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## Research Article

# Calcium Propionate Alleviates DSS-Induced Colitis and Influences Lipid Profile, Interferon Gamma, Peptidoglycan Recognition Protein 3 and Calprotectin in One-Year-Old Female and Male C57BL/6 Mice -

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## ABSTRACT

Although Dextran Sodium Sulphate (DSS)-induced colitis in a murine model is frequently used to investigate the health benefits of probiotic bacteria and Short-Chain Fatty Acids (SCFAs), there has been little research attempting to verify these effects - in particular those of calcium propionate - in middle-aged mice that are at higher risk because of their reduced immuno-competence and calcium signal generation, which is known to decline with age. Since patients with ulcerative colitis exhibit reduced Ca<sup>2+</sup> signalling and DSS-induced colitis results in colonic smooth muscle hyper contractility with increased Ca<sup>2+</sup> sensitization, we investigated the effects of Calcium Propionate (CP) in middle-aged mice under inflammatory conditions. Sixty female and male C57BL/6 mice were divided into four groups. Control Group 1 received drink water only, Inflamed Group 2 received DSS, positive control Group 3 received DSS plus Sodium Butyrate (SB), and Group 4 received DSS plus CP for 7 days in their drinking water *ad libitum*. Clinical signs of inflammation were monitored daily and histology of colons was examined on day 8. Plasma samples were analysed for key biomarkers of metabolic disorders and liver inflammation. Administration of CP ameliorated DSS-induced colitis/inflammation and attenuated the sum of histologic colitis scores. CP reduced plasma IFN- and calprotectin while enhancing PGlyRP3 in response to DSS treatment. Gender-specific and age-specific differences in immunological and metabolic responses of mice, following inflammation, to probiotics and enteric beneficial microbiome metabolites should be taken into account. In addition to immuno-competence, however, calcium signal generation may represent a novel mechanism by which gut microbiota regulates host metabolism.

**Key words:** Calcium Propionate; Colitis; Dextran; Sodium Sulphate; Ifn- $\gamma$ ; Old Age

## ABBREVIATIONS

CP: Calcium Propionate; DSS: Dextran Sodium Sulphate; ELISA: Enzyme-Linked Immunosorbent Assay; EFSA: European Food Safety Authority; GPCRs: G-Protein-Coupled Receptors Such As GPR41 and GPR43HDACs: Histone Deacetylases; FFAR3: Fatty Acid Receptor 3; IBD: Inflammatory Bowel Disease; IFN- $\gamma$ : Interferon gamma; mTOR: Mammalian Target of Rapamycin; Pglyrp3: Peptidoglycan Recognition Protein 3; PPAR- $\gamma$ : Peroxisome Proliferator-Activated Receptor Gamma; SB: Sodium Butyrate; SCFAs: Short-Chain Fatty Acids; STAT1: Signal Transducers and Activators of Transcription; TLRs: Toll-Like Receptors

## INTRODUCTION

Previous studies have provided molecular evidence for diet and gut microbiota dependency in age-health axis and revealed that alterations in intestinal microbiota composition are associated with several chronic conditions, including obesity and inflammatory diseases [1-7]. Other data suggest that the diversity of gut microbiota changes with age [8] and indicate an overall rearrangement in the aged-type microbiome of the pathways involved in the production of SCFAs [9]. SCFAs, primarily acetate, propionate, and butyrate, which are the major end products of carbohydrate metabolism in e.g. commensal bifidobacteria, have immune and metabolic roles like *in vitro* and *in vivo* anti-inflammatory and fat-burning properties [10,11]. It has been shown that SCFAs, by themselves, affect colonic inflammation and fat deposition and protect against high-fat diet-induced obesity [12-16]. SCFAs exert their biological effects on inflammation and immune/metabolic responses through direct and indirect engagement of different cellular receptors and signalling pathways like GPR41, GPR43, TLRs, PPAR- $\gamma$ , mTOR and two major SCFA signalling mechanisms, namely inhibition of HDACs and activation of G-GPCRs [13,17-21]

Several lines of evidence have indicated that age-related decrease in calcium signal generation plays a profound role in an age-dependent decrease in the proportion and function of T cells that may contribute to poor cell-mediated immune function and age-related immunodeficiency [22-25]. Another increasingly recognized age-related immune disorder is increased inflammatory response as a consequence of multiple defects at the early stages of the T cell signalling pathway, including calcium mobilization, MAPK activity

and NF- $\kappa$ B activation [26]. With respect to the involvement of T cells and Ca signalling in the Dextran Sodium Sulphate (DSS)-induced colitis, it has been reported that (i) T cells are a major contributor to the IL-10 pool in the gut during colitis [27], (ii) the migration of T cells to the colon is impaired during colitis [28], (iii) DSS-induced colitis results in colonic smooth muscle hyper contractility with increased Ca<sup>2+</sup> sensitization [29], (iv) dietary calcium decreases disease activity in mice with DSS-induced colitis [30], and suppresses adipose tissue oxidative and inflammatory stress in mice and humans [31] and (v) dietary compounds-induced activation of the extracellular calcium-sensing receptor, which is distributed throughout the gastrointestinal tract, reduce colonic inflammation and promote intestinal homeostasis in DSS-induced colitis [32].

