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Raspberry pomace alters cecal microbial activity and reduces secondary bile acids in rats fed a high-fat diet

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Running title: Raspberry pomace reduces the formation of secondary bile acids

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ABSTRACT

The profile of bile acids (BA) largely depends on the enzymatic activity of the microbiota, but this can be modulated by the dietary addition of biologically active compounds, e.g., polyphenols and polyunsaturated fatty acids. The aim of this study was to examine the effect of dietary raspberry pomace as a rich source of biologically active compounds on microbial activity and the BA profile in the caecum of rats fed a high-fat diet. Wistar rats were fed the standard diet AIN-93, a high-fat diet or a modified high-fat diet enriched with 7% different types of processed raspberry pomaces produced by standard grinding and fine grinding, with or without seeds. Rats fed the high-fat diet for eight weeks showed some disorders in liver function and cecal BA, as manifested by an increased concentration of cholesterol, total BA in the liver and cholic, deoxycholic, and β-muricholic acids in the cecal digesta. In general, irrespective of the type of raspberry pomace, these dietary preparations decreased liver cholesterol, hepatic fibroblast growth factor receptor 4, peroxisome proliferator-activated receptor alpha, cecal ammonia and favorable changed BA profile in the cecum. However, among all dietary pomaces, the finely ground preparation containing seeds had the greatest beneficial effect on the caecum by modulating bacterial activity and reducing the levels of secondary BA.

Keywords: cholesterol; bile acids; PPARα; ellagitannins; Wistar rat

1. INTRODUCTION

It is well known that a high-fat diet stimulates the secretion of bile acids (BA) into the intestinal lumen, leading to a higher risk of neoplastic changes in the lower gut [1-3]. The effect of dietary fat on BA metabolism results in microbial activity that promotes the deconjugation, dehydrogenation, and dehydroxylation of primary to secondary BA in the distal small intestine and colon, thus increasing the chemical diversity of the BA [4, 5]. The secondary BA are one of the factors associated with an increased risk of colon cancer [6-8]. Nevertheless, the modulation of the bacterial activity by the dietary addition of biologically active compounds, e.g., polyphenols and polyunsaturated fatty acids [9-11] might regulate the profile and concentration of the BA in the gastrointestinal tract. There is still little information regarding compounds that are able to regulate the BA profile in the gastrointestinal tract or their synthesis in the liver.

BA are synthesized from cholesterol in hepatocytes and then conjugated to glycine or taurine and released into the duodenum following the ingestion of a meal to facilitate the absorption of triglycerides, cholesterol, and lipid-soluble vitamins [12-14]. The synthesis of these compounds is regulated by at least 14 liver enzymes [15, 16]. The main enzymes responsible for BA synthesis are cholesterol 7-alpha-hydroxylase (CYP7A1) and sterol 12-alpha-hydroxylase (CYP8B1) [12, 15]. The hepatic expression of CYP7A1 and CYP8B1 is regulated by farnesoid X receptor (FXR), which is highly expressed in liver [17]. The activity of FXR can be regulated by BA, which are the main signaling endogenous ligands for this receptor, and through mechanisms that are dependent on the nuclear receptors small heterodimer partner (SHP) [15] and fibroblast growth factor 15/19 (FGF15/19) [18-20]. There is also molecular evidence for cross-talk between FXR and peroxisome proliferator-activated receptor α (PPARα), which is a nuclear receptor that mainly controls lipid and lipoprotein metabolism [21].

The BA synthesis in the liver seems to be modulated by biologically active compounds supplied in the diet e.g., diet enriched with ellagic acid increased expression of the CYP7A1 and CYP8B1 genes involved in bile acid synthesis [22]. Raspberries are popularly consumed fruits that are rich in biologically active compounds. These fruits are known as an excellent source of dietary antioxidants, largely due to their high level of phenolic compounds comprised primarily of cyanidins, anthocyanins, ellagitannins, and quercetin [23, 24]. During the processing of raspberries, particularly for the production of a concentrated juice, a significant portion of the compounds, mainly fiber and polyphenols, remains in the pomace. The nutritional and health-promoting properties of these bioactive compounds are not sufficiently understood. It is worth noting that more than 80% of the raspberry pomace consists of the seeds, which include approximately 23% oil and are a rich source of essential fatty acids [25, 26]. Unfortunately, seeds that have not been processed pass intact through the digestive system with all their biologically active compounds, thus reducing the biological value of raspberry pomace.

This study proposes two solutions -which have not been described previously in vivo- that may increase the health potential of the raspberry pomace in terms of the modulation of BA synthesis in the liver and the BA profile in the gut: (1) Separation of the seeds from the pulp fraction, thereby also increasing the concentration of biologically active compounds from the pulp that are readily available to the organism. (2) Fine grinding of the native pomace, damaging the seed coat and thus increasing the availability of the accumulated valuable seed compounds. The aim of this study was to examine whether dietary raspberry pomaces with or without seeds and their grinding level may favorably alter cecal microbial enzymatic activity and BA profiles in rats fed a high-fat diet.

2. MATERIALS AND METHODS

2.1 Preparation of raspberry pomaces

Dried raspberry press cake was obtained from a fruit transformation plant in the Masovia region in Poland. Four preparations were evaluated. Native press cake was characterized by a particle diameter smaller than 1.25 mm. To obtain other preparations, the press cake was ground up and sieved as follows. Fine-ground native press cake (containing seeds) was prepared using a Blixer 3 blender (Robot Coupe, France) with solid CO_2 at -78.5°C to obtain particles smaller than 0.65 mm. To prepare seedless press cake, the material was milled in a laboratory ball mill (own built, Łódź University of Technology, Poland) for 1 hour and sieved to a diameter smaller than 0.8 mm and larger than 0.65 mm. To obtain the nonstandard-ground fraction, a portion of the seedless press cake was further milled on the ball mill until granulation smaller than 0.65 mm was achieved.

