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The ratio of xylooligosaccharide to ferulic acid affects faecal ferulic acid content, short chain fatty acid output, and gut stress

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ABSTRACT

There have been contradicting observations regarding the prebiotic efficacy of feruloylated oligosaccharides (FOs) extracted from different varieties of cereals with varying oligosaccharides and ferulic acid (FA) levels. The present study was performed to determine whether the mass ratio of xylooligosaccharide (XOS) to FA influences their combined effects on faecal FA content, short chain fatty acid (SCFA) output, and gut stress of D-galactose-treated aging rats. The results show that there was no significant difference in the faecal FA levels of rats fed with 5:1 and 10:1 XOS:FA diet, although the FA level in the 5:1-supplemented diet was twice as much as in the 10:1 diet. More utilisation of FA decreased butyric acid and SCFA output in the faeces for diet 5:1 compared with diets 10:1 XOS:FA or XOS alone. Furthermore, compared with 10:1 XOS:FA or XOS alone treatments, the 5:1 XOS:FA diet resulted in increased 1-diphenyl-2-picrylhydrazyl activity and higher ratios of *Bifidobacterium* or *Lactobacillus* to *Escherichia coli* ($P < 0.05$), while not increasing the number of probiotic *Bifidobacterium* and *Lactobacillus*. These findings suggest that under the specific stress level set for this study, the sufficient amount of FA added to XOS (5:1) can stimulate FA utilisation to modify gut redox balance, while reducing faecal SCFA output.

KEYWORDS

xylooligosaccharide-to-ferulic acid mass ratios, ferulic acid, short chain fatty acid, gut stress, aging rat

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1. INTRODUCTION

Xylooligosaccharide (XOS) and ferulic acid (FA) are the main polysaccharides and polyphenols found in cereals, respectively. FA is mainly bound with XOS (Liu, 2007). Their chelate, feruloylated oligosaccharides (FOs), contains both non-digestible XOS and antioxidant FA. Some studies have revealed that FOs intake leads to significant increases in the ratio of *Firmicutes* to *Bacteroidetes* by 25.9-fold, compared with control (Gong et al., 2019); higher proportions of *Firmicutes* to *Bacteroidetes* are regarded as biomarkers of better colonic redox state. Conversely, Ou et al. (2016) found that oral administration of 300 mg kg^{-1} FOs from maize bran reduced the ratio of *Firmicutes* to *Bacteroidetes* from 477.7:1 to 55.1:1.

Based on these contradictory reports, we speculated that the ratio of XOS to FA may influence their combined effects. For our study, FOs were generally extracted from different varieties of cereals with varying FA levels and forms (free or bound) (Muralikrishna et al., 2011) rather than getting the pure XOS and FA alone as in previous studies, which may cause the ratio of XOS and FA being ignored.

Short chain fatty acids (SCFA) regulate gut health more directly than gut microbiota via activation of their specific receptors, GPR41 and GPR43 (G-protein-coupled receptor) (Sivaprakasam et al., 2016). Further research revealed that healthy guts exclude oxygen (Ash, 2017). Epithelial hypoxia helps to maintain a higher ratio of obligate anaerobic bacteria to facultative anaerobic bacteria (Litvak et al., 2018). This ratio shows a strong positive correlation with the colonic redox state (Qiao et al., 2013). Accordingly, SCFA production and gut redox state mainly determine gut homeostasis.

In order to cover the shortage of research on the effects of different ratios of polysaccharides to polyphenols, and to promote the utilisation of by-products to produce prebiotics containing both polysaccharides and polyphenols, this study compared the FA and SCFA contents and levels of oxidative stress biomarkers in the faeces and in the serum of D-galactose induced aging rats after feeding XOS plus FA at ratios of 5:1 and 10:1 as well as XOS alone.

2. MATERIAL AND METHODS

2.1. Chemicals and culture media

All chemicals used in this study were purchased from Beijing Kebio Biotechnology (Beijing, China), and all culture media were obtained from Beijing Land Bridge Technology (Beijing, China). The XOS used in this study contained 229.10 mg g^{-1} xylobiose, 141.22 mg g^{-1} xylotriose, 142.93 mg g^{-1} xylotetrase, 129.91 mg g^{-1} xylopentaose, and 28.86 mg g^{-1} xylohexaose.