As metabolic and immune-related pathways intersect at numerous levels [33], metabolism and body composition, followed by immune system, begin to change early in middle-age. For example, previous studies have shown that middle-aged adults often show visible signs of aging such as a 5-10 kg accumulation of body fat [34] and that fat mass peaks at middle age or early old age and then declines substantially in advanced old age [35]. This general acceleration in body fat accumulation, particularly middle age specific fat, is attributed mostly to changes in energy expenditure levels which are reduced physiologically with increasing age. However, under normal physiological conditions, the body metabolic system is able to manage extent and duration of availability of excess calories. But local or systemic inflammation negatively affects energy metabolic processes [2,7,16,26,32,36-41]. The functional consequence of an age-dependent up-regulation of the inflammatory response and weight gain is that inflammation increases body fat accumulation, and body fat accumulation further promotes inflammation. The result is a vicious cycle of metabolic lethargy and increased insulin resistance followed by weight gain and increased inflammation. Hence, when an individual starts to gain weight, it may become difficult to relieve the body from this constant inflammatory pathway [32,36,42,43]. Collectively and based on these data, besides age-related physiological low secretion of body metabolism-regulating hormones and changes in energy expenditure, low level of enteric beneficial microbiome metabolites, as consequences of intestinal dysbiosis, may contribute to body and abdominal fat accumulation, a feature of middle and advance adult age implicated in obesity and diabetes.



Several previous studies have shown effects of DSS on colitis-associated markers such as reduced body weight, colon shortening and epithelial damage in young 6-8-week-old mice [6,18,44].

Since the biological activities of calcium propionate have been studied and feeding Calcium Propionate (CP) alone to mice for 10 days at levels up to 300 mg/kg body/daily resulted in no significant effects on general health questionnaire [45], we in the frame of this pilot study particularly emphasized the investigation of effects of CP on global biochemical markers under inflammation conditions in middle (11-13 month) aged mice that are at higher risk, because of their decreased immuno-competence and calcium signal generation, which is known to decline with age [22,26,46-49].

## MATERIAL AND METHODS

Conventional C57BL/6 mice were obtained from Harlan (Rosscorf, Germany). The mice were bred and kept under conventional pathogen-free conditions in the same room in our facility to minimize the influence of differences in the environment. All animal experiments were approved by the Ministry of Environment, Nature Protection, and Agriculture (Kiel, Germany) and Use Committee. Sixty approximately one year-old ( $326.75 \pm 56.86$ - day-old, Mean  $\pm$  S.D) C57BL/6 mice (31 males and 29 females) were randomly selected and divided into four groups, each of these including 15 animals. Mice were single-housed in 12 cm x 30.5 cm x 12.5 cm polycarbonate cages in a 12:12 light cycle room. In order to minimize the impact of social isolation, the individually held mice were held so that they could communicate olfactorily and acoustically with each other in neighbouring cages. Each cage had enough bedding to cover the floor and bedding square. Food was available *ad libitum* throughout the experiment and the average daily consumption of feed and water was recorded.

Control Group 1 received drink water only. As colitic (inflamed) group, Group 2 received DSS (5% w/v from ROTH, Karlsruhe, Germany with MW 40 kDa), Group 3 received DSS (5% w/v) plus SB (3.85% w/v from Santa Cruz Biotechnology, Inc., Heidelberg, Germany, with MW 110.1) and Group 4 received DSS (5% w/v) plus CP (3.85% w/v from Merck, Darmstadt, Germany, with MW 186.22 g/mol) for 7 days in their drinking water *ad libitum*. SB was included as positive control, because it is known that SB attenuated DSS-induced colitis [44] and alleviated inflammation by IFN- $\gamma$ /STAT signalling in *in vivo* mouse model [50]. The molecular formula for CP is Ca (C<sub>2</sub>H<sub>5</sub>COO)<sub>2</sub>, which is the calcium salt of propionic acid. In accordance with Annex II of Regulation (EC) No 1333/2008 [51], it is listed as an authorized food additive (E282) in the Codex Alimentarius.

Throughout the experiment, the clinical signs of colitis and inflammation, including body weight, visible rectal bleeding, faeces consistency, faecal occult blood tests (hemoCARE, CARE diagnostica, Voerde, Germany), and mortality were monitored daily, whereas length and histology of the colons were examined on day 8. At the end of the experiments on the day 8, the physiological and pathological characteristics (bleeding from the anus or in faeces) of the mice were checked before anaesthesia. Fresh faecal samples were collected sterile and kept frozen at -80°C until analysis. Faeces samples (100 mg) were homogenised in extraction buffer (weight: volume 1:50) and faecal calprotectin levels were measured using an ELISA Kit (Immunodiagnostic AG, Bensheim, Germany) as described in the protocol of the kit. Then, all mice were anesthetized with an

intraperitoneal injection of Ketanest/ Rompun solution (8-10 $\mu$ l/g body weight) and subjected to intracardiac puncture. At the time of blood sampling the chest wall was cleansed with Baktolin<sup>®</sup> pure in spirit, and sterile syringes were used. Blood samples were collected into heparinized (20 unit/ml blood) sterile tubes and immediately transferred onto ice. Following centrifugation at 1000 x g (at 4°C) for 10 minutes, plasma was removed from the blood samples and stored in aliquots of 100  $\mu$ l at -80°C until analysis. Plasma concentrations of glucose, total cholesterol, triglycerides, Low-Density Lipoprotein Cholesterol (LDL-C), High-Density Lipoprotein-Cholesterol (HDL-C), Glutamat-Oxalat-Transaminase (GOT = AST also called Aspartat- Aminotransferase) and Glutamat-Pyruvat-Transaminase (GPT = ALT, also called Alanine aminotransferase) were measured enzymatically with an automatic analyser (Konelab<sup>™</sup> 20 Clinical Chemistry Analyzer, Thermo Fisher Scientific Inc., Waltham, MA, USA). Also, plasma IFN- $\gamma$  and Pglyrp3 levels were assessed using ELISA Kits (BD Biosciences, Heidelberg and Holzelt Diagnostika GmbH, Köln, Germany), respectively, as described in the protocols of the kits. Then, a midline laparotomy was performed to all animals immediately following blood sampling. Total lobes of the livers were excised immediately and weighed and liver to body weight ratios were determined. The abdominal cavities were observed for abdominal fat contents as well as visible liver and intestine inflammations. For histological assessment, whole colons were removed, measured in length (mm) from the rectum to the cecum and opened longitudinally. The colons were then Swiss-rolled and fixed in 10% buffered formalin for 24 h (at 4°C) and then in 1% buffered formalin at 4°C until paraffin embedding processes. Tissues were embedded into paraffin blocks and paraffin sections with five - micron ( $\mu$ m) thicknesses were prepared, placed on polylysine-coated slides, stained with Hematoxylin and Eosin (H&E). H&E-stained colonic sections were coded for blind microscopic assessment of inflammation and scored by two experienced pathologists. Histopathological indices, including the loss of epithelium, immune cell infiltration, loss of crypts and lesion of the entire mucosa, were used to grade the severity of colon inflammation. Severity of inflammation was scored as follows: 0, none; 1, low; 2, moderate and 3, clearly as described previously [44]. These indices were summated to generate the sum histologic colitis scores. Images were acquired using a Zeiss Axioskop 2 plus microscope (Carl Zeiss MicroImaging) equipped with a Polaroid DMC Ie camera (Polaroid).

