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Consumption of indigestible saccharides and administration of *Bifidobacterium pseudolongum* reduce mucosal serotonin in murine colonic mucosa

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Abstract

SCFA increase serotonin (5-hydroxytryptamine, 5-HT) synthesis and content in the colon *in vitro* and *ex vivo*, but little is known *in vivo*. We tested whether dietary indigestible saccharides, utilised as a substrate to produce SCFA by gut microbiota, would increase colonic 5-HT content in mice. Male C57BL/6J mice were fed a purified diet and water supplemented with 4 % (w/v) 1-kestose (KES) for 2 weeks. Colonic 5-HT content and enterochromaffin (EC) cell numbers were lower in mice supplemented with KES than those without supplementation, while monoamine oxidase A activity and mRNA levels of tryptophan hydroxylase 1 (*Tph1*), chromogranin A (*Chga*), *Slc6a4* and monoamine oxidase A (*Maoa*) genes in the colonic mucosa, serum 5-HT concentration and total 5-HT content in the colonic contents did not differ between groups. Caecal acetate concentration and *Bifidobacterium pseudolongum* population were higher in KES-supplemented mice. Similar trends were observed in mice supplemented with other indigestible saccharides, that is, fructo-oligosaccharides, inulin and raffinose. Intragastric administration of live *B. pseudolongum* (10^8 colony-forming units/d) for 2 weeks reduced colonic 5-HT content and EC cell numbers. These results suggest that changes in synthesis, reuptake, catabolism and overflow of 5-HT in the colonic mucosa are not involved in the reduction of colonic 5-HT content by dietary indigestible saccharides in mice. We propose that gut microbes including *B. pseudolongum* could contribute to the reduction of 5-HT content in the colonic mucosa via diminishing EC cells.

Key words: **Serotonin: 1-kestose: Bifidobacteria: Gut microbiota: SCFA**

Serotonin (5-hydroxytryptamine, 5-HT) is a bioactive monoamine that is synthesised in serotonergic neurons of the central nervous system and in enterochromaffin (EC) cells in the gastrointestinal epithelium of humans, and more than 90 % of total body 5-HT is synthesised in the latter[1–3]. Synthesis of 5-HT in EC cells relies on the rate-limiting enzyme, tryptophan hydroxylase 1 (TPH1), which metabolises the 5-HT precursor, L-tryptophan. Upon chemical and mechanical stimuli in the intestinal lumen as well as neural and endocrine inputs, 5-HT is released from secretory granules predominantly located at the basal surface of EC cells[1–3]. However, 5-HT release also occurs at the apical surface into the intestinal lumen[1,3,4]. The released 5-HT participates in various functions in the gut, which include peristalsis, secretion and vasodilation, by acting on a diverse range of receptors expressed on smooth muscle, enteric neurons and epithelial cells[1–3]. The extracellular 5-HT is subsequently transported into surrounding epithelial cells through the serotonin reuptake transporter and is then converted to 5-hydroxyindoleacetic acid (5-HIAA) by a monoamine oxidase-A (MAO-A)[2]. Otherwise, the extracellular 5-HT is transported to platelets to circulate.

To date, the gut serotonergic system has been studied using rodent models as well as human biopsy specimens[1–4]. Recent studies using rodent models have highlighted the involvement

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; Chga, chromogranin A; DW, deionised water; EC, enterochromaffin; FOS, fructo-oligosaccharides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KES, 1-kestose; MAO-A, monoamine oxidase-A; RT-qPCR, real-time quantitative PCR; TPH1, tryptophan hydroxylase 1.

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of gut microbiota in the gut serotonergic system[5–8]. Their effect has been demonstrated in germ-free mice displaying impaired 5-HT signalling and low 5-HT levels in the colon, faeces and serum, but not in the small intestine[5,6]. Accordingly, these reductions in 5-HT reflected a low rate of 5-HT synthesis, accompanied by lower TPH1 expression and a lower density of 5-HT-producing EC cells in the colonic mucosa[6]. The mechanisms underpinning the involvement of microbiota in the gut serotonergic system remain elusive, and the role of microbial metabolites, that is, SCFA, has been suggested. Fukumoto *et al.*[7] reported that intraluminal perfusion of a mixture of acetate, propionate and butyrate increases the luminal release of 5-HT in isolated rat colon. Reigstad *et al.*[5] showed that supplementation with acetate and butyrate for 24 h increases TPH1 mRNA levels in BON cells, a human neuroendocrine cell line that secretes 5-HT and various peptide hormones. Yano *et al.*[6] showed that supplementation with butyrate and propionate for 1 h promotes 5-HT release and TPH1 mRNA expression in RIN14B cells, which is another EC cell model derived from rat pancreatic islets. In line with these reports, our previous study demonstrated that TPH1 mRNA expression in murine colonic organoids was increased in response to supplementation with acetate, propionate and butyrate[8]. Acetate, in particular, contributed to increased 5-HT levels and EC cell numbers in the colonic organoids, thereby facilitating maturation of EC cells[8]. While SCFA appear to be important determinants for 5-HT levels *in vitro* and *ex vivo*, their *in vivo* effects on 5-HT levels in EC cells remain to be elucidated.

SCFA are the principal products of gut microbial fermentation of dietary indigestible saccharides including fructo-oligosaccharides (FOS). Our previous study showed that dietary supplementation with 1-kestose (KES), the smallest constituent of FOS, increased acetate and butyrate in the caecal contents of rats[9]. In addition, we also observed that KES supplementation increased the population of *Bifidobacterium* spp. in the caecum and faeces of rats, dogs and humans[9–13]. In light of the involvement of SCFA and gut microbiota in regulating colonic 5-HT levels, we postulated that dietary indigestible saccharides influence colonic 5-HT levels through an effect of SCFA and/or *Bifidobacterium* spp. on EC cells. The aim of this study was therefore to test this hypothesis in mice.

Materials and methods

Animals and diets

All study protocols were pre-approved by the Animal Use Committee of Hokkaido University (approval no. 14-0028 and 19-0017), and all mice were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Male C57BL/6J mice (age, 6 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled (21 to 25 °C) room under a 12 h light–12 h dark cycle. They were allowed free access to food and water. Mice were acclimatised for 1 week with a commercial AIN-93G pellet diet (D10012G; Research Diets).

Experimental design (supplementary materials)

In Expt 1, male C57BL/6J mice were randomly allocated to two groups (n 6 in each group) and fed the AIN-93G pellet diet *ad libitum* and allowed free access to either deionised water (DW) or DW supplemented with 4 % (w/v) KES (B Food Science). These two groups were referred to as KES− and KES+, respectively. Randomisation of mice to each group was carried out using a computerised random number generator (www.random.org), and investigators were not blinded to treatment. The dose of indigestible saccharides was set according to our previous study[14], in which 4 % KES supplementation of the drinking water increased caecal *Bifidobacterium* spp. in mice. Two weeks after starting the test diets, mice were anaesthetised by intraperitoneal injection of a mixed solution of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight) and killed by exsanguination from the carotid artery. Serum was separated from the blood samples, mixed with a 5-HT stabiliser (ascorbic acid, a component of 5-HT ELISA kit described below) and stored at −20°C for 5-HT measurement as described below. After a laparotomy, the proximal colon was excised and opened longitudinally and the luminal contents were collected with a small spatula, weighed and subjected to 5-HT measurement as described below. After washing with ice-cold PBS, a 1-cm section of colon tissue was excised and subjected to immunohistochemistry as described below. For the remainder of the colon, the mucosa was scraped with a glass slide, immediately plunged into liquid N_2 and then stored at −80°C for 5-HT and MAO-A activity measurements and RNA isolation as described below. The caecal contents were collected with a small spatula, weighed and then subjected to SCFA measurement and microbiota analysis as described below. In humans, the colon functions as a microbial fermentation vessel, whereas, in mice, this fermentation occurs in the caecum[15]. Therefore, the present study examined the caecal contents in an effort to extrapolate the data to the human condition.