2.2. Animals and diets

The declined gut microbiota can be observed in aging mice, indicating that aging could modify gut homeostasis (Li et al., 2021). Accordingly, rats with D-galactose induced aging were adopted to study the prebiotic efficiency of XOS and FA. Six-month-old female Sprague–Dawley rats, weighing 300 ± 5 g, were housed under a constant temperature (25 ± 2 °C) and humidity (50–70%) and a 12-h light–dark cycle. The rats had free access to water and a standard diet (Beijing

HFK Bioscience Co., Ltd., Beijing, China). All animal procedures were approved by the Animal Care and Use Committee of Beijing Vital River Laboratories.

After 1 week of acclimatisation, all animals were randomly divided into five groups of 10 rats each, including a D-galactose-treated control group (DC), in which each rat received daily a subcutaneous injection of 300 mg kg^{-1} D-galactose solution, and a normal control group that received the same volume of sterile water for 45 days (Li et al., 2016). To the remaining three groups the standard diet plus 2.5 mg kg^{-1} day^{-1} XOS only, 2.5 mg kg^{-1} day^{-1} XOS with 0.5 mg kg^{-1} day^{-1} FA ethyl ester (5:1 XOS:FA), or 2.5 mg kg^{-1} day^{-1} XOS with 0.25 mg kg^{-1} day^{-1} FA ethyl ester (10:1 XOS:FA) were orally administered. The *in vitro* study showed that there was no significant difference between 10:1 and 15:1 XOS:FA treatment in SCFA and stress biomarkers (Data not presented), thus only treatment with 5:1 and 10:1 XOS: FA was chosen in the *in vivo* research.

After 4 weeks, the rats were euthanised. Blood was collected from the neck and centrifuged (4,000 r.p.m. for 10 min at 4 °C) to obtain serum. Serum and colonic contents were flash frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. FA content analysis

Ten millilitres of supernatant centrifuged from faecal inoculum were mixed with 50 µL 6.75% hydrochloric acid and incubated for 10 min at room temperature, followed by passage through a 0.22-µm filter. The resulting filtrate was analysed using an HPLC instrument (LC-2010A; Shimadzu Corporation, Tokyo, Japan) with XDB-C18 column (250 × 4.6 mm) (Agilent Technologies, Santa Clara, CA, USA).

HPLC was performed as described previously with some modifications (Deng et al., 2010). The mobile phase consisted of methanol and 1.5% acetic acid (45:55 v/v) and was injected at a flow rate of 0.5 mL min^{-1} at 25 °C. Absorption was detected at 320 nm using the Shimadzu LC-2010 ultraviolet detector. Each sample was injected in a 10 µL volume. The FA content in the samples was determined with the help of a calibration curve.

2.4. SCFA content analysis

SCFA contents were analysed using the method of You et al. (2017) with some modifications. 500 mL supernatant centrifuged from faecal inoculum were mixed thoroughly with 0.5 mL 0.1 mmol L^{-1} sulphuric acid solution before passing through a microfilter (0.45 µm pore size); the filtrate was stored at –20 °C until analysis. Samples were analysed using the Shimadzu RID-10A detector, set at 217 nm, with an HPX-87H column (250 × 4.6 mm, 5 µm particle size; Bio-Rad, Hercules, CA, USA). The mobile phase was a 0.05 mol L^{-1} PBS buffer solution supplemented with methanol (90:10, v/v), and 50 µL were injected at a flow rate of 0.8 mL min^{-1} at 25 °C. The contents of the samples were determined with the help of a calibration curve.

2.5. DPPH-scavenging activity

Free radical-scavenging activity was determined using the DPPH model with some modifications (Oliveira et al., 2016). A 2 mL aliquot of the supernatant was mixed with 2 mL 0.2 mM DPPH in anhydrous ethanol, followed by incubation in the dark for 30 min at room temperature. To generate the blank, 2 mL of the sample were mixed with 2 mL ethanol, and to generate the control, 2 mL ethanol were mixed with 2 mL DPPH solution. The optical densities at 517 nm were recorded using a UV–visible spectrophotometer (SP-756, Spectrum Instruments Co., Ltd.,

Shanghai, China). The percentage of DPPH-scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity}\,(\%) = \left(1 - \frac{A_{sample} - A_{blank}}{A_{control}}\right) \times 100$$

where A_{sample} = the absorbance of the sample + DPPH, A_{blank} = the absorbance of the sample + ethanol, and $A_{control}$ = the absorbance of ethanol + DPPH.