## STATISTICAL ANALYSES

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the post-hoc tests and Multiple Comparison of histological results with Bonferroni post-test. Results are expressed as means  $\pm$  SD and are significant at a *P* value of less than 0.05.

## RESULTS

### CP and SB alleviate clinical signs of DSS-induced colitis

As expected, the DSS group showed marked pathophysiological symptoms of inflammation as evidenced by visible rectal bleeding, more loose faeces and positive faecal occult blood tests compared to control group (Table 1). In 53 cases, among 15 animals and during 7 days, the DSS group (2) showed positive faecal occult blood tests. In contrast, the DSS plus SB group (3) showed 9 cases positive faecal occult blood tests and the DSS plus CP group (4) showed only 5 cases positive faecal occult blood tests (Table 1).



**Table 1:** Physiological and pathophysiological characteristics of DSS and SCFAs - treated middle aged mice.

Group Nr.	Treatments	Mortality/n	Body weight (g)		Stool consistency	Number of observed positive cases of faecal occult blood tests (among 15 mice per group, during 7 days)	Colon length (cm)
			Day 0	Day 8			
1	Control	0/15	34.1 ± 5.7	35.8 ± 6.9	Normal	2	10.1 ± 1.7
2	DSS	0/15	33.5 ± 6.8	27.5 ± 4.7 * †	Loose stool	53*	7,1 ± 1.5*
3	DSS + SB	0/15	33.6 ± 4.3	31.5 ± 4.4 * † §	Normal	9 * §	8.9 ± 1.2§
4	DSS + CP	0/15	32.0 ± 5.1	32.4 ± 6.4	Normal	5 * §	9.5 ± 0.7§

\*  $p < 0.05$ , significant difference versus control

§  $p < 0.01$ , significant difference of group 3 and 4 versus group 2

†  $p < 0.05$ , significant difference of day 0 versus day 8

For faecal occult blood testing, fecal samples were obtained from all mice on each day of the study by taking individual mice out of their cage. The colon length was measured from the rectum to the cecum on day 8.

As shown in Table 1, no significant body weight differences were found between the four groups on day 0. The mice were weighed five times during the experimental period. In the colitic group (DSS alone), body weights were increased during the first four days and begin to decrease gradually from day 5 onward, so that their cumulative weight loss remained significantly through the 7 days of treatment compared to control group.

The body weights at day 8 of the DSS plus SB (3) and the DSS plus CP (4) groups were significantly higher than those of DSS group 2 ( $p < 0.05$ ). However, on day 8, no significant body weight differences were observed between the DSS plus SB (3) and the DSS plus CP (4) groups.

Liver weight and liver weight to body weight ratio were determined to assess the general health of the animals [52]. Consistent with the body weight data, the liver weights (Figure 1 A) and the liver weight to body weight ratios (Figure 1 B) were significantly lower for mice receiving DSS plus SB or DSS plus CP than for the DSS group (inflamed group). Interestingly, the DSS-treated group (2) appeared to have more abdominal fat than the DSS plus SB (3) and DSS plus CP (4) groups.

For each mouse, average daily food and water intakes were calculated. The mean food intake was  $2.81 \pm 0.33$  g for control mice whereas the food intakes in the inflamed group (DSS alone), DSS plus SB and DSS plus CP groups were lower, at  $2.41 \pm 0.56$  g,  $2.09 \pm 0.39$  g and  $2.11 \pm 0.44$  g. There was no significant difference in the volume of water consumed by each group of mice (data not shown).

The colonic mucosal injury and inflammation was further evaluated based on total colon length. The gross appearance of the organs from the DSS-treated mice showed obvious reddening and shortening of the colon, which are typical signs of intestinal inflammation. Consistent with the exacerbated clinical signs, DSS exposure alone caused significantly decreased colon lengths of mice killed on day 8. On the other hand, both SB and CP, concurrent with alleviations in pathophysiological indices of intestinal inflammation, significantly suppressed DSS-induced shortening of colon length, a marker for colitis. However, in this respect CP was more effective than SB (Table 1 and Figure 2).