2.2 Chemical composition of the raspberry pomaces

Dry matter, ash, crude protein, crude fat and total dietary fiber (TDF) were determined according AOAC official methods [27]: 920.151; 940.26; 920.152; 930.09; and 985.29. Carbohydrates were determined according to the following formula: carbohydrate = total solids – (protein + fat + ash). The proximate compositions of the raspberry pomaces are presented in Table 1. For the ellagitannin (ET) and anthocyanin (AC) measurements, the samples of raspberry pomaces and known standards were diluted with 50% (v/v) methanol, filtered through PTFE filters (0.45 µm) and introduced into high-performance liquid chromatography (HPLC) systems.

2.2.1 Quantification of Ellagitannins

The content of ETs was determined using a Smartline chromatograph (Knauer, Berlin, Germany) composed of degasser (Manager 5000), binary pump (P1000), an autosampler (3950), a thermostat and a detector PDA (2800). The ETs were separated on a 250 x 4.6 i.d.,

5-µm, Gemini C18 110A column (Phenomenex) by gradient elution with 0.05% (v/v) phosphoric acid in water (solvent A) and 83:17 (v/v) acetonitrile:water with 0.05% phospohoric acid (solvent B). The column temperature was set at 35°C, the flow rate was 1.25 mL/min, and the gradient program was as follows: 0 – 5 min, 5% (v/v) B; 5 – 10 min, 5 – 15% (v/v) B; 10 – 35 min, 15 – 40% (v/v) B; 35 – 40 min, 40 – 73% (v/v) B; 40 – 44 min, 73% (v/v) B; 44 – 46 min, 73 – 5% (v/v) B; 46 – 54 min, 5% (v/v) B. The injection volume was 20 µL. Data were collected using the ClarityChrom version 3.0.5.505 program (Knauer, Berlin, Germany). ETs were detected at 250 nm, and the standard curves generated using lambertianin C, sanguiin H-6, and ellagic acid were applied for quantification. Standards of lambertianin C and sanguine H-6 were isolated from raspberry extract as described by Sójka et al. (2013) [28]. Ellagic acid standard was purchased from Extrasynthese.

2.2.2 Quantification of flavan-3-ols (FLs)

The contents of FLs, i.e., the sum of proanthocyanidins and catechins, were determined using the method described by Sójka et al. (2013) [28]. For separation, the same column and conditions were used. For this analysis, the Shimadzu system equipped with a pump (LC-20AD), a degasser (DGU-20A5R), an autosampler (SIL-20ACHT), a thermostat (CTO-10ASUP), and a detector (RF-10AXL), and LabSolutions Lite version 5.52 software was used.

2.2.3 Quantification of Anthocyanins

HPLC coupled to a DAD and an electrospray ion (ESI) trap mass spectrometer was used to identify ACs. The HPLC system was equipped with a SCM1000 membrane solvent degasser (ThermoQuest, San Jose, CA, USA), a binary high-pressure gradient pump (1100 Series; Agilent Technologies, Santa Clara, CA, USA), an autosampler, and a column oven (Surveyor Series, Thermo-Finnigan, San Jose, CA, USA). A Gemini C18 110A

250 mm × 4.6 mm i.d. (Phenomenex) 5-µm column was used. The column temperature was 30°C, and the injection volume was 10 µL. Chromatographic data were collected using Xcalibur software, version 1.2 (Thermo-Finnigan, San Jose, CA, USA). The solvents and the gradient used for AC separations were as follows: solvent A, 0.25% (v/v) formic acid in water; solvent B, 0.25% (v/v) formic acid in acetonitrile, with a flow rate of 12 mL/min; the gradient program (time in min – % (v/v) was as follows B): 0–5, 2–5, 32–20, 37–70, 42–70, 45–5, and 55–5. The MS system coupled to HPLC was an LCQ DECA ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) equipped with an ESI source, which was used in negative mode. The phenol content was quantified using a KNAUER Smartline chromatograph (Berlin, Germany). Details about equipment, separation as well as quantification of the phenol content were previously described by Sójka et al. (2015) [29]. The polyphenolic compositions of the raspberry pomaces are presented in Table 2.

2.3 Animal study

This study was conducted in strict accordance with the recommendations of the National Ethic Commission (Warsaw, Poland). All procedures and experiments complied with the guidelines and were approved by the Local Ethic Commission of the University of Warmia and Mazury (Olsztyn, Poland, Permit Number: 68/2014) with respect to animal experimentation and the care of the animals under study, and all efforts were made to minimize suffering. The nutritional experiment was performed using 48 male Wistar rats, which were allocated to 6 groups of 8 animals each that were housed individually in plastic cages. The initial body weight was comparable among groups (154±6.5 g on average). For 8 weeks, each group was fed a modified version of the semi-purified rodent diet recommended by Reeves (1997) [30] (details provided in supplemental Table 1). Group C was fed a standard diet for laboratory rodents that consisted of 6% fat and 5% fiber (AIN-93 diet), and group HF received a high-fat diet containing 20% fat and 4.55% fiber. Another experimental

group was fed a high-fat diet enriched with 7% four different types of raspberry pomaces with standard grinding with or without seeds ($HFSG_s$ and HFSG, respectively) and fine grinding with or without seeds ($HFNG_s$ and HFNG, respectively). Details regarding the proportional composition of each group-specific diet are shown in supplemental Table 1. The diets were freshly prepared at weekly intervals, stored in hermetic containers at -20°C and administered ad libitum. The individual food intake was recorded on a daily basis. The animals were maintained individually in cages under a 12-hour light/dark cycle, a controlled temperature of 21–22°C and extensive room ventilation (15 times per hour), and they had free access to water.

2.3.1 Sample collection and analysis

At the end of the experiment, the rats were anesthetized with sodium pentobarbital according to the recommendations for the euthanasia of laboratory animals (50 mg/kg body weight). After laparotomy, blood samples were collected from the vena cava and stored in tubes. Immediately after euthanasia (ca. 10 min), the cecal and colonic pH values were measured directly in the intestinal segments (model 301 pH meter; Hanna Instruments, Vila do Conde, Portugal). The caecum and colon were removed and weighed. Fresh cecal content was used for determination of the ammonia concentration by the microdiffusion method in Conway's dishes. The short-chain fatty acid (SCFA) concentrations were measured using gas chromatography (Shimadzu GC-2010, Kyoto, Japan) and a capillary column (SGE BP21, 30 m × 0.53 mm; SGE Europe Ltd., Milton Keynes, UK) as previously described [10]. The activities of selected bacterial enzymes (α-galactosidase, α-glucosidase, β-galactosidase and β-glucosidase) released into the cecal environment were measured according to the rates of p- and o-nitrophenol release from their nitrophenyl glucosides according to a previously described method [31]. The concentration of total BA in the caecum was measured using a colorimetric kinetic method with a commercial BA assay kit.