2.6. Enumeration of the target bacteria by real-time PCR

DNA was extracted from each faecal sample using the TIANamp stool DNA kit (DP328, Tiangen Biotech Co., Ltd. Beijing, China). The forward primer (5′ AGCAGTAGGGAATCTTCCA3′) and reverse primer (5′CACCGCTACACATGGAG3′) for 16S rRNA of *Lactobacillus* were synthesised by Invitrogen Trading Co., Ltd. (Shanghai, China). The cycle conditions were 30 s at 94 °C, followed by 40 cycles of 5 s at 94 °C and 30 s at 60 °C. The primers for 16S rRNA of *Bifidobacterium* and *Escherichia coli* were generated in our previous study (Du et al., 2018). Ten-fold serial dilutions of target stain plasmid were used for the generation of a standard curve. The number of genome copies of each sample was extrapolated by comparing the detected *Ct* of the unknown sample with the standard curve.

2.7. Statistical analyses

All values were expressed as mean ± standard deviation, and triplicate assays were performed for each treatment. Statistical analysis was computed using IBM SPSS 20.0 (IBM Corp., Armonk, NY, USA). Significant differences were detected by one-way analysis of variance; means were compared between groups using the least significant difference test via Tukey's test. Differences were considered significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

After 45 days of D-galactose treatment, the body weight of the animals was significantly higher among the aging rats (525.8 ± 45.9 g) than the control group (504.8 ± 46.2 g; $P < 0.05$), whereas the DPPH-scavenging activity in faecal inocula was lower among the aging rats (77.82 ± 0.55%) than the control group (80.67 ± 12.68%; $P < 0.05$). This indicated that the aging rat model was established successfully.

3.1. FA and SCFA contents

Rats exhibited slightly different ($P < 0.05$) faecal FA levels after consuming a diet supplemented with XOS and FA at a mass ratio of 5:1 compared with 10:1, although the FA level in the 5:1-supplemented diet was twice as in the 10:1-supplemented diet (Fig. 1A). Much more FA was transferred into blood, resulting in significantly higher ($P < 0.05$) serum FA levels in rats fed the diet supplemented with 5:1 compared with 10:1 XOS:FA (Fig. 1B). The data reconfirmed that more FA was used by the host when comparing 5:1 with 10:1 XOS:FA treatments.

As shown in Fig. 2, the levels of all SCFA decreased to a greater extent in the faeces of rats fed the diet supplemented with 5:1 compared with 10:1 XOS: FA ($P < 0.05$). They were both lower

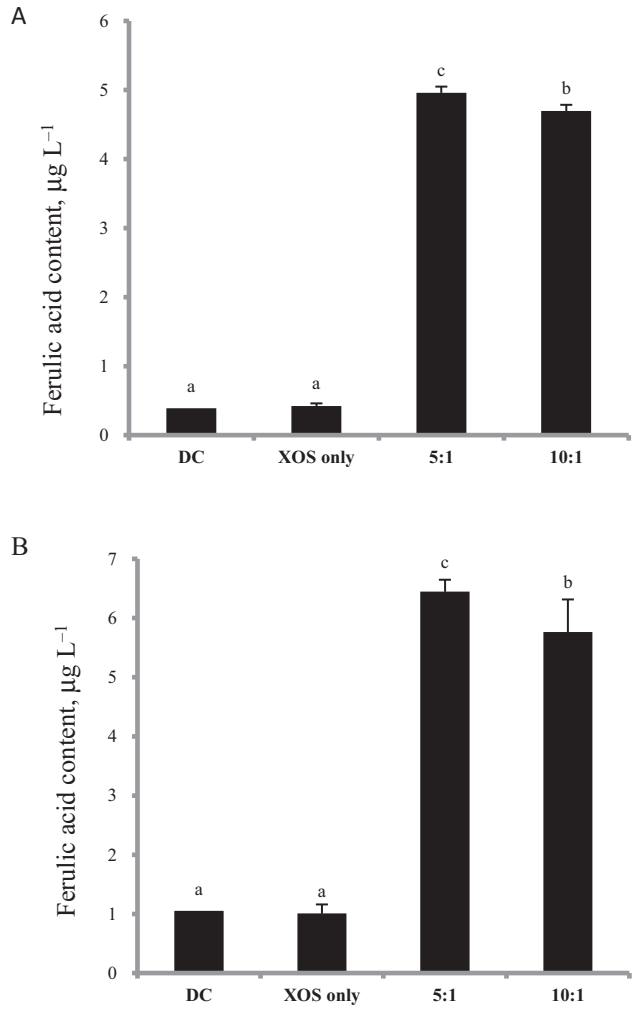

Fig. 1. Ferulic acid levels in faecal inocula (A) and blood (B) from aging rats after treated with different mass ratios of xylooligosaccharide to ferulic acid.
DC: the model group treated with D-galactose; XOS only: the group treated with single xylooligosaccharide; 5:1: the group treated with xylooligosaccharide + ferulic acid (5:1, m m^{-1}); 10:1: the group treated with xylooligosaccharide + ferulic acid (10:1, m m^{-1}). Mean values of ten replicates ± standard deviation are presented. Values with different letters are significantly different ($P < 0.05$)