**Evaluation key biomarkers of metabolic disorders and liver inflammatory markers**

As shown in Figure 3A-C, SB and CP reduced significantly markers of metabolic disorders in response to DSS. As shown in Figure 3A, mice treated with DSS plus SB and DSS plus CP showed

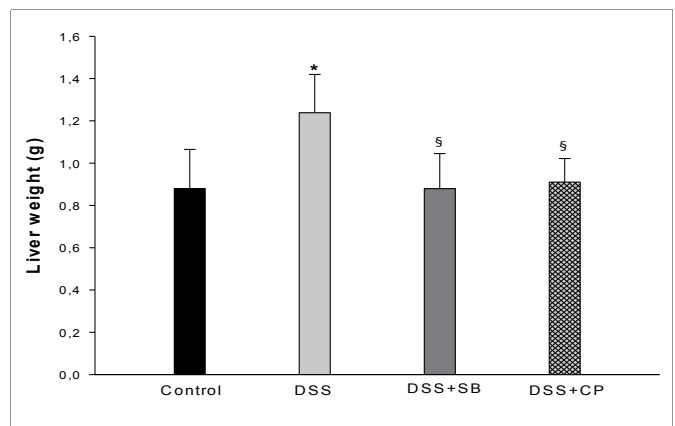


Figure 1A: Ghadimi et al.

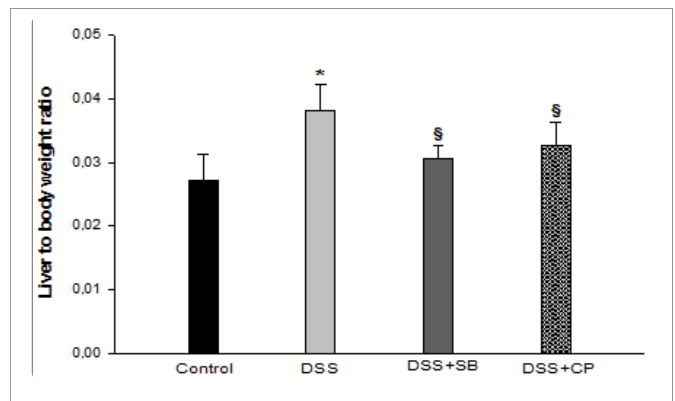


Figure 1B: Effect of SB and CP on the liver and liver to body weights ratio of middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control), or treated with DSS alone, DSS plus SB or CP for 7 days. On day 8, after euthanizing, total lobe of the livers were excised immediately and weighed (A) and liver to body weight ratios were determined (B). The results are expressed as mean values ± SD (n = 15 in each group). \*  $p < 0.05$  vs. control, §  $p < 0.05$  vs. DSS alone, namely colitic (inflamed) group.

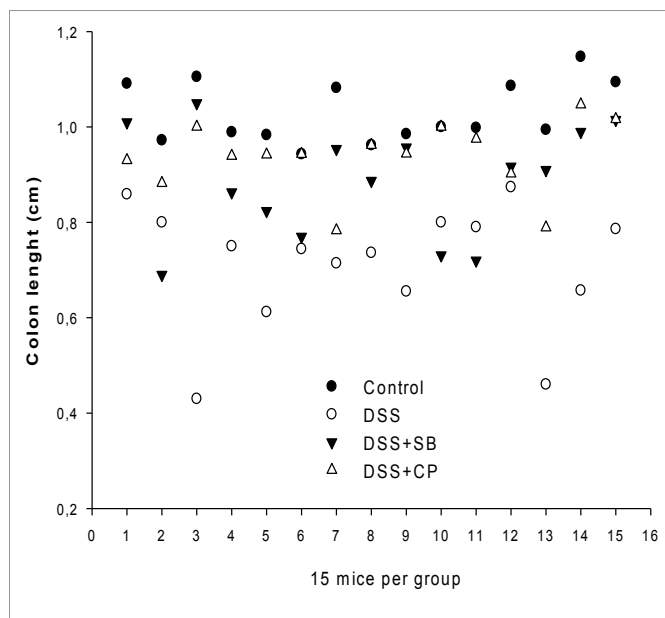
significantly lower plasma levels of glucose, triglycerides, cholesterol and LDL, compared with mice that received DSS alone ( $p < 0.05$ ). However, reduction in cholesterol did not reach statistical significance in the case of CP. Moreover, mice treated with DSS plus CP showed significantly higher plasma levels of HDL (Figure 3 B). This increase is reflected in a reduced ratio of total cholesterol to HDL and ratio of LDL to HDL (Figure 3 C).