In Expt 2, male C57BL/6J mice were randomly allocated to five groups (n 7 in each group), fed the AIN-93G pellet diet *ad libitum* and allowed free access to either DW or DW supplemented with either 4 % (w/v) KES, FOS (Meioligo P, Meiji), inulin (INU) (Fuji FF, Fuji Nihon Seito) or raffinose (RAF) (Nippon Beet Sugar Manufacturing) as drinking water. These five groups were referred to as CON (control), KES, FOS, INU and RAF, respectively. To generalise from KES to the indigestible saccharides, the present study employed FOS, INU and RAF. A number of previous studies have investigated these saccharides in food and nutritional sciences for both humans and animals. Randomisation of mice to each group was carried out using a computerised random number generator (www.random.org), and investigators were not blinded to treatment. Two weeks after starting the test diets, mice were anaesthetised by inhalation of sevoflurane and killed by cervical dislocation and the colonic and caecal samples were obtained as described in Expt 1.

In Expt 3, male C57BL/6J mice were allocated to four groups (n 8 in each group) such that the average abundance of *Bifidobacterium* spp. in faeces was similar among the groups. The abundance of *Bifidobacterium* spp. was estimated by real-time quantitative PCR (RT-qPCR) targeting 16S rRNA genes

as described below. Investigators were not blinded to treatment. Mice were fed the AIN-93G pellet diet *ad libitum* and intragastrically administered 200 μl of either PBS or PBS supplemented with 1×10^8 colony-forming units of *Bifidobacterium pseudolongum* or heat-killed *B. pseudolongum* once daily and allowed free access to DW or DW supplemented with 4 % (w/v) KES as drinking water. Thus, mice in the four groups received PBS, *B. pseudolongum*, heat-killed *B. pseudolongum* and KES, which were referred to as CON, BIF, HBIF and KES, respectively. Preparation of *B. pseudolongum* is described below. Two weeks after starting the treatments, mice were anaesthetised by inhalation of sevoflurane and killed by cervical dislocation and the colonic and caecal samples were obtained as described in Expt 1.

Measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

Colonic contents were homogenised in PBS supplemented with a 5-HT stabiliser, while colonic mucosa samples were homogenised in a cell lysis buffer composed of 50 mM Tris-HCl (pH 7·5), 1 % (w/v) Triton X-100, 0·15 M NaCl, 0·1 % (w/v) SDS, 1 % (w/v) sodium deoxycholate and 5-HT stabiliser as described previously[8]. After centrifugation, 5-HT in the supernatants was determined using a Serotonin ELISA Kit (Cat no. KA2518; Abnova) according to the manufacturer's instructions. In the colonic mucosa, 5-HIAA in the supernatants was also determined using a 5-Hydroxyindoleacetic acid ELISA Kit (Cat no. KA1881; Abnova) according to the manufacturer's instructions and both 5-HT and 5-HIAA concentrations were normalised to the protein concentration in the supernatants, as determined by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. 5-HT concentration in serum samples and *B. pseudolongum* culture medium obtained as described below were also measured using the Serotonin ELISA Kit.

Measurement of monoamine oxidase A activity

Colonic mucosa samples were homogenised in a buffer composed of 0·1 M phosphate buffer (pH 7·4) and 10 % (w/v) glycerol. After centrifugation, MAO-A activity in the supernatants was measured using a Monoamine Oxidase Assay Kit (Cat no. EMAO-100; BioAssay Systems) according to the manufacturer's instructions. The activity was normalised to the protein concentration in the supernatants, as determined by a Pierce BCA Protein Assay Kit according to the manufacturer's instructions.

Preparation of Bifidobacterium pseudolongum *for intragastric administration*

B. pseudolongum isolated previously from the faeces of BALB/c mice[16] was routinely cultured in a basal medium, that is, de Man, Rogosa and Sharpe (MRS) broth supplemented with 0·05 % (w/v) L-cysteine HCl, using the AnaeroPack system (Mitsubishi Gas Chemical) at 37°C for 72 h[17]. The bacterial culture was washed with sterile PBS before intragastric administration. Meanwhile, viable cell counts were determined by applying serial dilutions of the bacterial culture supplemented with anaerobic phosphate buffer, composed of 2 % (w/v) buffered peptone water, 0·05 % (w/v) L-cysteine HCl, 0·1 % (w/v) Tween 80 and 0·1 % (w/v) BL agar (Nissui Pharmaceutical), onto MRS agar plates and by counting colonies formed after incubation at 37°C for 72 h under anaerobic conditions[18]. Heat-killed *B. pseudolongum* preparation was done by autoclaving at 121°C for 15 min.

Bifidobacterium pseudolongum *culture experiments*

B. pseudolongum was routinely cultured as described above. Stationary-phase cells were cultured in the basal medium supplemented with 2 μg/ml 5-HT HCl (Sigma) using the AnaeroPack system at 37°C, and the concentration of 5-HT and 5-HIAA in the medium was measured at 0, 12, 24 and 36 h of culture period using the respective ELISA kits as described above. In a separate experiment, exponentially growing cells were cultured in the basal medium supplemented with different concentrations (0, 1, 10, 100 and 1000 μg/ml) of 5-HT HCl using the AnaeroPack system at 37°C for 48 h and the growth was monitored with a spectrophotometer (BioPhotometer, Eppendorf) by absorbance at 600 nm.

Measurement of SCFA in the caecal contents

Caecal SCFA were measured by HPLC according to the internal standard method[19].

mRNA expression analysis

mRNA expression was analysed as previously described[8,20]. Total RNA was extracted from colonic mucosa samples using a ReliaPrep RNA Tissue Miniprep System (Promega Japan) according to the manufacturer's instructions. The RNA concentration was monitored with a spectrophotometer (Nanodrop Lite; Thermo Fisher Scientific) by absorbance at 260 nm (A260). An A260 of 1·0 was considered 40 μg/ml of the extracted RNA. The ratio of A260:A280 nm was measured, and the samples with a ratio of 1·8:2·0 were used for reverse transcription. Total RNA (1 μg) was reverse-transcribed to generate first-strand cDNA using a ReverTra Ace qPCR RT Master Mix (Toyobo) according to the manufacturer's instructions. The reaction was run in a thermal cycler (Life Eco; Bioer Technology) with a thermal profile of 37°C for 15 min followed by 5 min at 95°C, and the obtained cDNA was stored at −20°C for subsequent PCR. Murine TPH1, serotonin reuptake transporter, chromogranin A (ChgA), MAO-A, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are encoded by *Tph1, Slc6a4, Chga, Maoa, Actb* and *Gapdh* genes, respectively. Since *Tph1*, *Slc6a4* and *Maoa* genes are associated with the synthesis, reuptake and catabolism of 5-HT, respectively[1–3], mRNA level of these genes was determined to test whether changes in the colonic 5-HT content were mediated by 5-HT synthesis, reuptake and/or catabolism. The *Chga* gene was analysed as an enteroendocrine cell marker[21]. To compare the steady-state levels of these mRNA, RT-qPCR was performed using a GeneAce SYBR qPCR Mix α No ROX (Nippon Gene) with a Thermal Cycler Dice TP800 (Takara Bio). The thermal profile was adjusted to denaturation at 95°C for 10 min, followed by forty-five cycles of denaturation

at 95°C for 30 s, annealing and extension at 60°C for 60 s. A melting curve analysis was performed after amplification to assess the specificity of RT-qPCR. The data were calculated through $2^{-\Delta\Delta Ct}$ method with the geometric mean of 2 endogenous reference genes, that is, *Actb* and *Gapdh*. RT-qPCR was carried out in duplicate. The method recommended in Minimum Information for the Publication of Quantitative Real-Time PCR Experiments was strictly followed[22]. The sequences of primers used for RT-qPCR are described in online Supplementary Table 1.