than that of XOS only and the aging rat model control group, except the propionic acid in the 10:1 XOS:FA treatment group. In particular, the lowest faecal butyric acid output was observed for 5:1 treatment, indicating that FA increased butyric acid (one kind of SCFA) consumption in the colonic lumen. However, there was no significant difference of faecal butyric acid output

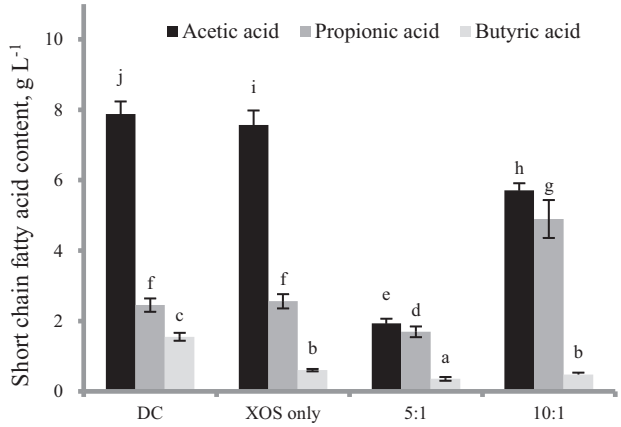

Fig. 2. Short chain fatty acid contents of aging rats faecal inocula treated with different mass ratios of xylooligosaccharide to ferulic acid.
DC: the model group treated with D-galactose; XOS only: the group treated with single xylooligosaccharide; 5:1: the group treated with xylooligosaccharide + ferulic acid (5:1, m m^{-1}); 10:1: the group treated with xylooligosaccharide + ferulic acid (10:1, m m^{-1}). Mean values of ten replicates ± standard deviation are presented. Values with different letters are significantly different ($P < 0.05$)

between XOS and 10:1 treatment. Similar results were found for total faecal SCFA, which was lower in lean subject than in the obese subject group (Schwiertz et al., 2009).

From the FA (Fig. 1B) and SCFA (Fig. 2) results, it appears that faecal SCFA release decreased with increasing serum FA levels. The precise mechanism leading to this phenomenon cannot yet be explained. The most probable explanation is that FA being antioxidant, could activate antioxidant pathway Nrf2 (nuclear factor erythroid 2-related factor 2) (Lampiasi and Montana, 2018) that has been shown to control the expression levels of PPAR-γ (peroxisome proliferator activated receptorsγ) (Cho et al., 2010), which is a butyrate specific receptor that regulates the luminal bioavailability of oxygen by driving the energy metabolism of butyrate towards β-oxidation and improving gut butyrate consumption (Byndloss et al., 2017).

Based on these observations, we can speculate that only adding enough FA to XOS (5:1) could promote FA utilisation to enhance butyric acid and SCFA utilisation in the lumen instead of losses in the faeces.

3.2. Antioxidant activity

DPPH-scavenging activities in rat faecal inocula are shown in Fig. 3. The DPPH-scavenging activity was significantly higher ($P < 0.05$) in the XOS alone treatment group than in the D-galactose treatment group, but lower ($P < 0.05$) than in the XOS and FA combined treatment groups. Typically, the 5:1 XOS:FA group showed the highest ($P < 0.05$) DPPH-scavenging activity. It may be worth noting that FA could react directly with ROS to repair colonic oxidative damage (Perez-Ternero et al., 2017). Supplementation with sinapic acid (another type of phenolic acid) reduced ROS levels by 27.0% in the colonic tissues (Yang et al., 2019). It can be