The BA in the caecum were assessed using an Agilent 1200 rapid resolution LC system (Stockport, U.K.) coupled to an AB Sciex 4000 QTrap triple-quadrupole mass spectrometer (Warrington, U.K.). Chromatographic separation was conducted using a Supelco Ascentis Express C18, 15 cm x 4.6 mm, 2.7-µm column (Sigma Aldrich) by gradient elution with 0.012% (v/v) formic acid in methanol (solvent B) and 5 mM ammonium acetate in water (solvent A). The column temperature was set at 40°C, the flow rate was 0.6 mL/min, and the gradient program was as follows: 0 – 2 min, 50% (v/v) B; 2 – 20 min, 50 – 95% (v/v) B; 20 – 22 min, 95% (v/v) B; 22– 23 min, 95– 50% (v/v) B; 23– 40 min, 50% (v/v) B. The injection volume was 5 µL. Mass data acquisitions were performed using Analyst 1.6.2 software (AB Sciex). The internal standards d4-cholic, d4-glycochenodeoxycholic, d4-glycocholic, d4-litocholic, d4-deoxycholic, and d4-chenodeoxycholic acids, and the relative response factor, were used to quantify the BA.

The liver fat mass was determined shortly after dissection by time-domain nuclear magnetic resonance (Minispec LF 90II, Bruker). After storage of the liver at -80°C, FGF19, FGFR4, FXR, PPARα and SHP-1 were determined using commercial ELISA kits (Cloud-Clone Corp). The concentration of total BA in the liver was estimated using a commercial BA assay kit (Blue Gene Biotech, Shanghai, China). Total hepatic cholesterol was measured in liver using standard diagnostic kits (Alpha Diagnostics) after lipid extraction by the Folch method [32].

2.3.2 Quantification of ellagitannin metabolites

The ET metabolites were evaluated in the colon and serum. Details concerning the sample preparation have been previously described [10]. The metabolites were determined using HPLC (Knauer Smartline system with a photodiode array detector, Berlin, Germany) coupled to a Gemini C18 column (110 Å, 250×4.60 mm; 5 µm, Phenomenex). The separation

conditions were the same as those used to determine the ETs in the dietary extracts. The ET metabolites were identified by comparisons of the UV spectra with the available data in the literature [33]. Furthermore, to confirm the results, the samples were dissolved in methanol and analyzed using HPLC-ESI-MS with a Dionex UltiMate 3000 UHPLC and a Thermo Scientific Q Exactive series quadrupole ion trap mass spectrometer. The ET metabolites were separated using a Kinetex C18 column (110 Å, 150×2.1 mm; 2.6 μm, Phenomenex) and a binary gradient of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B) at a flow rate of 0.5 mL/min as follows: stabilization for 1.44 min with 5% B, 5-15% B for 1.44-2.98 min, 15-40% B for 2.98-10.1 min, 40-73% B for 10.1-11.5 min, 73% B for 11.55-12.7 min, 73-5% B for 12.7-13.28 min, and 5% B for 13.28-18 min. Urolithin-A isolated from human urine via semipreparative HPLC was used as the standard to quantify the ET metabolites. The detailed procedure used for the isolation of urolithin-A has been described elsewhere [33].

2.4 Statistical analysis

STATISTICA software (version 10.0; StatSoft Corp., Krakow, Poland) was used to determine whether variables differed among the treatment groups. The results were calculated statistically using one-way analysis of variance (ANOVA) and Duncan's multiple range test. Differences were considered significant at $P<0.05$. The results are presented as the mean values ± the standard error of the mean (SEM), except for the phenolic composition of raspberry pomaces expressed as the mean and the standard deviation (SD) of the mean. In order to present a reliable value for the phenolic composition the SD was calculated from 3 samples.

3. RESULTS

After an 8 week feeding period, the preparations of raspberry pomaces did not affect dietary intake or animal growth (data not shown). Cecal/colonic tissue and the relative mass and pH of the digesta in the intestinal segments were comparable among all experimental groups (Table 3). All of the animals fed high-fat diets displayed a significant increase in fat accumulation in the liver ($P<0.05$ vs. C). The control high-fat diet (group HF) had the highest cecal ammonia concentration, but treatment with HFSG and HFNGs reduced these values to levels similar to those in the C group (Table 4). There were no significant differences in the cecal activity of selected microbial enzymes or the concentration of SCFAs between the HF and C group. The highest cecal activity of α-galactosidase ($P<0.05$ vs. C and HF) and β-glucosidase ($P<0.05$ vs. all groups except HFSG) and the highest total SCFA cecal concentration ($P<0.05$ vs. C and HFSG) were noted in the HFNG group. Additionally, both dietary treatments with native pomaces (with seeds, irrespective of grind type) significantly increased the cecal activity of bacterial α-galactosidase in comparison to the HF group. Only HFNG treatment significantly elevated content of butyric acid in the cecal digesta ($P<0.05$ vs. all other groups). Interestingly, both finely ground raspberry pomaces significantly increased the concentration of acetic acid in the cecal digesta ($P<0.05$ vs. C). Irrespective of the grind type, the preparation of seedless raspberry pomace significantly increased the blood serum concentration of ellagic acid dimethyl ether glucuronide (DMEAG) (Table 5).