Gut microbiota analysis

Gut microbiota was analysed as previously described[14,16]. DNA was isolated from a piece of fresh faeces and whole caecal contents in each mouse using a QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions. DNA samples (5 ng) served as a template for RT-qPCR of the 16S rRNA gene fragment to estimate the number of total bacteria, *Bifidobacterium* spp. and *B. pseudolongum* as previously described[16]. RT-qPCR was performed using a GeneAce SYBR qPCR Mix α No ROX with a Thermal Cycler Dice TP800. The reaction conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 30 s and 60°C for 60 s. A melting curve analysis was performed after amplification to assess the specificity of RT-qPCR. The sequences of primers used for RT-qPCR are described in online Supplementary Table 2. For RT-qPCR standards, subcloned 16S rRNA gene fragments were prepared from *Blautia coccoides* (JCM 1395^T), *Bifidobacterium animalis* (JCM 1190^T) and *B. pseudolongum* subsp. *pseudolongum* (JCM1264) for total bacteria, *Bifidobacterium* spp. and *B. pseudolongum*, respectively, as previously described[16]. RT-qPCR was carried out in duplicate. The method recommended in Minimum Information for the Publication of Quantitative Real-Time PCR Experiments was strictly followed[22].

Immunohistochemistry

The tissue samples were fixed in a fixating agent (Yufix; Sakura Finetek Japan) for 3 h at room temperature. The samples were then dehydrated through a graded ethanol series, embedded with paraffin and sliced at 5-μm thickness on a microtome (REM-710; Yamato Koki). After deparaffinisation and rehydration, the sections were heated in 10 mM citrate buffer (pH 6·0) for 40 min at 98°C for antigen retrieval. The sections were blocked with 5 % bovine serum albumin/PBS for 1 h and then incubated with anti-serotonin rat monoclonal antibody (Cat no. ab6336; Abcam, RRID AB_449517) and anti-ChgA rabbit polyclonal antibody (Cat no. ab45179; Abcam, RRID AB_726879) at dilutions of 1:100 and 1:200, respectively, at 4°C overnight. After washing with PBS, the sections were stained with Alexa Fluor 488-conjugated donkey anti-rat IgG (Cat no. A-21208; Thermo Fisher Scientific, RRID AB_141709) and Alexa Fluor 568-conjugated donkey anti-rabbit IgG (Cat no. A-10042; Thermo Fisher Scientific, RRID AB_2534017) at a dilution of 1:1000 at room temperature for 1 h. These primary and secondary antibodies have been used in our previous study[23]. After washing with PBS, the slides were counterstained with a ProLong Gold antifade reagent with DAPI (Life Technologies) and viewed under a fluorescence microscope (BX40; Olympus). For each section, ten fields viewed per section were photographed and the number of ChgA- and 5-HT-positive EC cells was counted in each field. All cell counts were performed by a single observer who was blinded to the mouse treatments.

Statistical analyses

The sample size was calculated based on the experimental design (unpaired *t* test in Expt 1 and one-way ANOVA in Expt 2 and 3) determining the colonic 5-HT content as the primary outcome measure. We used G*Power software (version 3.1.9.4)[24] for the power analysis with an α probability of 0·05 and a power of 0·80, and the effect size of 1·8 was estimated based on the results from preliminary experiments (unpublished results). Hence, the required sample size was six, seven and eight mice per group in Expt 1, 2 and 3, respectively.

Results are presented as standard box plots with median and interquartile ranges with minimum and maximum or means with their standard errors. In Expt 1, variance homogeneity was examined using the F test, and significant differences between mean values of two groups were determined using an unpaired *t* test. In Expt 2 and 3 and in culture experiments, variance homogeneity was examined using the Brown–Forsythe test. To compare the mean values of five and four groups, Dunnett's *post hoc* test was applied when a significant effect was found by one-way ANOVA. Correlations between parameters were assessed by Pearson's correlation test. Data were analysed using GraphPad Prism software for Macintosh (version 8; GraphPad Software). *P* values of $< 0{\cdot}05$ were considered to be statistically significant.

Results

Dietary 1-kestose reduced colonic 5-hydroxytryptamine and increased caecal acetate and Bifidobacterium pseudolongum *(Expt 1)*

The final body weight did not differ between mice in the KES− and KES+ groups (online Supplementary Table 3). In the colonic mucosa, the 5-HT content was significantly lower in the KES+ group than in the KES− group (Fig. 1(a)). Serum 5-HT concentration was the same between the two groups (Fig. 1(b)). The wet weight of colonic contents tended to be higher in the KES+ group than in the KES− group (Fig. 1(c)), and no difference was observed in the total 5-HT content in the colonic contents between the two groups (Fig. 1(d)). Accordingly, the 5-HT concentration in the colonic contents was significantly lower in the KES+ group than in the KES− group (Fig. 1(e)). The MAO-A activity in the colonic mucosa did not differ between the two groups (Fig. 1(f)). Likewise, the mRNA levels of *Tph1*, *Chga*, *Slc6a4* and *Maoa* genes did not differ between the two groups (Fig. 1(g)–(j), respectively). Immunohistochemistry showed ChgA and 5-HT signals in the colons of KES− and KES+ mice (Fig. 2(a)). The number of ChgA-positive cells was significantly lower in the KES+ group than in the KES− group (Fig. 2(b)). Likewise, the number of ChgA/5-HT-double positive cells, which are regarded as EC cells, was significantly lower in the KES+ group than in the KES− group (Fig. 2(c)). The wet weight of caecal contents was significantly higher in the KES+ group than in the KES− group (Fig. 3(a)). In the caecal contents, acetate

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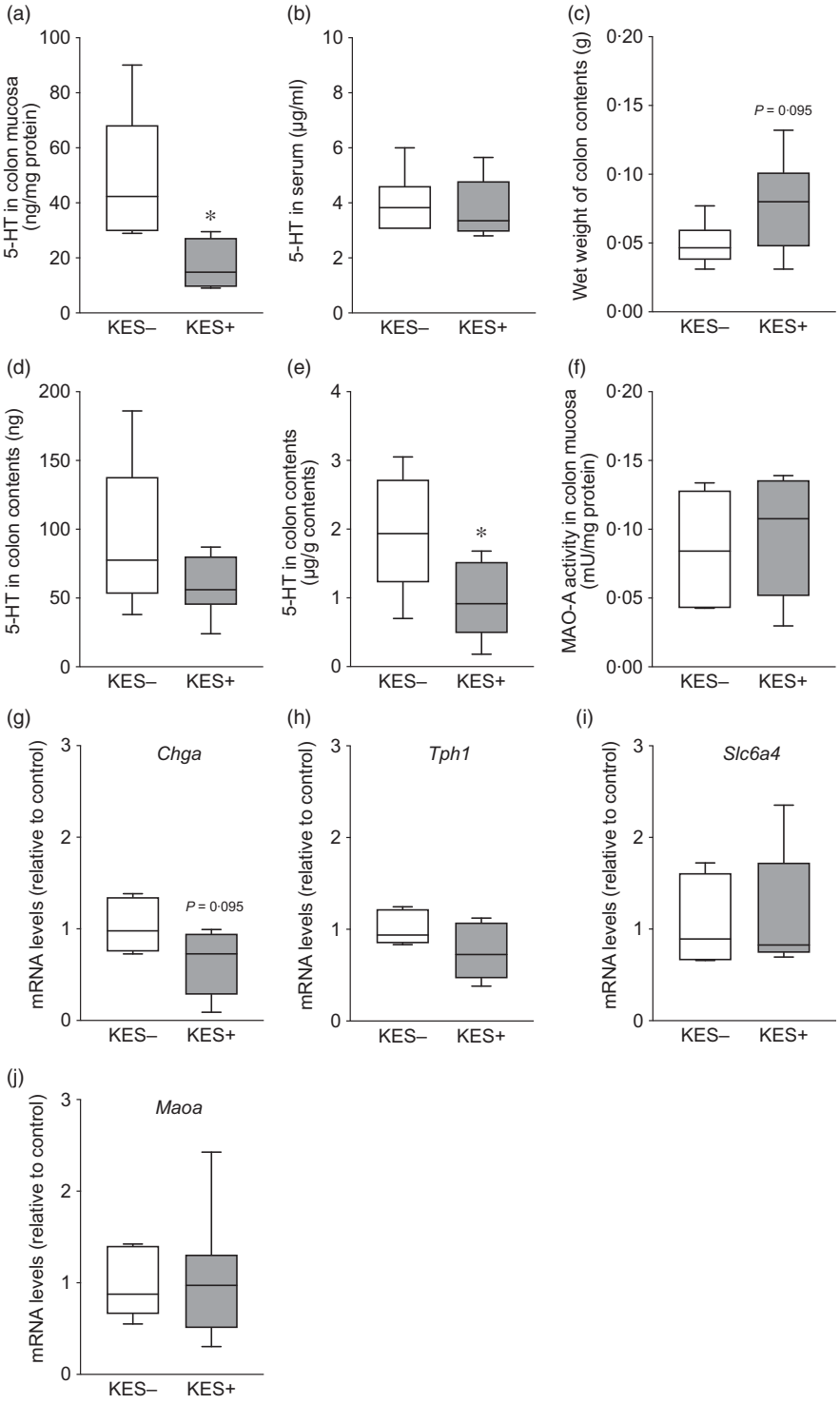