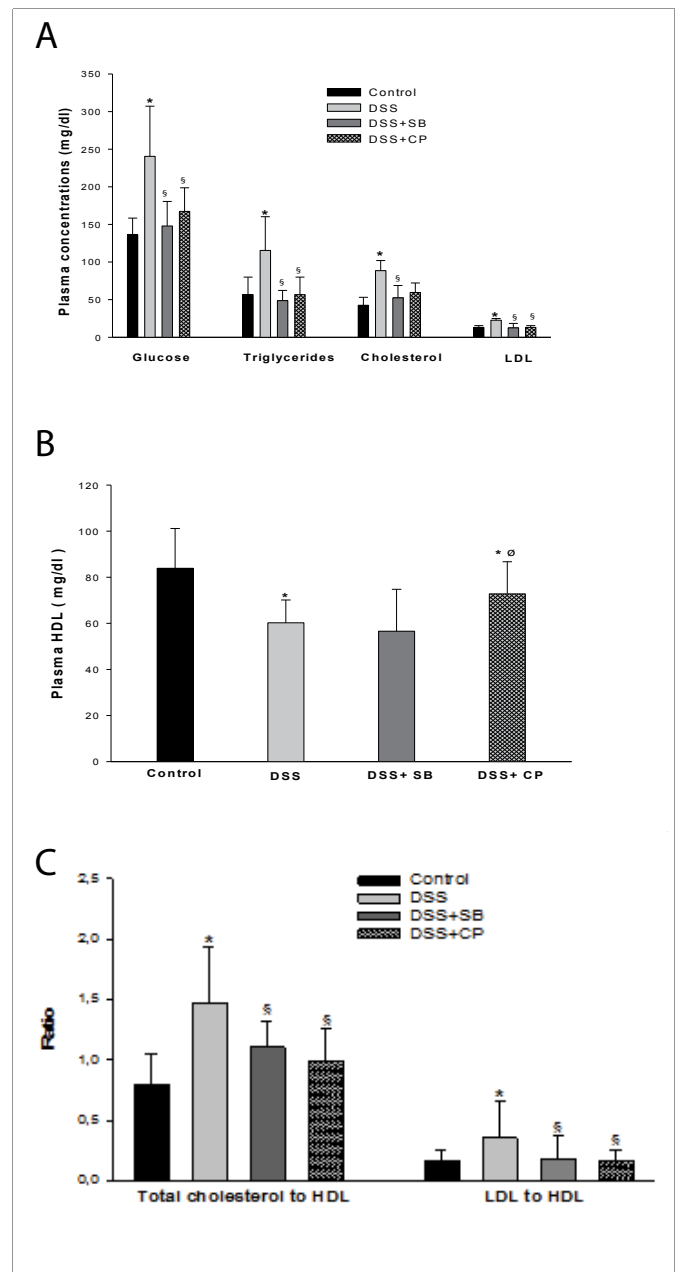
Furthermore, to assess the degree of liver inflammation or damage, we examined blood plasma GOT and GPT levels, which are regarded as indices for liver function. In inflammatory conditions affecting the liver, ALT (GPT) is characteristically as high as or higher than AST (GOT), and the GOT/GPT ratio, which normally is < 1, becomes bigger than unity [53]. Normal C57BL/6 mice have a plasma GOT ranging from 77.2 U/l ± 6 SD and GPT 21U/l ± 2.6 SD [54]. As shown in table 3, mice treated with DSS alone had significantly lower abnormal plasma ALT levels (47.92 U/l ± 13.53 SD) and significantly higher abnormal AST levels (100.73 U/l ± 13.41 SD). Abnormal values of these two enzymes are reflected in increased AST/ALT ratio > 2. Conversely, mice given DSS together with CP had significantly lower AST/ALT ratio (< 1 = 0.99) than mice given DSS alone, indicating that CP counteracts the inflammatory activity of DSS and recovers the DSS-impaired AST/ALT ratio.

### Histological evaluation of colonic inflammation

Five-micron colonic sections were H&E stained, coded for blind microscopic assessment of inflammation and evaluated in a blinded fashion by two clinical experienced pathologists for loss of epithelium, immune cell infiltration, loss of crypts and lesion of the entire mucosa (Figure 4). Severity of inflammation was scored as follows: 0, none; 1, low; 2, moderate and 3, clearly. These indices were summarized to generate the histologic colitis scores. H&E-stained day 8 colon sections of DSS-treated mice showed severe lesions throughout the mucosa, alteration of epithelial structure, high-level neutrophil and lymphocyte infiltration into the mucosal and submucosal areas, and loss of crypts (Figure 4C, D). In H&E-stained sections, morphological changes of the colon were lower in DSS plus SB group (Figure 4E, F) compared with DSS group. Interestingly, histological damages of the colon were not observed in CP group which improved completely the histopathological indices of colonic inflammation compared with colitic group (Figure 4G, H). All alterations observed were combined



**Figure 2:** A scatter diagram graph showing influence of SB and CP on the colonic length of middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control), or treated with DSS alone, DSS plus SB or CP for 7 days. The colon length was measured from the rectum to the cecum on day 8. The results are expressed as mean values (n = 15 in each group) connected to table 1.



**Figure 3:** Effects of SB and CP on biomarkers of metabolic disorders and liver inflammation markers in middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control), or treated with DSS alone (inflamed group), DSS plus SB or DSS plus CP in drinking water for 7 days (n = 15 per group). On day 8, the mice were euthanized and blood plasma samples were analyzed for biomarkers of metabolic disorders including glucose, triglycerides, cholesterol and LDL (A), HDL (B), ratio of total cholesterol to HDL and ratio of LDL to HDL(C). The results are expressed as mean values ± SD (n= 15 in each group). \* p< 0.05 vs. control, § p < 0.05, significant difference of group 3 (DSS plus SB) and 4 (DSS plus CP) versus group 2 (DSS alone).

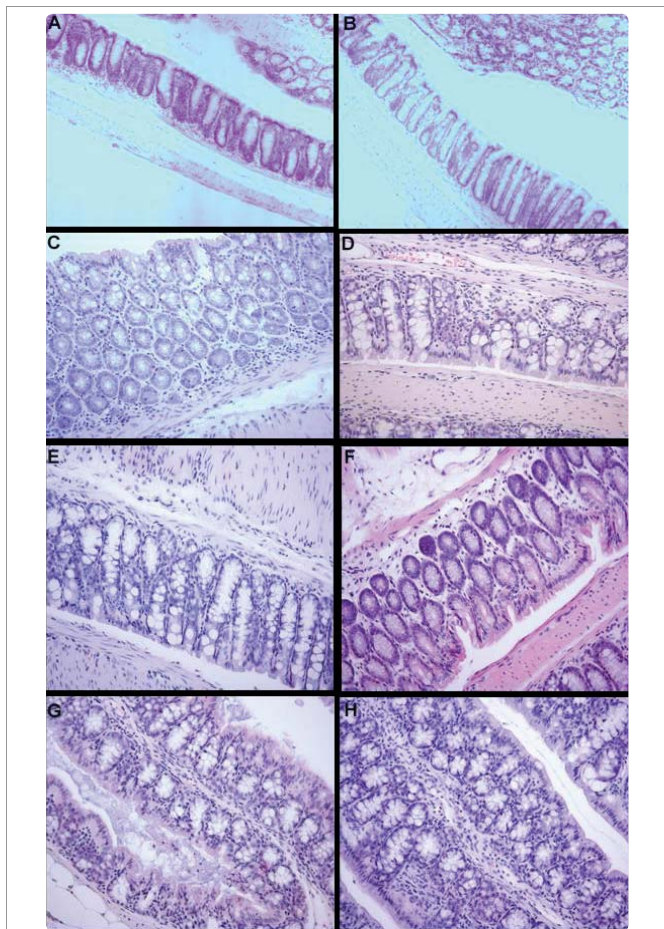
to obtain histological scores (Table 2). The histological damage score in DSS plus SB and DSS plus CP groups was significantly decreased as compared with the colitic group (DSS alone); however, the score was significantly lower in DSS plus CP group than in DSS plus SB group. The histologic colitis scores calculated after DSS plus SB and DSS plus CP treatments were significantly lower than that of colitic group mice (DSS alone); 41 versus 15 and 0, respectively. Also, the difference between DSS plus SB and DSS CP groups was significant (Table 2).