The effect of the dietary raspberry pomaces on the BA profile in the cecal digesta is shown in Figure 1. The high-fat diet used in this study significantly increased the cecal concentration of cholic (CA), deoxycholic (DCA) and β-muricholic (β-MCA) acids, compared with the control C group. However, for all the aforementioned acids, this effect was reduced by dietary application to the high-fat diet of both raspberry pomaces with seeds. Treatments with seedless pomaces caused a significant decrease in CA and β-MCA, but not in the DCA cecal concentration in comparison to the HF group. The fine grinding of dietary

raspberry pomace with seeds (group HFNGs) resulted in a significant reduction of litocholic (LCA) and ω-muricholic acid (ω-MCA) concentrations in the cecal digesta ($P<0.05$ vs. all groups including C). In the case of the fine grinding of seedless raspberry pomace, such manipulation significantly reduced the cecal concentration of MCA ($P<0.05$ vs. all other groups fed high-fat diets). The highest cecal concentration of chenodeoxycholic acid (CDCA) was found in the HFSGs rats ($P<0.05$ vs. C and HFNG). The raspberry pomaces examined also modulated the profile of conjugated BA in the rat caecum (Figure 2). The dietary HFNGs treatment resulted in a significant decrease in the cecal concentrations of glycocholic (GCA; $P<0.05$ vs. other groups), glycolithocholic (GLCA; $P<0.05$ vs. C), taurodeoxycholic (TDCA; $P<0.05$ vs. other groups), taurochenodeoxycholic (TCDCA; $P<0.05$ vs. C and HF) and tauroursodeoxycholic (TUDC; $P<0.05$ vs. other groups except HFSG) acids. Concomitantly, the HFNGs group was characterized by the highest concentrations of glycochenodeoxycholic (GCDCA; $P<0.05$ vs. C) and taurolithocholic (TLCA; $P<0.05$ vs. all other treatments with raspberry pomaces) acids in the cecal digesta. The highest cecal concentration of tauro-α-muricholic (T-α-MCA) and tauro-β-muricholic (T-β-MCA) acids was noted following HFNG treatment ($P<0.05$ vs. HFSG and HFNGs, as well as $P<0.05$ vs. all other groups, respectively).

The total BA concentration in the cecal digesta was comparable among all groups (Table 6). However, the control high-fat diet (without pomace application) displayed significantly ($P<0.05$) elevated total BA concentrations in the liver in comparison to group C. Excluding HFSG, all treatments with raspberry pomaces effectively reduced the liver values of total BA to the levels observed in C rats. The highest cholesterol concentration in the liver was noted in the HF group ($P<0.05$ vs. all other treatments except HFNG). Among the groups fed high-fat diets, the HFNGs rats were characterized by the lowest liver cholesterol concentration ($P<0.05$ vs. all other groups fed high-fat diets). The two groups fed diets with

seedless pomaces had the highest levels of SHP-1 ($P<0.05$ vs. HF and HFNGs groups). The dietary treatments with pomaces containing seeds caused significant reductions in liver FGFR4 levels compared with HF, and both treatments with seedless pomaces resulted in levels comparable to that in group HF ($P>0.05$ vs. HF). The highest levels of hepatic FGF19 and PPARα were noted in the HFSG group, while the lowest levels of those parameters were detected in the HFNGs group.

4. DISCUSSION

The results of the present study indicated that raspberry pomaces were a valuable source of not only dietary fiber but also fat and polyphenols, most of which were ETs, FLs and ACs. Other studies of raspberry pomaces have reported a very wide range of these compounds, especially polyphenols [34, 35]. The concentrations of these substances are strongly influenced by many factors, e.g., genotype and extrinsic factors such as agricultural practices, the environment and maturity [36]. Differences in the chemical composition of the examined raspberry pomaces were also caused by the level of grinding and the presence of seeds in the pomace. Most of the polyphenols accumulate in the seedless fraction of the pomace [37]; consequently, an increased concentration of these bioactive compounds was observed after the removal of seeds from the raspberry pulp. Additionally, the disruption of complexes between fibers and polyphenols by fine grinding increased the concentration of these bioactive compounds. Moreover, approximately 80% of the raspberry pomace comprises seeds [38], which consist of approximately 23% oil enriched with polyunsaturated fatty acids (PUFAs) [25, 26]. The fine grinding of the native raspberry pomace released the accumulated lipid fraction from the seeds and thus increased the level of available fat in the examined raspberry pomace.

After feeding with a high-fat diet, a significantly higher concentration of ammonia was detected in the cecal digesta of the rats. A high concentration of this compound can destroy cells, alter nucleic acid synthesis and increase viral infections in the lower bowel [39]. HFSG and $HFNG_s$ treatments reduced the unfavorably high levels of ammonia in the caecum. Cecal ammonia synthesis is mostly modulated by microbial activity which may be changed by diet enriched with polyphenols. HFNG treatment significantly elevated the production of bacterial SCFAs, especially acetic and butyric acid. Butyrate in the colonic enterocytes is rapidly absorbed and metabolized, whereas acetic acid is converted to acetyl-CoA contributing to lipogenesis [40]. HFNG treatment also significantly increased the activity of bacterial α-galactosidase and β-glucosidase in the caecum. These microbial enzymes assist in the hydrolysis of indigestible oligosaccharides. Thus, higher activity of galactosidases may decrease colonic fermentation and gas production, which is undesirable, especially in people with irritable bowel syndrome [41]. However, some authors have suggested that galactosidases could be responsible for the generation of detrimental metabolites, e.g., from kaempferol-3-O-galactoside-rhamnoside-7-O-rhamnoside [42]. The observed changes in activity of the bacterial enzymes might be associated with the type and amount of polyphenols, as well as dietary fat in the diet. The raspberry pomaces used in this study were a rich source of ETs and ACs, which have antibacterial properties against certain groups of intestinal bacteria such as Clostridium leptum, Campylobacter spp., and E. coli CM 871 [10, 11, 43, 44]. A previous study of rats fed a diet supplemented with raspberry polyphenol extracts obtained from pomace, showed that the intensity of the cecal microbiota activity might be related to the dosages and profiles of dietary polyphenols, e.g., the ET to FL ratio [45]. The inhibitory effects of plant phenols could be caused by their covalent attachment to reactive nucleophilic sites in an enzyme, such as free amino and thiol groups or tryptophan residues [46]. A diet high in saturated fat can also affect the microbial diversity, which may

be due to an overflow of dietary fat into the distal parts of the intestine [47]. Our previous studies have shown that obesogenic diet rich in saturated fatty acids decreased microbial glycolytic activity and metabolite formation in the distal intestine [9, 48]. The modulation of bacterial activity may also be associated with the secretion of BA into the small intestine, which are potent antimicrobial agents both alone and in conjunction with diet-derived fatty acids [49].