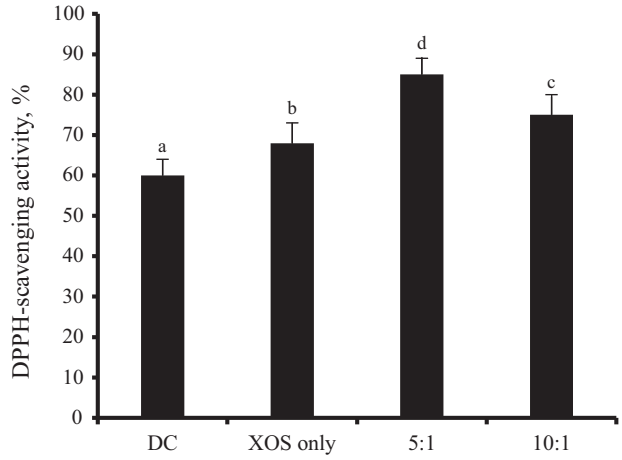

Fig. 3. DPPH-scavenging activity in faecal inocula treated with different mass ratios of xylooligosaccharide to ferulic acid.

DC: the model group treated with D-galactose; XOS only: the group treated with single xylooligosaccharide; 5:1: the group treated with xylooligosaccharide + ferulic acid (5:1, m m^{-1}); 10:1: the group treated with xylooligosaccharide + ferulic acid (10:1, m m^{-1}). Mean values of ten replicates ± standard deviation are presented. Values with different letters are significantly different ($P < 0.05$)

clearly seen that the addition of sufficient FA to XOS (mass ratio of 5:1) better promotes the gut redox state in terms of DPPH-scavenging activity compared with the XOS: FA 10:1 group.

The ratios of *Bifidobacterium* to *E. coli* and *Lactobacillus* to *E. coli* are presented in Fig. 4. Compared with the group that received D-galactose treatment, all XOS:FA combined treatment groups showed significantly (P <0.05) higher ratios of *Bifidobacterium* to *E. coli* and *Lactobacillus* to *E. coli*, with markedly higher in the 5:1 than in the 10:1 XOS:FA by 1.57- and 1.76-fold, respectively (Fig. 4A and B). This is because FA could improve gut redox state resulting in a higher ratio of obligate anaerobic bacteria to facultative anaerobic bacteria. Thus, in the present study, a sufficient amount of FA added to XOS (5:1) promoted the ratio of *Bifidobacterium* and *Lactobacillus* to *E. coli*. This result provided additional evidence that XOS:FA 5:1 promoted better the gut redox state compared with XOS:FA 10:1 group.

However, real time PCR analysis revealed that although feeding rats a blend of the two prebiotics could enhance the ratio of *Bifidobacterium* or *Lactobacillus* to *E. coli*, the addition of FA could not stimulate the growth of probiotic *Bifidobacterium* and *Lactobacillus* species, and resulted in significantly ($P < 0.05$) lower numbers of *Bifidobacterium* and *Lactobacillus* in 5:1 and 10:1 XOS:FA groups compared with the XOS alone group. The number of genome copies of *E. coli* in the faeces decreased after rats were fed a blend of XOS and FA (5:1 and 10:1) compared with the XOS group and the aging rat control group ($P < 0.05$) (Table 1).

This result is partially consistent with findings that polyphenolic acids and quercetin could repress the growth of *E. coli* (Prasad et al., 2014), and that polyphenols from seed coats exhibited good antibacterial activity against *E. coli* (Lu et al., 2018). This implied that FA also has antibacterial activities. Treatment with FA alone or higher ratio of FA might promote antibacterial activity, which could hinder the growth of gut probiotics.