Fig. 1. Serotonin (5-hydroxytryptamine (5-HT)) content and gene expression in male C57BL/6J mice fed diet supplemented with or without 1-kestose (KES) for 2 weeks (Expt 1). Charts (a) and (b) show the colon mucosal 5-HT content and serum 5-HT concentration, respectively. Charts (c), (d) and (e) show the wet weight, total 5-HT content and 5-HT concentration in the colonic contents, respectively. Chart (f) shows the activity of monoamine oxidase A (MAO-A) in the colonic mucosa. Charts (g), (h), (i) and (j) show the mRNA level of chromogranins A (*Chga*), tryptophan hydroxylase 1 (*Tph1*), serotonin reuptake transporter (*Slc6a4*) and monoamine oxidase-A (*Maoa*) genes in the colonic mucosa, respectively. In charts (g), (h), (i) and (j), data for KES+ are shown relative to levels in KES−, which were set to 1. Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, n 6. White and grey boxes represent mice fed diet without supplementation (KES−) and with KES supplementation (KES+), respectively. Mean values between groups were compared using an unpaired t test. $^{*}P < 0{\cdot}05$ *v.* KES−.

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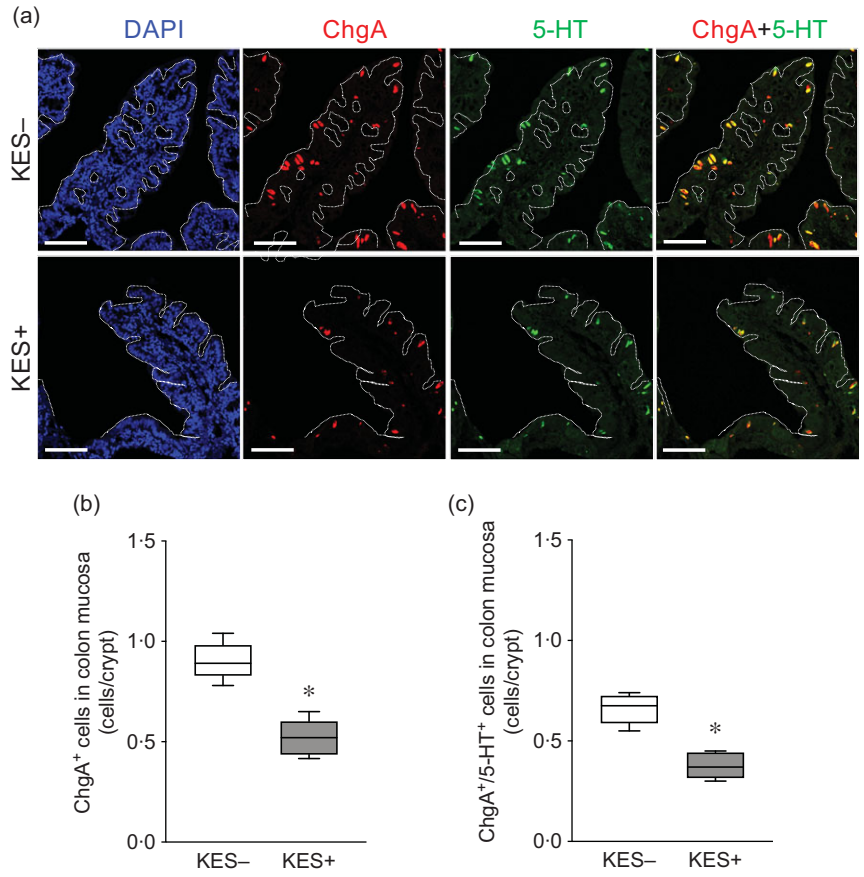

Fig. 2. Enterochromaffin (EC) cells in the colon of male C57BL/6J mice fed diet supplemented with or without 1-kestose (KES) for 2 weeks (Expt 1). Chart (a) shows the representative immunohistochemistry for chromogranin A (ChgA) and serotonin (5-hydroxytryptamine (5-HT)) with DAPI counterstaining. White dotted line and white bar represent tissue outline and 100 μm, respectively. Charts (b) and (c) show the number of ChgA-positive cells and ChgA/5-HT-double positive cells, that is, EC cells, respectively, in each crypt. Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, n 6. White and grey boxes represent mice fed diet without supplementation (KES–) and with KES supplementation (KES+), respectively. Mean values between groups were compared using an unpaired t test. *$P < 0{\cdot}05$ *v.* KES–.

concentration was significantly higher in the KES+ group than in the KES– group, and butyrate concentration tended to be higher in the KES+ group than in the KES– group, whereas there were no differences in propionate concentration between the groups (Fig. 3(b)). The numbers of *Bifidobacterium* spp. and *B. pseudolongum* were significantly higher in the KES+ group than in the KES– group (Fig. 3(c) and (d), respectively), and it appears that *B. pseudolongum* constitutes the majority of *Bifidobacterium* spp. in the caecal contents. No difference was observed in the number of total bacteria in the caecal contents between the two groups (Fig. 3(e)).

A significant positive correlation was observed between the number of *B. pseudolongum* and the concentration of acetate, but not propionate and butyrate, in the caecal contents (Fig. 4(a)–(c), respectively). The 5-HT content in the colonic mucosa did not correlate with the concentration of acetate, propionate and butyrate (Fig. 4(d)–(f), respectively). The caecal number of *B. pseudolongum* negatively correlated with the 5-HT content in the colonic mucosa and the number of EC cells (Fig. 4(g) and (h), respectively), while the 5-HT content in the colonic mucosa positively correlated with the number of EC cells (Fig. 4(i)).

Because laboratory mice of the same strain purchased from different suppliers reportedly harbour different microbial communities[25], we examined the colonic 5-HT content and caecal *B. pseudolongum* in C57BL/6J mice purchased from another supplier, that is, Charles River Laboratories Japan (online Supplementary Methods). The final body weight did not differ between mice in the KES– and KES+ groups (online Supplementary Table 3). KES supplementation tended to reduce the colonic 5-HT content (online Supplementary Fig. 1A), whereas the mRNA levels of *Tph1*, *Chga*, *Slc6a4* and *Maoa* genes did not differ between the two groups (online Supplementary Fig. 1b, c, d and e, respectively). The wet weight of caecal contents was significantly higher in the KES+ group than in the KES– group (online Supplementary Fig. 1f). In the caecal contents, acetate concentration tended to be higher in the KES+ group than in the KES– group and propionate concentration was significantly higher in the KES+ group than in the KES– group, whereas there were no differences in butyrate concentration between the groups (online Supplementary Fig. 1g). The number of caecal *B. pseudolongum* was significantly higher in the KES+ group than in the KES– group (online