**Assessment of specific markers (IFN- $\gamma$ , calprotectin, PGLYRP3)**

As shown in figure 5, IFN- $\gamma$  was drastically induced in DSS alone-treated mice. In contrast, the plasma level of IFN- $\gamma$  was significantly lower in mice given DSS plus SB and, particularly, DSS plus CP. When measuring plasma levels of Pglyrp3, mice treated with DSS plus SB or CP generated significantly higher levels of Pglyrp3 than the colitic group of mice treated with DSS alone ( $p < 0.05$ ) (Figure 6).

Next we measured fecal calprotectin concentrations because, on one hand, calprotectin is a calcium-binding protein, which is expressed in neutrophils, monocytes and early stage macrophages and its degranulation inside the intestinal lumen occurs as a response to local inflammation, and correlates positively and strongly with fecal



**Figure 4:** Effects of SB and CP on histological features of DSS-induced colitis in middle aged C57BL/6 mice. Mice were given drink water alone (control) or treated with DSS alone (inflamed group), DSS plus SB, or DSS plus CP in drinking water for 7 days ( $n = 15$  per group). On day 8, the mice were euthanized and their entire colons were removed, longitudinally opened, Swiss-rolled, and embedded in paraffin. Histopathological indices, including the loss of epithelium, immune cell infiltration, loss of crypts and lesion of the entire mucosa, were used to grade the severity of colon inflammation. Representative photomicrographs (two individual mice from each group) are of the middle colon of control mice (A and B), inflamed mice treated with DSS alone (C and D), of mice treated with DSS plus SB (E and F) and of mice treated with DSS plus CP (G and H). Typical representative section of destroyed colon from DSS alone-treated mice showing loss of crypts and epithelial integrity as well as severe inflammatory cell infiltration. Representative sections from the colons of DSS plus SB and DSS plus CP treated mice showing the deep, even crypts observed in a section of normal colonic mucosa. Photomicrographs of H & E-stained sections, x 20.

**Table 2:** Histological grading of colitis. Histopathological indices, including the loss of epithelium, immune cell infiltration, loss of crypts and lesion of the entire mucosa, were used to grade the severity of colon inflammation. Severity of inflammation was scored as follows: 0, none; 1, low; 2, moderate and 3, clearly. These indices were summed to generate the sum histologic colitis scores.

Group Nr.	Treatments (n = 15 in each group)	Histological characterization of colon				The sum histologic colitis scores
		15 cases of score 0	15 cases of score 0	15 cases of score 0	15 cases of score 0	
1	Control	15 cases of score 0	15 cases of score 0	15 cases of score 0	15 cases of score 0	0
2	DSS	9 cases of score 0 1 case of score 1 4 cases of score 2	1 cases of score 0 7 cases of score 1 7 cases of score 2	8 cases of score 0 6 cases of score 1 1 case of score 2	11 cases of score 0 3 cases of score 1 1 case of score 2	41*
3	DSS + SB	14 cases of score 0 1 case of score 1	4 cases of score 0 9 cases of score 1 1 cases of score 2	14 cases of score 0 1 case of score 2	14 cases of score 0 1 case of score 1	15* §
4	DSS + CP	15 cases of score 0	15 cases of score 0	15 cases of score 0	15 cases of score 0	0§

Description of Scores: 0 = None, 1 = Low, 2= Moderate and 3 = Clearly; \* Significant versus Control ( $p < 0.05$ ); § Significant difference of group 3 and 4 versus group 2 ( $p < 0.05$ ).

**Table 3:** Effects of SB and CP on liver enzymes of mice treated with DSS.

Groups	GOT(AST) (IU/L)	GPT(ALT) (IU/L)	AST: ALT
Control	116.42 ± 11.19	119,45 ± 12,53	0.97 ± 0.89
DSS	100.73 ± 13.41	47.92 ± 13.53*	2-10 ± 0.99*
DSS + SB	104.00 ± 18.97	52.48 ± 17.72*	1.98 ± 1.07*
DSS + CP	110.36 ± 16.43	110.45 ± 12.53 §	0.99 ± 1.31§

Results are expressed as mean values of 15 mice per group ( $\pm$  SD;  $n = 15$ ). \* Significant versus Control ( $p < 0.05$ ), § Significant versus inflamed group 2 (DSS alone) ( $p < 0.05$ ).