BA are synthesized from cholesterol in the liver and further metabolized by the gut microbiota into secondary BA, e.g., DCA and LCA. A high-fat diet is known to stimulate the secretion of BA into the intestinal lumen [1, 2]. Secondary BA are considered to be cytotoxic to colonic crypt cells, resulting in an increase in compensatory proliferation of colonic epithelial cells, which is associated with an increased risk of colon cancer [6-8]. In this study, a high-fat diet significantly increased the concentration of CA and DCA in the cecal digesta. The high levels of these BA were reduced after enriching the diets with raspberry pomace containing seeds. The concentration of DCA in the gastrointestinal tract might by modulated by bacterial deconjugation and dehydroxylation of CA from the small intestine, forming new DCA throughout the length of the colon [50]. Addition to the high-fat diet of finely ground raspberry pomace with seeds considerably reduced the concentration of CA conjugated to glycine (GCA), potentially explaining the lower level of CA and DCA in the cecal digesta. The reduced concentration of DCA after treatment with a finely ground preparation containing seeds may also be associated with a lower concentration of DCA conjugated to taurine (TDCA). Ngamukote et al. (2011) [51] suggested that gallic acid, catechin, and epicatechin might bind to TDCA and thus modulate the concentration of that BA in the gastrointestinal tract. Another study conducted in rats indicated that some dietary polyphenols, e.g., catechin, might reduce fecal BA, DCA and LCA [3]. Moreover, some intestinal microbes are able to utilize taurine as an electron acceptor for anaerobic respiration

and thus reduce the concentration of taurine-conjugated BA [52]. It can be assumed that the addition of raspberry polyphenols to the diet can selectively modulate the composition as well as the activity of the microbiota, thus altering the metabolism of the primary BA. Bravo et al. (1998) [53] have shown that the addition of mono- or PUFAs to the diet increased BA synthesis compared with saturated fatty acids in rats. In the present study the lipid fraction (rich in PUFAs) released from raspberry seeds reduced the BA concentration in the liver as well as the DCA, LCA, β-MCA and ω-MCA concentrations in the cecal digesta. These results raise the possibility that the compounds released from raspberry seeds in cooperation with polyphenols have the strongest effect on the synthesis of hepatic BA and their profile in the cecal digesta.

In the ileum and the colon, anaerobic bacteria promote the 7-alpha-dehydroxylation of the primary BA and the formation of secondary BA. These BA are more hydrophobic and more potent activators of FXR than the primary BA [54-56]. It is generally recognized that FXR negatively regulates BA synthesis directly in the liver by inducing the expression of SHP-1. In the present study, the high-fat diet had no significant effect on the hepatic levels of FXR and SHP-1. However, addition to the diet of a finely ground preparation without seeds markedly increased the levels of SHP-1, thereby reducing the concentration of BA in the liver. Sayin et al. (2013) [56] demonstrated that the microbiota exerts its effects not only within the gut but also in other parts of the enterohepatic system, for example, by regulating BA synthesis in the liver via an increase in the FXR-dependent activation of FGF19 in the ileum due to reduced levels of T-β-MCA. FGF19 binds to FGFR4 and suppresses the synthesis of BA in the liver [18, 20]. In the present study, HFNG treatment markedly increased the concentration of T-β-MCA, but there were no significant differences in the hepatic levels of FGF19 and FGFR4. A significant reduction of these two hepatic factors as well as the lower BA concentrations in the liver were observed in the group fed a diet

supplemented with finely ground raspberry pomace containing seeds. The BA synthesis might be also regulated by PPARα [21]. This receptor regulates intra- and extracellular lipid metabolism and stimulates reverse transport of cholesterol [57], which is essential for BA synthesis. Therefore, higher level of the PPARα may increase concentration of hepatic cholesterol and synthesis of BA. In this study, the high-fat diet increased the level of PPARα and also the concentration of cholesterol and BA in the liver. The enrichment of the high-fat diet with the finely ground preparation containing seeds significantly reduced the level of PPARα and all of the changes described in the liver. PPARα ligands may also modulate the BA profile by increasing and decreasing the synthesis of CA and CDCA in the bile, respectively [58]. Indeed, $HFNG_s$ treatment reduced the concentration of GCA and increased the concentration of GCDCA in the caecum. Additionally, some derivatives of ET metabolites that are transported in the blood may regulate the levels and proportions of the cholesterol fractions [59, 60], thereby regulating hepatic BA synthesis. In this study, we observed a significantly higher concentration of DMEAG in the serum of rats fed a diet supplemented with seedless raspberry pomaces.

In conclusion the high-fat diet used in this study led to disorders in the gastrointestinal tract and liver function and thus the BA profile in the rat caecum. Nevertheless, addition of finely ground raspberry pomaces containing seeds to the high-fat diet reduced the cecal ammonia concentration and improved the metabolism of BA in the caecum by decreasing the concentration of secondary BA, DCA and LCA. Moreover, the same raspberry preparation reduced the concentration of cholesterol and BA in the liver. The molecular mechanisms responsible for regulating hepatic BA synthesis after treatment with finely ground raspberry pomaces containing seeds might be associated with a reduction of FGF19, FGFR4 and PPARα levels in the liver. In contrast, finely ground seedless raspberry pomace increased activity of the β-glucosidase and production of butyric acid but did not change concentration

of ammonia as well as the undesirably high levels of DCA and LCA in the caecum. Furthermore, seedless raspberry pomace activated different molecular mechanisms regulating hepatic BA synthesis as manifested by increased levels of SHP-1 and PPARα. In summary, the presence of seeds and the grinding level of the raspberry pomace had significant effects on cecal microbial activity, BA profile and also hepatic BA synthesis.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

CONTRIBUTORS

BF, JJ and ZZ designed the research. BF, JJ, MS, KK, NR and AM conducted the research. BF and AJ analyzed the data and wrote the paper. BF has primary responsibility for the final contents.