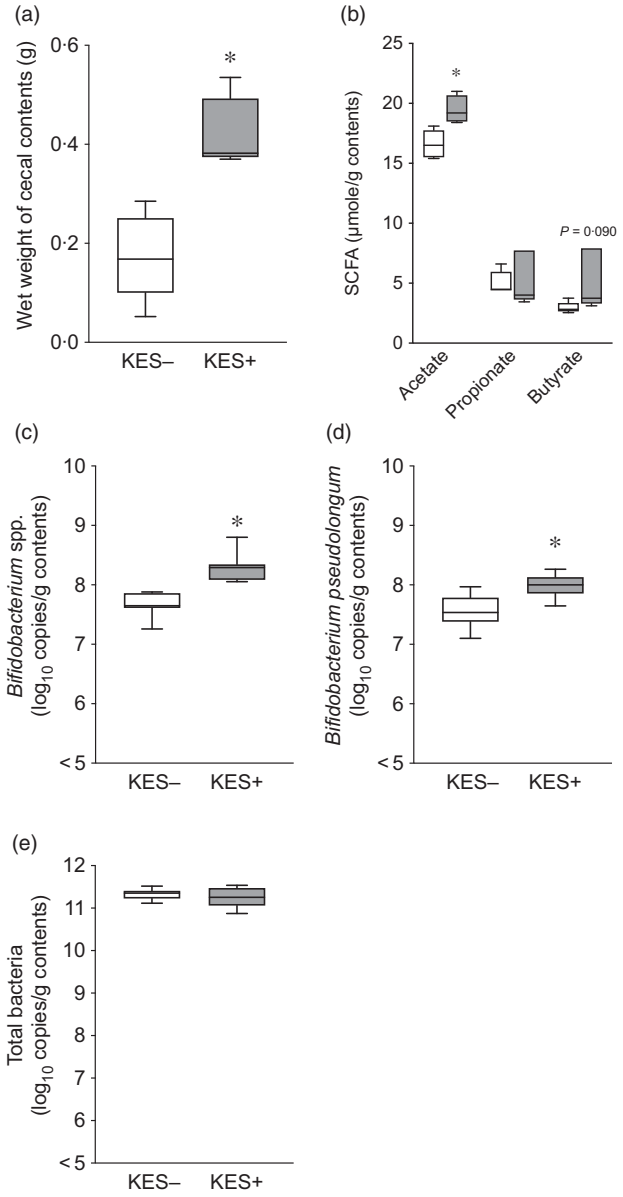

Fig. 3. Wet weight of caecal contents (chart (a)), caecal concentration of SCFA (chart (b)), caecal *Bifidobacterium* spp., *B. pseudolongum* and total bacteria (charts (c), (d) and (e), respectively) in male C57BL/6J mice fed diet supplemented with or without 1-kestose (KES) for 2 weeks (Expt 1). Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, *n* 6. White and grey boxes represent mice fed diet without supplementation (KES–) and with KES supplementation (KES+), respectively. Mean values between groups were compared using an unpaired *t* test. **P* < 0·05 *v.* KES–.

Supplementary Fig. 1h) and tended to correlate positively with the concentration of acetate in the caecal contents (online Supplementary Fig. 2a). No correlation was observed between the number of *B. pseudolongum* and the concentration of propionate and butyrate in the caecal contents (online Supplementary Fig. 2b and c, respectively). The 5-HT content in the colonic mucosa did not correlate with the concentration of acetate, propionate and butyrate (online Supplementary Fig. 2d, 4e and f, respectively). The caecal number of *B. pseudolongum* negatively correlated with the 5-HT content in the colonic mucosa (online Supplementary Fig. 2g).

Other indigestible saccharides, that is, fructo-oligosaccharides, INU and RAF, reduced colonic 5-hydroxytryptamine and increased caecal Bifidobacterium pseudolongum *(Expt 2)*

The final body weight of mice did not differ between the CON, KES, FOS, INU and RAF groups (online Supplementary Table 3). As compared with mice in the CON group, the wet weight of caecal contents and the number of *B. pseudolongum* in the caecum were significantly higher in mice in the KES, FOS, INU and RAF groups (Fig. 5(a) and (b), respectively). In the colonic mucosa, 5-HT content was significantly lower in the KES, FOS, INU and RAF groups than in the CON group (Fig. 5(c)). Likewise, 5-HIAA content was significantly lower in the KES, INU and RAF groups and tended to be lower in the FOS group than in the CON group (Fig. 5(d)). There were no differences in 5-HIAA:5-HT ratio among the groups (Fig. 5(e)). In the colonic mucosa, the mRNA level of *Tph1* gene was significantly higher in the RAF group than in the CON group (Fig. 5(f)) and the mRNA level of *Chga* gene was significantly lower in the KES and FOS groups than in the CON group (Fig. 5(g)). No differences were observed in the mRNA level of *Slc6a4* and *Maoa* genes among the groups (Fig. 5(h)).

Administration of live Bifidobacterium pseudolongum *reduced colonic 5-hydroxytryptamine (Expt 3)*

The final body weight of mice did not differ between the CON, BIF, HBIF and KES groups (online Supplementary Table 3). The wet weight of caecal contents did not differ among the CON, BIF and HBIF groups, whereas the KES group showed a significantly higher weight of caecal contents as compared with the CON group (Fig. 6(a)). Faecal *Bifidobacterium* spp. numbers at the start of the experiment were the same: 4·17 (0·23), 4·02 (0·31), 4·10 (0·28) and 4·09 (0·24) $\log_{10}$ copies/g faeces in the CON, BIF, HBIF and KES groups, respectively. The numbers of *Bifidobacterium* spp. and *B. pseudolongum* in the caecal contents were significantly higher in the BIF and KES groups than in the CON group (Fig. 6(b) and (c), respectively). In the colonic mucosa, the 5-HT content and the number of EC cells were significantly lower in the BIF and KES groups than in the CON group (Fig. 6(d)).

Bifidobacterium pseudolongum *does not metabolise 5-hydroxytryptamine*

When *B. pseudolongum* was cultured in the medium supplemented with 5-HT HCl (2 μg/ml), the concentration of 5-HT and 5-HIAA in the medium was not changed throughout the culture period (about 36 h, Fig. 7(a)). Detection of 5-HIAA in the medium seems due to the cross-reactivity (5·5 %) of ELISA with 5-HT according to the manufacturer. The growth of *B. pseudolongum* as monitored by absorbance at 600 nm was significantly higher in the medium supplemented with 100 μg/ml 5-HT HCl than in the medium without supplementation, whereas

