serum lipid level of CMC treated mice was altered.

4. Discussion

In the present study, mice were treated with 1% w/v CMC solution for 8 weeks. Body weight of CMC treated mice were higher than that of control mice, but the difference is not significant. It was reported that two common dietary emulsifiers consumption increase body weight and also induce higher serum lipid level and blood glucose level [22]. Mice used in the present study is Kunming mice which is not inclined to develop obesity or metabolic syndrome under normal diet condition. Food intake and water intake showed no difference among the two groups means that CMC has little impact on appetite.

Chitosan could decrease serum cholesterol level, liver triglyceride level, and serum LDL level [1,19]. But as suggested in the previous reports, many factors influence on the biological effects of dietary fiber including molecular weight and viscosity. In one research, authors found that deacetylated konjac glucomannan is not as effective as konjac glucomannan to reduce dietary induced hyperlipidemia and hepatic steatosis [23]. Molecular mass and oligomer structure are important parameters for barley β-glucan to affect microbial composition of hypercholesterolemic rats [24]. CMC is obtained by carboxymethylation of chitosan, changing its molecular structure and biological effects at the same time. It is still not clear how CMC impacts on the serum lipid profile, but one possible way is through influence on microbiota and inflammation. Pro-inflammation cytokines including IL-6, IL-1β and TNF-α showed little difference. But CMC treatment decrease the level of IL-10 in the fat tissue. IL-10 is one important anti-inflammation cytokines. High fat diet induced obesity mice also displayed lower IL-10 level as pointed out in the previous study [25]. The decreased level of IL-10 should be the result of CMC altered inflammation profile which is similar to those of obese mice.

As mentioned by previous publications, CMC open-up the tight junction protein in the intestinal track which induce higher absorption ratio of drugs [12]. Several factors may impact on tight junction opening ability of CMC such as molecular weight and solution viscosity. It is speculated that ionic interaction between CMC and tight junction protein contribute to the configuration change which lead to its opening up. But the exact mechanism is not clear and need further investigation. In our present study, it was found that CMC decrease the expression level of two important junction proteins, occludin and ZO-1. Decreased occludin and ZO-1 is characteristic of obesity as demonstrated by previous study [25]. Hence, it is deduced that lower level of junction protein may promote metabolic disorders of glucose and lipid as showed by our results.

Several chronic diseases like obesity, low-degree inflammation, diabetes etc. are highly related to colon microbiota imbalance. It is important to understand how common carbohydrate polymers like chitosan and its derivatives impact on colon microbiota profile considering their wide scope of applications. It was reported that chito-oligosaccharides with high number of deacetylated units tend to induce a colonic microbiota imbalance demonstrated by decreasing the bacterial population of *Bifidobacterium* spp., *Eubacterium rectale*, *Clostridium coccoides*, *C. Histolyticum* and *Bacteroides/Prevotella* [26]. However, it was suggested that chitosan oligosaccharides with polymerization degrees of 2–6 improve glucose metabolism by reversing the dysbiosis of gut microbiota in diabetic