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FIGURE CAPTIONS

Figure 1. Bile acid profile in the rat caecum. Values are means ± SEM. Mean values within a column with different superscript letters (a, b, c) were significantly different by Duncan's post hoc test ($P \leq 0.05$). CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, litocholic acid; MCA, muricholic acid; α-, β-, ω-MCA, α-, β-, ω-muricholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid.
C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and finely ground native raspberry pomace, respectively.

Figure 2. Profile of conjugated bile acids in the rat caecum. Values are the means ± SEM. The mean values within a column with different superscript letters (a, b, c) were significantly different by Duncan's post hoc test ($P \leq 0.05$). GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GLCA, glycolitocholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TLCA, taurolitocholic acid; T-α-, T-β-MCA, tauro-α-, β-muricholic acid; TUDC, tauroursodeoxycholic acid.
C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and finely ground native raspberry pomace, respectively.

Table 1. Chemical composition of the raspberry pomaces, %.

	Raspberry pomace, standard granulation	Raspberry pomace, fine granulation	Raspberry pomace seedless fraction, standard granulation	Raspberry pomace seedless fraction, fine granulation
Dry matter	93.13 ± 0.00	93.77 ± 0.05	93.23 ± 0.01	93.70 ± 0.02
Ash	1.68 ± 0.00	1.75 ± 0.02	2.74 ± 0.01	2.73 ± 0.05
Protein	11.20 ± 0.31	11.65 ± 0.12	19.64 ± 0.60	20.38 ± 0.14
Fat	11.44 ± 0.10	12.46 ± 0.17	9.19 ± 0.05	9.09 ± 0.08
Carbohydrates*	65.90	64.72	55.26	54.58
TDF	64.76 ± 0.15	61.98 ± 0.06	48.90 ± 0.49	47.00 ± 0.40
SDF	0.92	0.46	1.80	3.13
LM	1.14	2.74	6.36	7.58
Total polyphenols	2.91 ± 0.04	3.19 ± 0.05	6.40 ± 0.00	6.92 ± 0.04

*Carbohydrates=dry matter–(ash+protein+fat+polyphenolics).

TDF, total dietary fiber; SDF, soluble dietary fiber; LM, low-molecular carbohydrates.

Table 2. Polyphenol composition of raspberry pomaces.

	Raspberry pomace, standard granulation	Raspberry pomace, fine granulation	Raspberry pomace seedless fraction, standard granulation	Raspberry pomace seedless fraction, fine granulation
Ellagitannins (ET)				
Minor ET*	195.9 ± 6.5	215.8 ± 11.0	534.4 ± 3.4	569.9 ± 0.5
Lambertianin C	1322.4 ± 29.0	1509.8 ± 39.0	3744.7 ± 21.6	4080.3 ± 23.6
Sanguiin H-6	754.5 ± 20.4	844.6 ± 21.6	1510.8 ± 3.9	1631.2 ± 12.6
Ellagic acid	61.1 ± 3.1	70. 0 ± 1.9	103.0 ± 1.3	108.7 ± 1.4
Total ET	2333.9 ± 58.9	2640.2 ± 73.4	5892.9 ± 16.3	6390.0 ± 37.4
Flavan-3-ols (FL)				
Total FLAVA	544.4 ± 33.3	517.4 ± 17.7	425.7 ± 15.0	434.6 ± 14.5
(+)-catechin	7.6 ± 0.5	8.2 ± 0.1	12.9 ± 0.2	12.9 ± 0.2
(-)-epicatechin	101.3 ± 1.4	98.7 ± 0.8	64.8 ± 0.6	65.0 ± 3.1
Proanthocyanidins	435.5 ± 34.5	410.5 ± 18.0	348.0 ± 15.8	356.6 ± 13.6
Extension units (%)				
(+)-catechin	47.6 ± 1.0	47.5 ± 0.6	41.3 ± 0.2	39.3 ± 0.5
(-)-epicatechin	4.3 ± 0.2	5.5 ± 0.6	17.2 ± 1.6	18.6 ± 3.2
Terminal units (%)				
(+)-catechin	5.6 ± 0.2	5.6 ± 0.1	4.3 ± 0.2	5.6 ± 0.5
(-)-epicatechin	42.5 ± 1.2	41.5 ± 0.6	37.2 ± 1.5	36.5 ± 2.3
mDP	2.1 ± 0.0	2.1 ± 0.0	2.4 ± 0.1	2.4 ± 0.2
Anthocyanins (AC)**				
Cy-3-soph	13.8 ± 0.4	14.8 ± 0.4	35.2 ± 1.0	39.0 ± 3.7
Cy-3-glu-rut	1.0 ± 0.0	1.0 ± 0.0	2.3 ± 0.1	2.6 ± 0.2
Cy-3-glu	12.2 ± 0.4	13.3 ± 0.5	32.5 ± 1.2	35.9 ± 3.4
Cy-3-rut	1.0 ± 0.0	1.1 ± 0.1	3.0 ± 0.1	3.3 ± 0.3
Minor ACY	0.6 ± 0.0	0.7 ± 0.0	1.3 ± 0.1	1.4 ± 0.1
Total ACY	28.6 ± 0.9	30.9 ± 0.9	74.3 ± 2.5	82.2 ± 7.7
TPH	2908.9 ± 44.5	3193.6 ± 54.3	6400.9 ± 4.0	6917.8 ± 34.5

Values are expressed as the mean ± standard deviation (mg/100 g) ; Cy-3-spoh – cyanidin-3-O-spohoroside; Cy-3-glu-rut – cyanidin-3-O-glucosyl-rutinoside; Cy-3-glu – cyanidin-3-O-glucoside; Cy-3-rut – cyanidin-3-O-rutinoside; mDP – mean degree of polymerization; TPH – total polyphenols; * - the contents of these substances were calculated based on the sanguiin H-6 standard; ** - the contents of anthocyanins were calculated based on the cyanidin-3-O-glucoside standard.

Table 3. Tissue and digesta mass of the hindgut, as well as the liver mass and fat accumulation, in rats fed the experimental diets.