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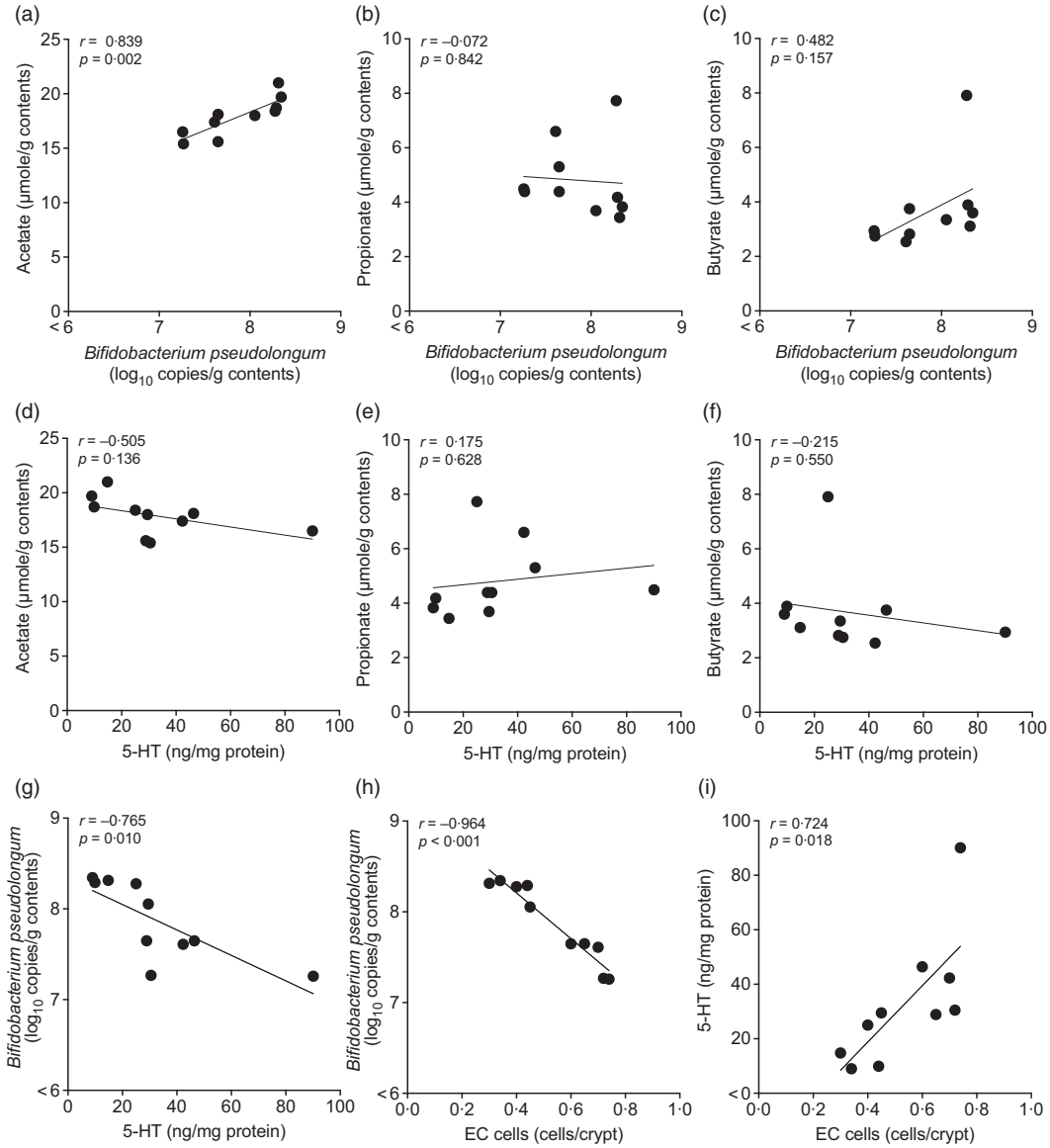

Fig. 4. Relationship between caecal concentrations of SCFA, caecal numbers of *Bifidobacterium pseudolongum*, colonic serotonin (5-hydroxytryptamine (5-HT)) content and colonic enterochromaffin (EC) cell numbers in male C57BL/6J mice fed diet supplemented with or without 1-kestose (KES) for 2 weeks (Expt 1). Pearson's correlation coefficient (*r*) was used to evaluate the relationships.

supplementation with 1, 10 and 1000 μg/ml 5-HT HCl had no influence on the growth (Fig. 7(b)).

Discussion

In the present study, indigestible saccharides including KES were supplemented in the drinking water at a concentration of 4 % (w/v). Given that the normal daily consumption of drinking water is approximately 5 ml in mice, the daily intake of indigestible saccharides in the present study would correspond approximately to 200 mg. According to Nair & Jacob[26], the human equivalent dose of 200 mg per mouse is calculated as approximately 0·7 g/kg body weight. Roberfroid[27] has described that the dose of indigestible inulin-type fructans varied in human intervention studies. For instance, Kleessen *et al.*[28] employed 40 g/d inulin, while Buddington *et al.*[29] administered 4 g/d FOS. Therefore, the daily intake of indigestible saccharides at a dose of approximately 0·7 g/kg body weight is not necessarily irrational in humans.

Contrary to our initial expectation, we observed that the consumption of indigestible saccharides including KES reduced the 5-HT content in the murine colonic mucosa. Previous studies including ours have shown that SCFA including acetate and butyrate increased 5-HT release, 5-HT content and gene expression of TPH1, the rate-limiting enzyme for 5-HT synthesis, in colon tissues *ex vivo* and EC cell lines[5–8]. Our previous study showed that supplementation with KES increased acetate and

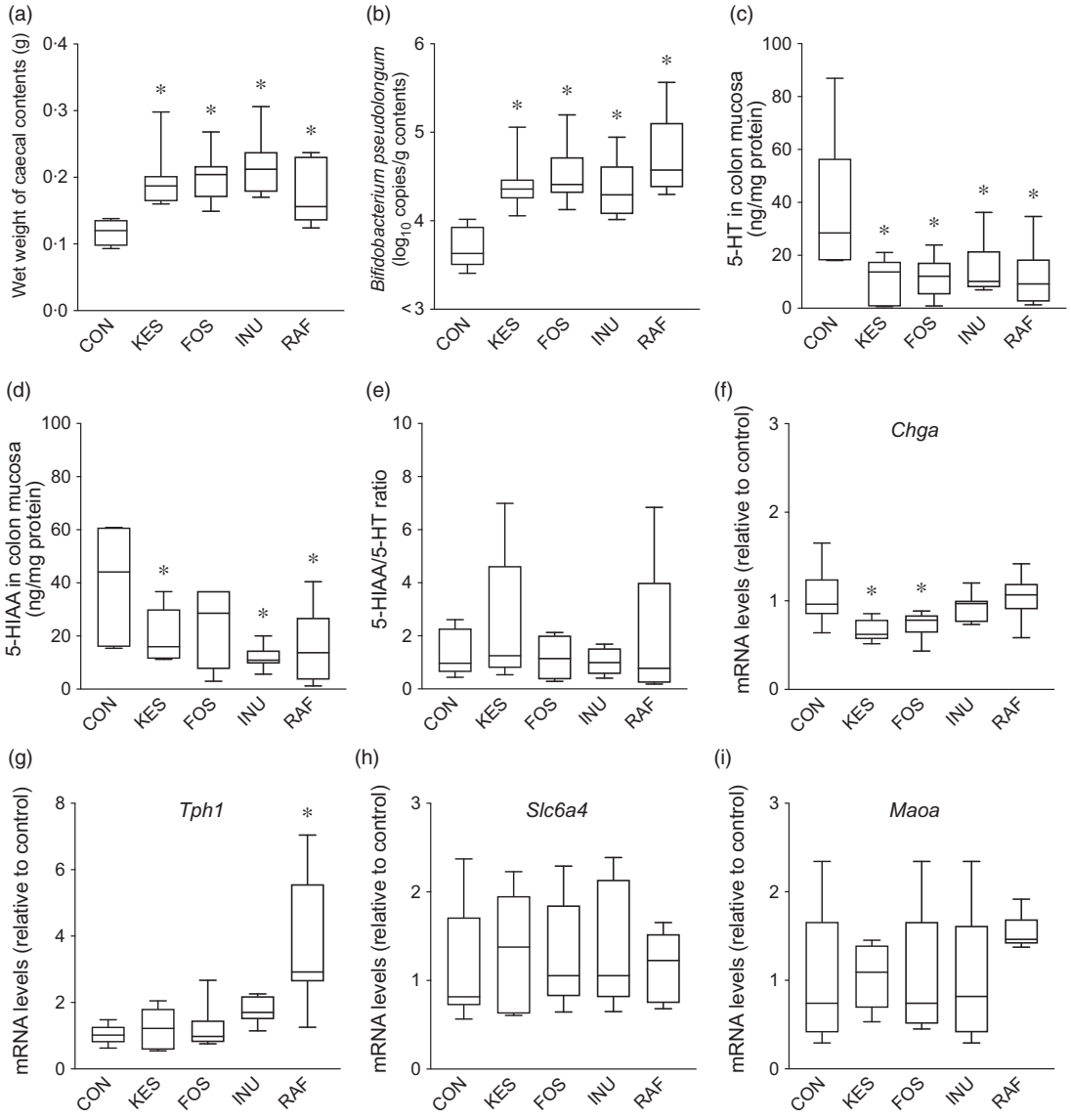

Fig. 5. Wet weight of caecal contents (chart (a)), caecal population of *Bifidobacterium pseudolongum* (chart (b)), colonic contents of serotonin (5-hydroxytryptamine (5-HT), chart (c)) and 5-hydroxyindoleacetic acid (5-HIAA, chart (d)), 5-HIAA:5-HT ratio (chart (e)) and colonic gene expression (charts (f)–(h)) in male C57BL/6J mice given drinking water supplemented with either 1-kestose (KES), fructo-oligosaccharides (FOS), inulin (INU) or raffinose (RAF) or without supplementation (CON) for 2 weeks (Expt 2). Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, *n* 7. In charts (f)–(h), data for KES, FOS, INU and RAF are shown relative to levels in CON, which were set to 1. Mean values between groups were compared using Dunnett's *post hoc* test following one-way ANOVA. $^{*}P < 0{\cdot}05$ *v.* CON.