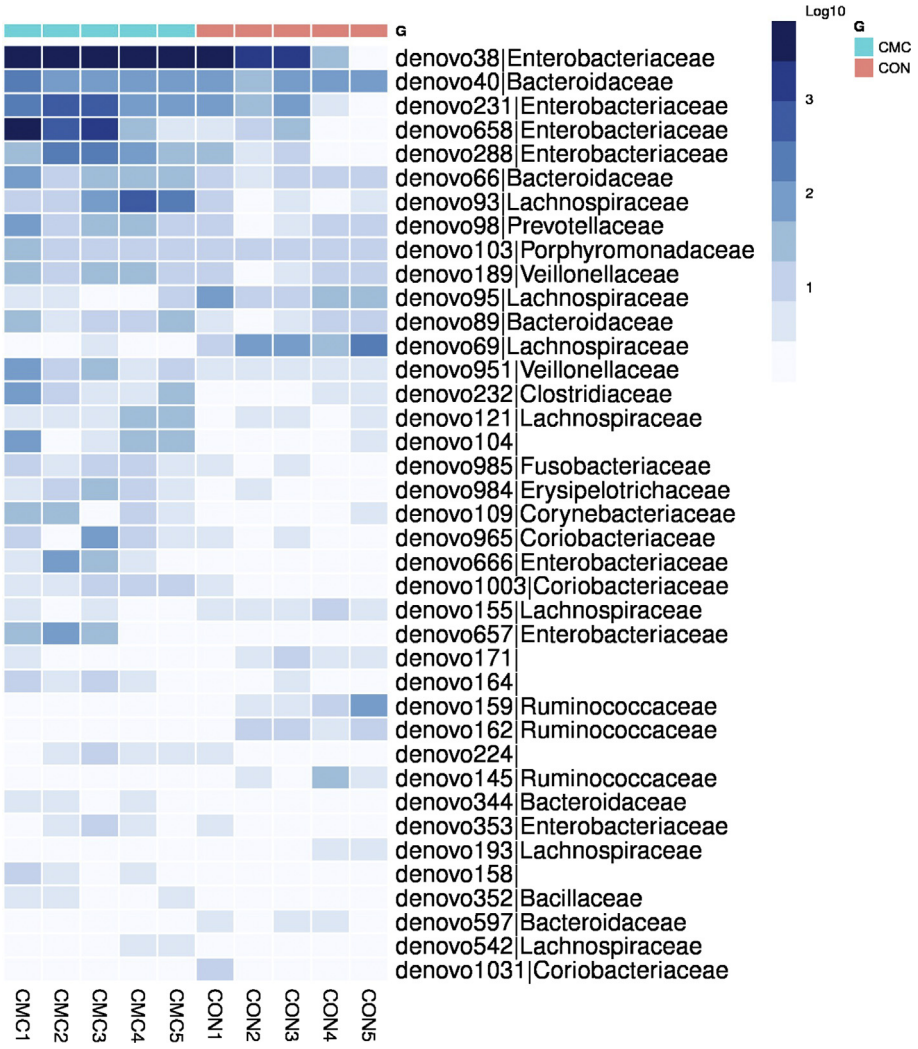

Fig. 4 – Heatmap of colon microbiota species with significant difference of CMC treated mice (CMC) and control (CON).

mice [27]. In our study, CMC treated mice revealed lower OTUs than that of control one, suggesting lower microbial diversity which is also a prominent trait of obesity. PCA analysis distinguish CMC treated mice with control mice, meaning that CMC treatment significantly alter microbiota profile from the normal one.

Recent studies indicated that higher level of *Prevotella* copri decrease other beneficial microbes and has strong correlation with rheumatoid arthritis [28]. Moreover, *Prevotella* copri increase the inflammation reaction, insulin resistance, and stimulate the secretion of many pro-inflammation cytokines [29,30]. *Citrobacter* family belongs to the order of *Enterobacteriaceae*. Several genera of this family associate with intestinal infection. For instance, *Citrobacter* rodentium cause hyperplasia and inflammation in mice, and *Citrobacter* freundii is an opportunistic pathogen which could cause infection to human [31,32]. It has been reported that high cholesterol diet increase *Parabacteroides* sp. which is negative correlated with IL-10 level, and tight junction protein level such as occludin and ZO-1 [21]. It confirmed our observation that the level of IL-10 in the serum of CMC treated mice was significantly lower than that of control ones.

5. Conclusion

In conclusion, CMC is a commonly used biopolymer for food, cosmetic and pharmaceutical applications. For the first time, the impact of its long-term consumption on major health parameters including glucose level, serum lipid level, inflammation and microbiota profile were investigated in this study. It was found that CMC treatment interferes with the serum lipid level, decrease IL-10 concentration in fat tissue. CMC has strong antibacterial ability which may responsible for the disturbed microbiota profile. Tight junction protein level was also reduced by CMC treatment. Our work indicated that CMC functions similar with factors which induce metabolic syndrome or obesity and but the exact mechanism need further studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.07.002.

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