	Cecum			Colon			Liver	
	Tissue mass	Digesta mass	pH	Tissue mass	Digesta mass	pH	Tissue mass	Fat
	g/100 g BW	g/g cecal tissue		g/100 g BW	g/g cecal tissue		g/100 g BW	%
C	0.54 ± 0.02	1.27 ± 0.22	7.19 ± 0.08	0.88 ± 0.04	0.37 ± 0.08	6.84 ± 0.23	8.86 ± 0.29	18.52^{b} ± 1.30
HF	0.54 ± 0.02	1.01 ± 0.07	7.19 ± 0.06	0.98 ± 0.06	0.43 ± 0.12	6.87 ± 0.02	8.46 ± 0.28	23.46^{a} ± 0.70
HFSG	0.56 ± 0.03	1.41 ± 0.13	7.21 ± 0.07	0.94 ± 0.02	0.32 ± 0.10	6.76 ± 0.11	8.04 ± 0.23	23.04^{a} ± 1.15
HFNG	0.58 ± 0.03	1.27 ± 0.17	7.09 ± 0.04	0.94 ± 0.04	0.28 ± 0.05	7.04 ± 0.08	8.34 ± 0.34	23.89^{a} ± 1.32
$HFSG_S$	0.52 ± 0.02	1.18 ± 0.08	7.23 ± 0.06	0.87 ± 0.04	0.28 ± 0.03	6.58 ± 0.09	7.92 ± 0.31	26.05^{a} ± 1.60
$HFNG_S$	0.58 ± 0.02	1.11 ± 0.14	7.20 ± 0.07	0.86 ± 0.03	0.25 ± 0.06	6.75 ± 0.17	8.51 ± 0.25	24.07^{a} ± 1.06
P (ANOVA)	NS	NS	NS	NS	NS	NS	NS	**<0.01**

Values are the means ± SEM. The mean values within a column with different superscript letters (a, b, c) were significantly different by Duncan's post hoc test ($P \leq 0.05$). NS, nonsignificant data, $P > 0.05$.

C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and finely ground native raspberry pomace, respectively.

Table 4. Activity of microbial enzymes and concentration of SCFAs and ammonia in the cecal digesta of rats fed the experimental diets.

	Ammonia	α-gal	β-gal	α-glu	β-glu	SCFAs			
						acetic	propionic	butyric	Total
	mg/100 g digesta	μmol/h/g digesta				μmol/g digesta			
C	24.61^{bc} ± 1.62	8.62^{bc} ± 1.04	60.29 ± 7.75	19.63 ± 2.66	2.93^{b} ± 0.32	31.27^{b} ± 1.76	7.92 ± 0.74	3.26^{b} ± 0.34	45.61^{b} ± 2.87
HF	32.65^{a} ± 1.70	6.49^{c} ± 1.10	73.47 ± 10.92	19.59 ± 2.25	2.09^{b} ± 0.49	36.40^{ab} ± 2.06	9.21 ± 0.43	4.26^{b} ± 0.33	53.23^{ab} ± 2.64
HFSG	23.02^{c} ± 1.33	9.61^{abc} ± 1.07	56.50 ± 11.76	17.60 ± 3.57	3.43^{ab} ± 0.63	35.42^{ab} ± 1.07	8.68 ± 0.38	4.41^{b} ± 0.37	51.35^{b} ± 1.31
HFNG	31.24^{a} ± 1.26	12.29^{a} ± 1.22	60.84 ± 8.32	20.62 ± 2.95	4.54^{a} ± 0.46	40.89^{a} ± 0.93	10.17 ± 0.59	5.54^{a} ± 0.23	59.50^{a} ± 1.46
$HFSG_S$	29.83^{ab} ± 2.75	10.09^{ab} ± 1.22	82.62 ± 11.82	19.40 ± 5.44	2.86^{b} ± 0.50	36.53^{ab} ± 2.47	8.88 ± 0.56	3.85^{b} ± 0.27	52.02^{ab} ± 3.17
$HFNG_S$	26.25^{bc} ± 2.49	10.15^{ab} ± 0.71	59.59 ± 11.08	15.07 ± 3.13	2.70^{b} ± 0.64	36.94^{a} ± 1.73	10.04 ± 0.67	3.78^{b} ± 0.58	53.43^{ab} ± 3.06
P (ANOVA)	**<0.05**	**<0.05**	NS	NS	**<0.01**	**<0.05**	NS	**<0.01**	**<0.05**

Values are the means ± SEM. The mean values within a column with different superscript letters (a, b, c) were significantly different by Duncan's post hoc test ($P \leq 0.05$). α-gal, α-galactosidase; α-glu, α-glucosidase; β-gal, β-galactosidase; β-glu, β-glucosidase; NS, nonsignificant data, $P > 0.05$; SCFAs, short chain fatty acids.

C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and fine ground native raspberry pomace, respectively.

Table 5. Ellagitannin metabolite profiles in the colon digesta and serum of rats fed the experimental diets.

	Colon		Serum
	Nasutin	Urolithin A	Ellagic acid dimethyl ether glucuronide
	µg/g		µg/ml
C	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
HF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
HFSG	19.50 ± 35.43	8.72 ± 5.63	0.07^{a} ± 0.02
HFNG	29.13 ± 41.47	8.77 ± 4.71	0.08^{a} ± 0.02
$HFSG_S$	19.19 ± 10.75	6.69 ± 3.15	0.03^{b} ± 0.01
$HFNG_S$	7.55 ± 12.53	2.26 ± 6.40	0.03^{b} ± 0.01
P (ANOVA)	NS	NS	**<0.01**

C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and finely ground native raspberry pomace, respectively.

Table 6. Bile acids in the cecal digesta and liver and the concentration of cholesterol and indicators that regulate the synthesis of bile acids in the liver of rats fed the experimental diets.