butyrate in the rat caecal contents[9]; thus, it would have been rational to presume that KES consumption could promote 5-HT availability in the colon via SCFA production *in vivo*. Nevertheless, the present study showed that the 5-HT content in the murine colonic mucosa was reduced by KES supplementation despite the increase of acetate and butyrate in the caecal contents in Expt 1. We therefore have to consider that the increase of 5-HT content in the colonic mucosa by SCFA would fail to be reproduced *in vivo*.

In the present study, consumption of KES produced no significant changes in the mRNA expression of *Tph1*, *Slc6a4* and *Maoa*, which encode the 5-HT synthesising enzyme, 5-HT reuptake transporter and 5-HT catabolising enzyme, respectively. In addition, the colonic activity of MAO-A was not affected by KES consumption in Expt 1. Furthermore, the colonic 5-HT content was reduced by the consumption of different indigestible saccharides, and a similar trend was observed for colonic 5-HIAA content in Expt 2. Hence, the 5-HIAA:5-HT ratio, which reflects mucosal 5-HT turnover[30], was unchanged in the colonic mucosa of mice fed the different indigestible saccharides. Taken together, these results suggest that changes in synthesis, reuptake and catabolism of 5-HT in the colonic mucosa are not involved in the reduction of the colonic 5-HT content induced by dietary indigestible saccharides including KES.

Dietary KES reportedly increases not only SCFA but also bifidobacteria in the gut. Our previous studies demonstrated

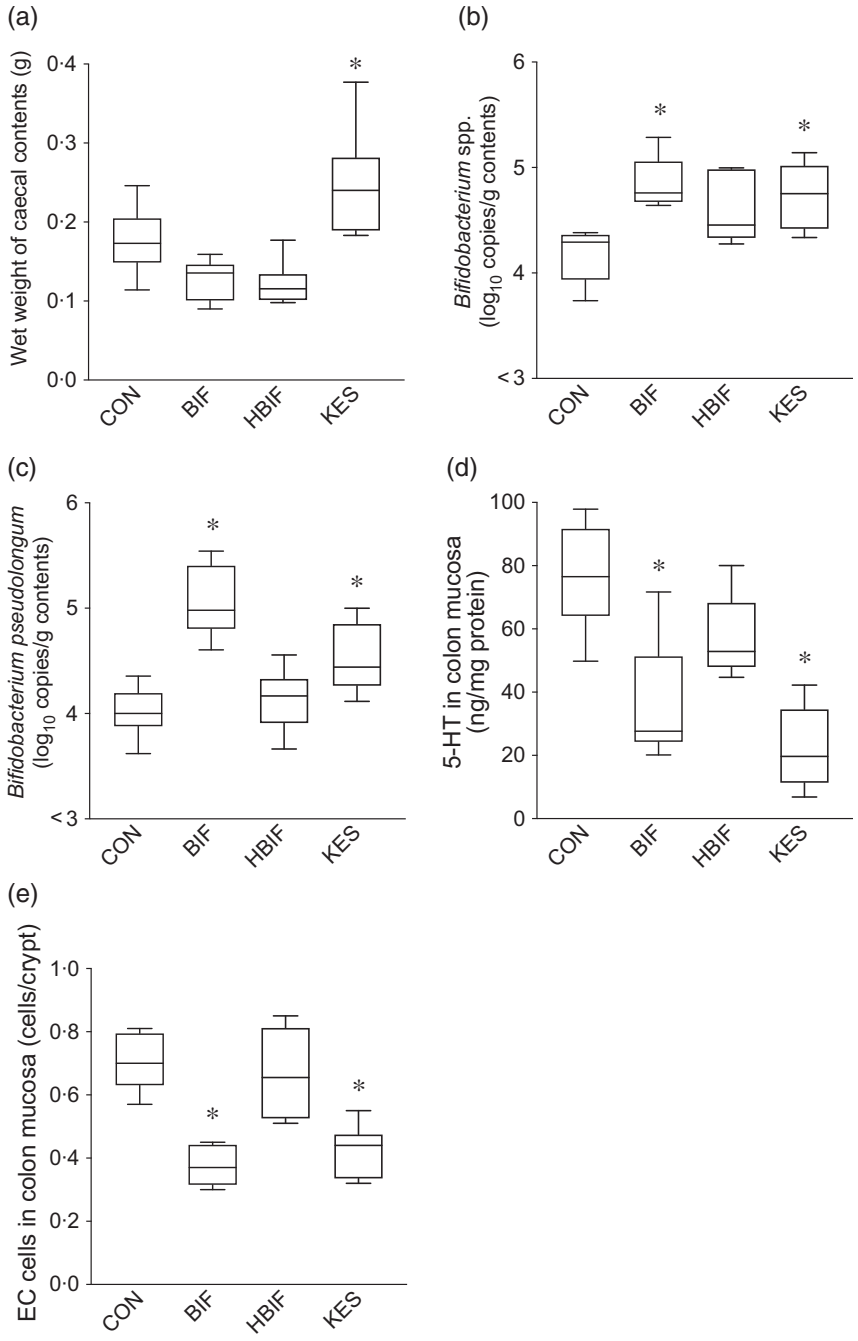

Fig. 6. Wet weight of caecal contents (chart (a)), caecal populations of *Bifidobacterium* spp. (chart (b)) and *B. pseudolongum* (chart (c)), colonic content of serotonin (5-HT, chart (d)) and colonic enterochromaffin (EC) cell numbers (chart (e)) in male C57BL/6J mice administered either live (BIF) or heat-killed (HBIF) *B. pseudolongum* or given drinking water supplemented with 1-kestose (KES) for 2 weeks (Expt 3). Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, *n* 8. Mean values between groups were compared using Dunnett's *post hoc* test following one-way ANOVA. $^*P < 0{\cdot}05$ *v.* CON.

that KES supplementation increased the population of *Bifidobacterium* spp. in the caecum and faeces of rats, dogs and humans[9–13]. In the present study, we observed that populations of *Bifidobacterium* spp. and *B. pseudolongum* in murine caecal contents were increased by dietary KES in Expt 1. *B. pseudolongum* is reportedly the most predominant bifidobacterial species in various mammals[31]. Indeed, our results indicate that *B. pseudolongum* constitutes the majority of *Bifidobacterium* spp. in the caecal contents. In Expt 1, we observed a significant positive correlation between the number of *B. pseudolongum* and the concentration of acetate in the caecal contents, suggesting that *B. pseudolongum* is responsible for the elevated acetate production by KES consumption. In addition, we observed a significant negative correlation between the colonic 5-HT content and the caecal *B. pseudolongum*, suggesting the involvement of *B. pseudolongum* in the reduction of colonic