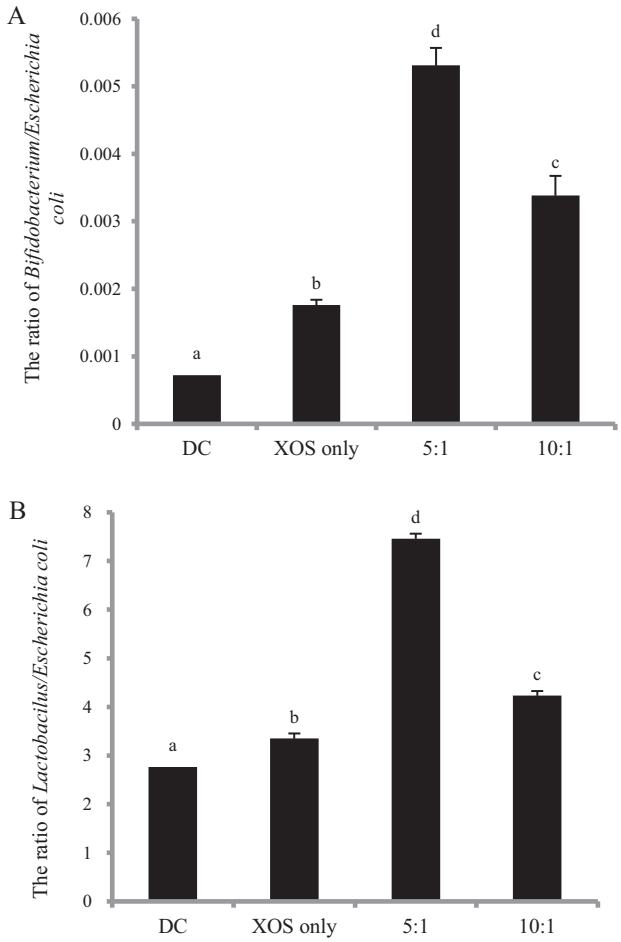

Fig. 4. The *Bifidobacterium* to *E. coli* (A) and *Lactobacillus* to *E. coli* (B) ratios of faecal inocula of aging rats treated with different mass ratios of xylooligosaccharide to ferulic acid.
DC: the model group treated with D-galactose; XOS only: the group treated with single xylooligosaccharide; 5:1: the group treated with xylooligosaccharide + ferulic acid (5:1, m m^{-1}); 10:1: the group treated with xylooligosaccharide + ferulic acid (10:1, m m^{-1}). Mean values of ten replicates ± standard deviation are presented. Values with different letters are significantly different ($P < 0.05$)

The key factor that determines whether FA promotes antioxidant or antibacterial functions is whether FA enters the epithelium or stays in the faeces. When FA inhibits ROS production of colonic epithelial cells, then FA has antioxidant activity. Conversely, if FA stays in the colonic lumen, it will act as an antimicrobial agent. The ability of FA to transport into the epithelium depends on carrier-mediated transport. Interestingly, monocarboxylate transporters (MCT1), a FA uptake transporter, is stimulated by butyric acid, and in a previous study, 1 M of butyrate significantly modulated FA uptake (Ziegler et al., 2016). The results presented here indicated that XOS and FA should maintain a good balance so as to enhance the antioxidant activity of FA

Table 1. Real-time PCR quantification of genome copies in three representative species of aging rat faecal inocula treated with different mass ratios of xylooligosaccharide to ferulic acid

Treatment	*Bifidobacterium*	*Escherichia coli*	*Lactobacillus*
DC	190 ± 11a	263,000 ± 20,817e	727,000 ± 21,000f
XOS only	493 ± 48b	280,000 ± 34,395e	938,000 ± 31,000g
5:1	209 ± 36a	39,300 ± 2,200c	293,000 ± 21,079e
10:1	194 ± 18a	57,200 ± 5,600d	242,000 ± 6,082e

Notes: Values (number of genome copies/g wet weight of faeces) are reported as means ± standard deviation ($n = 10$). Within a given column, treatments with different letters are statistically different from each other ($P < 0.05$).
DC: the model group treated with D-galactose; XOS only: the group treated with single xylooligosaccharide; 5:1: the group treated with xylooligosaccharide + ferulic acid (5:1, m m^{-1}); 10:1: the group treated with xylooligosaccharide + ferulic acid (10:1, m m^{-1}). Mean values of ten replicates ± standard deviation are presented.

rather than the antibacterial property that could disrupt the balance of intestinal microflora. In practice, it is impossible to have too much FA in FOs. The mass ratio of XOS to FA is approximately 10:1 in maize bran FOs (Huang et al., 2018). For this reason, it is more important to increase the amount of FA to XOS in the preparation of prebiotics such as FOs.

4. CONCLUSIONS

Two novel findings were provided in this study: (1) the addition of a sufficient concentration of FA to XOS promoted faecal FA utilisation to enhance butyric acid consumption in the lumen instead of losses in the faeces. Treatment with XOS:FA in a mass ratio of 5:1 promoted FA consumption and markedly reduced SCFA release with the faeces of aging rats compared with the 10:1 XOS:FA and XOS alone treatments. (2) Treatments with all XOS:FA ratios, as well as XOS alone, caused some improvement in the gut redox state, with the 5:1 XOS:FA ratio showing the best antioxidant effect. However, the addition of FA to XOS increased the ratios of *Bifidobacterium* to *E. coli* and *Lactobacillus* to *E. coli*, but did not improve the numbers of probiotic *Bifidobacterium* and *Lactobacillus*. Taken together, our data revealed that the sufficient amount of FA added to XOS (5:1) is essential for maintaining gut homeostasis, and is better than 10:1 XOS: FA or XOS alone.

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