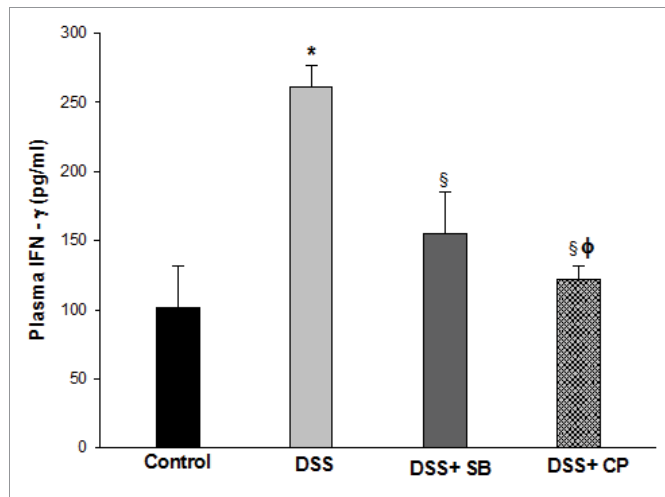
occult blood and, on the other hand, age-related decrease in calcium signal generation plays a profound role in age-dependent decrease in the proportion and function of T cells [24,55]. Consistent with the IFN- $\gamma$  data, fecal calprotectin was produced abundantly in mice treated with DSS alone. This augmented fecal calprotectin production was abrogated significantly when mice were administrated with DSS plus SB or DSS plus CP (Figure 7).

**DISCUSSION**

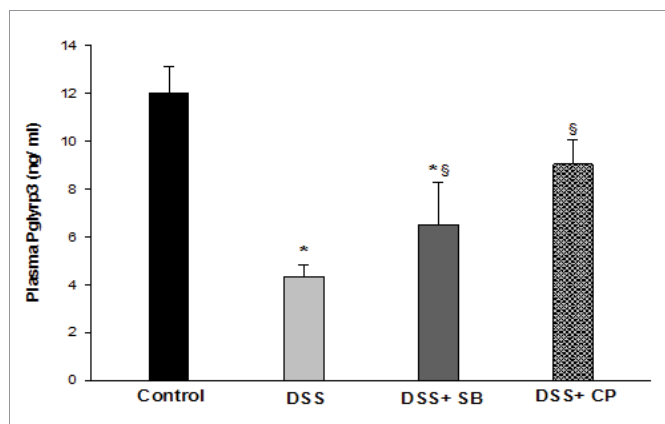
The DSS-induced colitis murine model is commonly used to test the potential beneficial health effects of probiotic bacteria, their products and soluble factors or SCFAs like SB [20,44,56-59], but the majority of these studies have included a single gender and used younger 6-8 weeks old mice, which do not accurately represent the age of typical age-related metabolic and immunity disorders [22,26,46-49]. It has been shown that the combination of obesity and chronic ulcerative colitis (DSS- induced severe inflammation) reciprocally exacerbates adipose tissue and colon inflammation [60] and patients



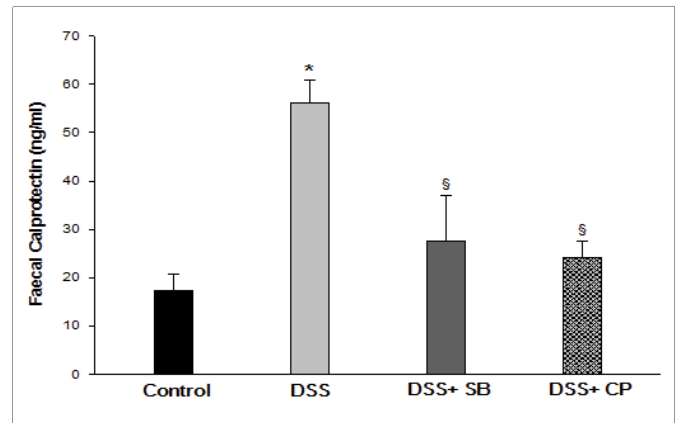
with ulcerative colitis exhibit reduced Ca<sup>2+</sup> signaling [61]. However, it is unknown whether gender influences clinical presentations and markers in DSS-induced inflammatory intestinal disease. Hence, in the present study, we sought to investigate the effects of CP in middle-aged male and female mice under inflammatory conditions. We show here that SB and particularly CP alleviate DSS-induced colitis in middle-aged mice. Liver weight and visceral fat content have been seldom reported in DSS treated young mice [6,18,44]. The interesting result, however, is the difference between liver weight and abdominal fat content in our DSS treated middle-aged mice. Although during treatment with DSS alone inflammation was enhanced, as shown by rises in the extent of diarrhoea and rectal bleeding, and DSS alone reduced body weight in the DSS group (2), but there was significant difference on liver weight and liver weight to body weight ratio between groups in the end of study. DSS would, therefore, seem also to disrupt metabolic processes and the prime cause of body weight loss is probably from dehydration in DSS alone-treated middle aged mice.



**Figure 5:** Influence of SB and CP on the plasma IFN-γ levels in middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control) or treated with DSS alone, DSS plus SB or CP for 7 days. The results are expressed as mean values ± SD (n = 15 in each group). \* p < 0.05 vs. control, § p < 0.05 vs. DSS alone, φ p < 0.05 vs. DSS plus SB.



**Figure 6:** Influence of SB and CP on the plasma Pglyrp3 levels in middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control) or treated with DSS alone, DSS plus SB or CP for 7 days. The results are expressed as mean values ± SD (n = 15 in each group). \* p < 0.05 vs. control, § p < 0.05 vs. DSS alone.



**Figure 7:** Influence of SB and CP on the faecal calprotectin levels in middle aged C57BL/6 mice treated with DSS. Mice were given drink water alone (control) or treated with DSS alone, DSS plus SB or CP for 7 days. Faeces (100 mg), collected on day 8, were homogenised in extraction buffer (weight: volume 1:50) and faecal calprotectin levels were measured using an ELISA Kit (Immunodiagnostic AG, Germany) as described in the protocol of the kit. The results are expressed as mean values ± SD (n = 15 in each group). \* p < 0.05 vs. control, § p < 0.05 vs. DSS alone.