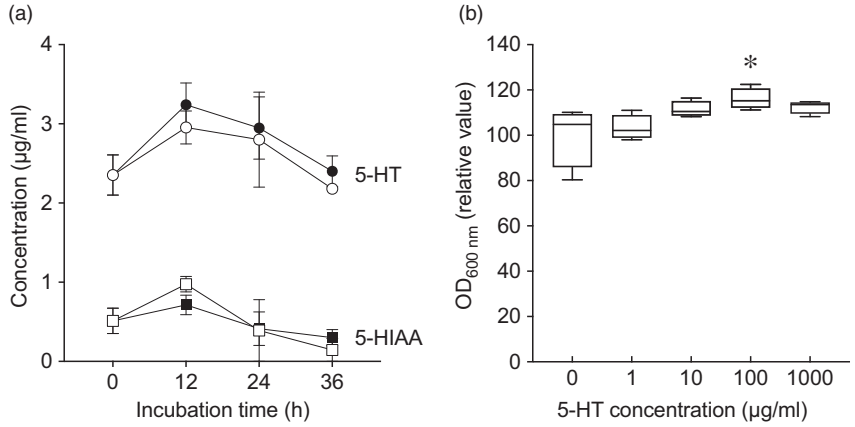

Fig. 7. Culture experiments of *Bifidobacterium pseudolongum*. Chart a shows the time-course changes in the concentration of serotonin (5-hydroxytryptamine (5-HT)) and 5-hydroxyindoleacetic acid (5-HIAA) in the culture medium of *B. pseudolongum* supplemented with 2 μg/ml 5-HT HCl. Closed and open symbols represent the medium incubated with and without *B. pseudolongum*, respectively. Results are presented as means with their standard error, *n* 3. Mean values between groups were compared using Dunnett's *post hoc* test following the Brown–Forsythe test. Chart B shows the growth of *B. pseudolongum,* as estimated by absorbance at 600 nm, cultured in the medium supplemented with the graded concentrations of 5-HT HCl for 48 h. Results are presented as standard box plots composed of median and interquartile range with minimum and maximum, *n* 4. Mean values between groups were compared using Dunnett's *post hoc* test following one-way ANOVA. **P* < 0·05 *v.* 0 μg/ml 5-HT HCl.

5-HT content by KES consumption. In this context, someone may suspect that acetate produced by *B. pseudolongum* would be involved in the KES-induced reduction of colonic 5-HT content. However, we observed no correlation between the colonic 5-HT content and the caecal acetate; thus, it is unlikely that acetate mediates the reduction of colonic 5-HT content by KES consumption. The present study also showed that KES reduced ChgA/5-HT-double positive EC cell numbers in the colon, raising the possibility that the KES reduction of colonic 5-HT content would reflect the reduced differentiation and/or promoted disappearance of EC cells in the colon. Indeed, we observed in Expt 1 that the caecal number of *B. pseudolongum* negatively correlated with the number of colonic EC cells and that the number of EC cells positively correlated with the 5-HT content in the colonic mucosa, suggesting that *B. pseudolongum* contributes to the reduction of 5-HT content in the colonic mucosa via diminishing EC cells.

To explore the causal relationship between *B. pseudolongum* and colonic 5-HT content, we administered *B. pseudolongum* to mice in Expt 3. We observed that intragastric administration of live but not heat-killed *B. pseudolongum* increased the caecal population of *B. pseudolongum*; thus, it seems possible that the administered bacteria might proliferate in the caecum. The present study also showed that intragastric administration of live *B. pseudolongum* reduced colonic 5-HT content, and the effect was comparable to that of KES consumption, whereas heat-killed *B. pseudolongum* had no effect. In addition, intragastric administration of live *B. pseudolongum* reduced the number of EC cells in the colonic mucosa. Again, these results strongly suggest that *B. pseudolongum* is involved in the reduction of colonic 5-HT content, which is attributable to the decrease of EC cells, by indigestible saccharides in mice. Nevertheless, it cannot be denied that reduced colonic content of 5-HT by indigestible saccharides resulted in the increased population of *B. pseudolongum*. Kwon *et al.*[32] showed that gut microbiota structure was different between *Tph1*−/− and *Tph*+/− mice and that 5-HT supplementation influenced the growth of commensal bacteria *in vitro*, suggesting that apically secreted 5-HT shapes the gut microbiota. However, the present culture experiment showed that the growth of *B. pseudolongum* was not influenced by 5-HT supplementation at the physiologically relevant concentrations, that is, 1 and 10 μg/ml; the present study showed that the concentrations of 5-HT in the colonic contents were up to approximately 3 μg/g. Thus, it is unlikely that the reduction of colonic 5-HT content results in the increase of *B. pseudolongum*.

The mucosal 5-HT content reflects leakage of 5-HT into the extracellular space; such elevated extracellular 5-HT can be consequently taken up by not only adjacent epithelial cells but also circulating platelets[2], and the 5-HT release also occurs at the apical surface into the intestinal lumen[1,3,4]. Fukumoto *et al.*[7] reported that intraluminal perfusion of SCFA increases the luminal release of 5-HT in isolated rat colon; thus, it may be possible that dietary KES-induced increase of SCFA would stimulate the luminal release of 5-HT in the colon, which results in the reduction of 5-HT content in the colonic mucosa. However, the present immunohistochemical analysis in the colon showed that, similar to the number of ChgA/5-HT-double positive cells, the number of ChgA-positive cells was decreased by KES consumption; thus, it is unlikely that the decrease of ChgA/5-HT-double positive cells would reflect the increased release of 5-HT in the EC cells. In addition, we observed in Expt 1 that the 5-HT concentration in the serum and total 5-HT amount in the colonic contents were not affected by KES consumption, suggesting that 5-HT overflow into both the circulation and colonic lumen is not responsible for the reduction of colonic 5-HT content by KES consumption. Nevertheless, someone may suspect that gut microbes could consume luminally released 5-HT, which in turn reduces the 5-HT in the colonic contents. In fact, Fung *et al.* demonstrated that *Turicibacter sanguinis*, a member of murine gut microbiota, incorporates 5-HT[33]. In the present culture experiment, however, the concentration of 5-HT and 5-HIAA in the medium was not changed, when *B. pseudolongum*

was cultured in the medium supplemented with 5-HT HCl. Thus, it is unlikely that *B. pseudolongum* could metabolise 5-HT in the colonic lumen.

A discussion of the limitations of the present study is warranted. Although the present findings suggest that *B. pseudolongum* contributes to the reduction of 5-HT content in the colonic mucosa via diminishing EC cells, we observed no changes in the mRNA expression of *Chga* and *Tph1*, which are expressed in the EC cells, by dietary indigestible saccharides including KES. In addition, considering that the turnover rate of intestinal EC cells is reportedly much slower than that of the surrounding enterocytes (16–150 d *v.* 3–4 d)[34], it seems difficult to assume that an altered turnover of EC cells influences the colonic 5-HT content during the present study's experimental period of only 2 weeks. Further studies are needed to clarify these inconsistencies. A second limitation is concerning a possibility that other bacterial species than *B. pseudolongum* would reduce the 5-HT in the colonic lumen. Clearly, indigestible saccharides could influence the growth of several bacterial groups. Our previous study showed that dietary KES supplementation increased the population of *Bacteroides* spp. and *Clostridium* cluster XIVa in addition to *Bifidobacterium* spp. in rat caecum[9]. Thus, it could be that *Bacteroides* spp. and *Clostridium* cluster XIVa mediated dietary KES-induced reduction of 5-HT in the colonic mucosa. Further studies are required to test this possibility. Third, cellular and molecular mechanisms by which *B. pseudolongum* reduces colonic 5-HT content and EC cell numbers remain unclear. We are currently investigating the effect of intra- and extracellular substances from *B. pseudolongum* on the 5-HT content and EC cell numbers in murine colonic organoids. Last, we need to mention the lack of blinding in the present study; thus, the investigators needed to be blinded to treatment assignment in the animal experiments.

In conclusion, the present results suggest that changes in synthesis, reuptake, catabolism and overflow of 5-HT in the colonic mucosa are not involved in the reduction of colonic 5-HT content by dietary indigestible saccharides in mice. We propose that gut microbes including *B. pseudolongum* could contribute to the reduction of 5-HT content in the colonic mucosa via diminishing EC cells. Further studies are needed to elucidate cellular and molecular mechanisms by which gut microbes reduce colonic 5-HT and EC cells.

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Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114521001306

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