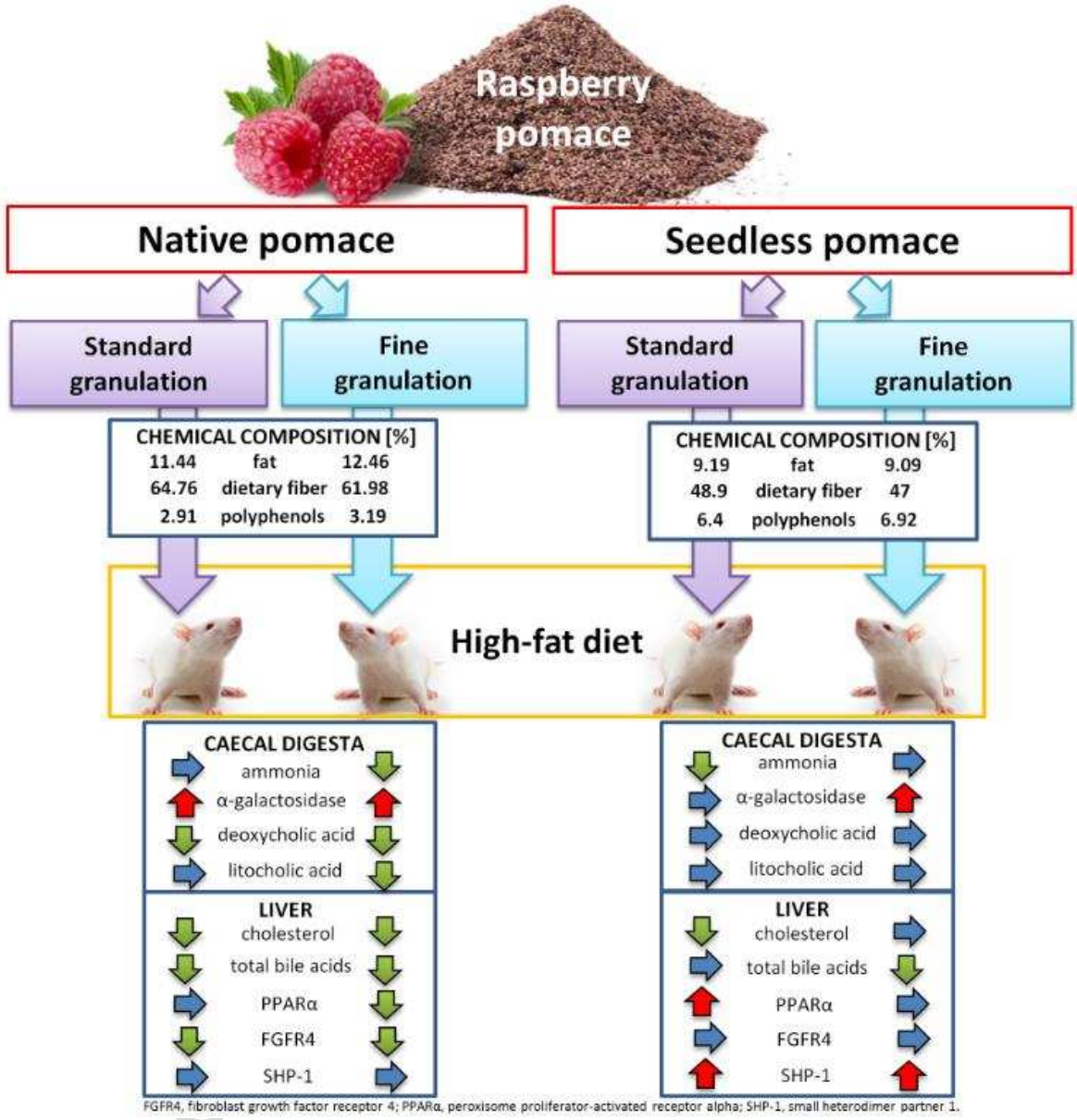
	Total bile acids in the caecum	Total bile acids in liver	Cholesterol	SHP-1	FGFR4	FGF19	PPARα	FXR
	µmol/g digesta	nmol/g liver	mg/g liver	ng/g liver	ng/g liver	ng/g liver	µg/g liver	ng/g liver
C	19.39 ± 2.17	1.72^{b} ± 0.12	3.61^{d} ± 0.27	1820^{ab} ± 207	1606^{b} ± 148	41.27^{bc} ± 3.12	21.13^{c} ± 0.88	1699 ± 111
HF	28.31 ± 1.92	2.83^{a} ± 0.19	8.16^{a} ± 0.25	1362^{b} ± 172	2353^{a} ± 158	51.42^{abc} ± 9.73	22.51^{b} ± 1.43	1764 ± 160
HFSG	24.09 ± 2.29	2.81^{a} ± 0.15	7.16^{b} ± 0.35	4487^{a} ± 644	2526^{a} ± 199	60.66^{a} ± 4.43	26.55^{a} ± 0.60	2027 ± 115
HFNG	21.75 ± 2.27	1.61^{b} ± 0.16	7.41^{ab} ± 0.38	4655^{a} ± 748	2449^{a} ± 176	54.96^{ab} ± 4.79	24.93^{ab} ± 0.66	2109 ± 107
$HFSG_S$	24.20 ± 1.86	1.81^{b} ± 0.28	7.10^{b} ± 0.29	1977^{ab} ± 255	1759^{b} ± 172	44.27^{abc} ± 3.71	22.84^{bc} ± 1.08	1972 ± 137
$HFNG_S$	19.97 ± 0.99	2.15^{b} ± 0.21	4.92^{c} ± 0.30	1472^{b} ± 205	1416^{b} ± 156	34.95^{c} ± 2.84	20.9^{c} ± 1.60	1727 ± 148
P (ANOVA)	NS	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.05**	**<0.01**	NS

Values are the means ± SEM. The mean values within a column with different superscript letters (a, b, c) were significantly different by Duncan's post hoc test ($P \leq 0.05$). FGF19, fibroblast growth factor 19; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid X receptor; NS, nonsignificant data, $P > 0.05$; PPARα, peroxisome proliferator-activated receptor alpha; SHP-1, small heterodimer partner 1.

C and HF were fed a control and high-fat diet, respectively; HFSG and HFNG were fed a high-fat diet containing 7% standard and finely ground seedless fraction of raspberry pomace, respectively; $HFSG_S$ and $HFNG_S$ were fed a high-fat diet containing 7% standard and finely ground native raspberry pomace, respectively.

Figure 1

Figure 2



Graphical abstract

Highlights

- Diet enriched with raspberry pomace reduces concentration of cholesterol in the liver.
- Fine ground raspberry pomace with seeds reduced cecal ammonia concentration.
- Raspberry pomace containing seeds reduced the formation of secondary bile acids.
- Fine ground preparation with seeds reduced levels of FGF19 and PPARα in the liver.