Interestingly, the mice in the DSS plus CP group showed lower abdominal fat compared to mice treated with DSS plus SB or with DSS alone. This effect may be due to increased blood calcium concentration, which increases faecal fat and energy excretion or more dehydration, which is typical of this model of colitis [62]. However, the reduced abdominal fat seen in the DSS plus CP group may be due to recovery of inflammation-induced disruption of metabolism, since dietary compounds-induced activation of the extracellular calcium-sensing receptor, which is distributed throughout the gastrointestinal tract, reduces colonic inflammation and promotes intestinal homeostasis in mouse model of DSS-induced colitis [63]. Moreover, these effects may result from improved calcium signals in T lymphocytes, because age-related declining in calcium signal generation contributes to functional immunodeficiency of old age in mice [48].

As complication of systemic extension of colitis, we also evaluated liver inflammation, affecting the metabolism of the body, based on key metabolic biomarkers including blood plasma levels of glucose, triglyceride, cholesterol, HDL and LDL as well as GOT and GPT liver enzymes that rise especially early in the disease. Of note, besides affecting gut inflammation, SB and, particularly, CP recovered the impaired liver function and body metabolism in middle aged mice treated with DSS.

Although DSS colitis is associated with severe inflammation, which is not comparable to the one occurring in obesity, our findings are, indeed, in line with previous studies showing the relationship between DSS-induced colitis and obesity as aggravating factors for each disease, with increased inflammation in the colon and adipose tissue and systemic alterations observed in the spleen, lymph nodes and bloodstream [60]. However, we confirmed, for SB and CP, previous studies showing that principle SCFAs protect against diet-induced obesity, by reducing body fat percentage [6,10,64-66]. Lastly, there are evidently differences in production of various hormones between genders in middle-age. For example, we observed that female mice treated with DSS alone had longer colon, lower decrease in body weight, and lower liver weight compared to males. Overall, our analysis reveals that female mice show less signs of inflammation and develop DSS-induced colitis. This protection seems to be mediated



by estradiol [57]. However, the use of a single gender may be more appropriate.

Because PglyRP3 protects mice from experimental colitis by preventing induction of IFN- $\gamma$  [19,21] and to further molecularly confirm the exacerbated clinical signs, we measured plasma levels of PglyRP3 and IFN- $\gamma$ . At the molecular levels, the inflammation-alleviating effects of SB and CP were reflected in blood and faeces. In blood as well as faeces, DSS plus SB and DSS plus CP abolished significantly the DSS alone-induced plasma levels of IFN- and faecal calprotectin levels as hallmarks of inflammation [19]. In blood, however, treatment with DSS plus SB or DSS CP resulted in significantly higher concentrations of PglyRP3 as compared with DSS alone. This result confirms a previous study, suggesting intestinal mucosal homeostasis and protection roles of Pglyrp3 against colitis [67] and that Pglyrp3 may mediate the benefits of enteric microbiome metabolites. Together, these data indicate that calcium propionate protects against DSS-induced colitis and related disruption of body metabolism in middle aged mice. Its exact mechanism is unclear, but two possibilities seem reasonable: (1) calcium, as a signal molecule, in combination with propionate, as SCFA produced naturally in intestine by bifidobacteria, may regulate body metabolism via anti-inflammatory effect and influence of calcium signalling. Calcium signalling and calcium-sensing receptor activation by high Ca levels by itself protects against DSS-induced colitis in mice via modulating mucosal apoptosis of the distal large intestine and protecting against constitutive epithelial cell damage [30,68]; and (2) luminal propionate itself, as a resident gut commensal bifidobacteria growth promoting nutrient, attenuates DSS-associated disruption of tight junction proteins and the intestinal barrier. The DSS model of acute inflammation is based on disruption of tight junction proteins and the intestinal barrier, resulting in translocation and dissemination of luminal bacteria and activation of local and systemic innate and adaptive proinflammatory immune responses [59,68]. Whether these observations can be extended to patients with colitis deserves consideration.

Regarding biological activity of calcium propionate and its target cells, both immune cells and epithelial cells seem to be involved. In case of inflammation, immune cells interact with endothelial cells, they are captured, they roll, are activated and arrested, which is followed by strengthening adhesion between immune cells and endothelial cells, and the immune cells finally migrate through the endothelial layer in a paracellular or transcellular manner and can thus be enriched in inflamed tissues [69]. Plasma/ fecal concentrations of PglyRP3 (from epithelial cells), IFN- $\gamma$  (from both activated T lymphocytes and epithelial cells) and calprotectin (from neutrophils) are original indications for this process.

Recently, Tong et al. 2016 [70] have shown that sodium propionate ameliorates DSS-induced colitis by improving intestinal permeability and reducing inflammation in young mice. However, we included Na-Butyrate as positive control and there are several reasons why we used Ca-propionate instead of Na-propionate and didn't include a group Na-propionate: (i) Ca-propionate, as an antifungal authorized food additive, is directly added to foods (especially for bread and other baked products); (ii) In contrast to Ca-propionate, Na-propionate is a chemical compound that is not directly added to foods; rather it is used as a preservative for silage and grain storage, as highly digestible energy and source of sodium for high-yielding dairy cows and as chloride-free sodium source in laying hens; and (iii) patients with ulcerative colitis exhibit reduced Ca<sup>2+</sup> signalling [61].

Collectively, our results suggest that gender-specific and age-specific differences in immunological and metabolic responses of mice, following inflammation, to probiotics and enteric beneficial microbiome metabolites should be taken into account. In addition to immunocompetence, however, calcium signal generation may represent a novel mechanism by which gut microbiota regulates host metabolism.

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