PAPER

Lowering of cholesterol bioaccessibility and serum concentrations by saponins: *in vitro* and *in vivo* studies†

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Using an *in vitro* digestion model, we studied the effect of six saponin extracts on the bioaccessibility of cholesterol and saturated fatty acids (SFAs). In the absence of saponins, around 78% of the available cholesterol was solubilized in the simulated intestinal fluids. The addition of two extracts, Quillaja Dry (QD) and Sapindin (SAP), was found to decrease cholesterol bioaccessibility to 19% and 44%, respectively. For both extracts, the main mechanism of this effect is the displacement of cholesterol molecules from the bile salt micelles, leading to formation of cholesterol precipitates that cannot pass through the mucus layer of the intestine. QD decreased strongly the SFA bioaccessibility as well, from 69 to 9%, due to formation of calcium–SFA precipitates, while SAP had no effect on SFA. We studied the *in vivo* activity of QD and SAP extracts by measuring serum cholesterol in mice fed with experimental diets within a 7-day period. Both extracts were found to prevent dietary hypercholesterolemia in mice fed on a cholesterol-rich diet. The other saponin extracts did not show any significant effect *in vitro* and, therefore, were not studied *in vivo*. The cholesterol lowering ability of Sapindin extract is reported for the first time in the current study.

1. Introduction

Saponins are biosurfactants composed of a rigid hydrophobic structure of a steroid or triterpenoid type which is linked to one, two or three hydrophilic sugar chains.[1] Saponins are known to possess a wide range of biological activities, such as enhancing cell membrane permeability, regulating nutrient uptake in the intestine, reducing protein digestibility, decreasing serum cholesterol, *etc*.[2–4]

The worldwide spread of atherosclerosis and its link to high serum cholesterol levels has motivated a thorough search for cholesterol-lowering saponin extracts.[5–14] Experiments with animals revealed that several saponins possess such hypocholesterolemic effects: alfalfa,[5–8] soy bean,[9–11] Quillaja,[9–11] Yucca,[12] Karaya[13] and digitonin.[14] As the saponins are very

poorly absorbed during digestion, the principal location of their action is believed to be in the intestine.[15]

There are two main mechanisms proposed in the literature to explain the lowering of serum cholesterol by saponins.[3] The first mechanism implies that saponins form insoluble complexes with cholesterol, thus inhibiting its intestinal absorption. This mechanism is supported by animal studies, which report that dietary intake of alfalfa saponins increases the fecal cholesterol output.[5] Furthermore, model experiments demonstrate the formation of insoluble alfalfa saponin–cholesterol aggregates when cholesterol crystals are placed in contact with alfalfa saponin solutions.[6]

The second mechanism suggests that saponins form large aggregates with bile salts (BS) in the intestine and thus inhibit ileal BS reabsorption. The latter effect triggers an increased synthesis of BS from cholesterol in the liver, which leads to depletion of serum cholesterol. Examples of this mechanism are soya saponins, which were observed to form large mixed aggregates with BS, molecular mass >10^6 a.u., which were not absorbed by rat intestine *in vivo*.[9]

Both mechanisms share the same key feature: they involve the formation of large, non-absorbable aggregates in the gastro-intestinal tract (saponin + cholesterol or saponin + BS), which directly or indirectly lower the serum cholesterol. One of the approaches for studying aggregation during gastro-intesti-

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nal digestion is to use *in vitro* digestion models.[16,17] These models allow determination of the morphology and chemical composition of the different aggregates formed during digestion, including the colloidal aggregates of BS – the so-called "dietary mixed micelles" or DMM.[18–21] Other advantages of the *in vitro* studies, compared to *in vivo* experiments, are that they offer better control over the experimental conditions, good reproducibility, lower cost and lack of ethical issues.

The DMM are small enough to pass through the intestinal mucus layer and are able to deliver nutrients or other substances to the vicinity of the enterocytes. Thus, the fraction of a substance that is present in small DMM is usually considered as the fraction that is available for absorption and is bioaccessible for the body. Bioaccessibility is believed to be proportional to bioavailability when the aqueous solubility of the substance is very low and the absorption is diffusion-limited, as is the case with cholesterol.[22,23] Therefore, the *in vitro* studies of cholesterol bioaccessibility are very suitable for preliminary screening of potential cholesterol-lowering saponins and for analyzing the mechanism of the respective saponin action.

So far, there have been no systematic studies on the relation between the effect of saponin extracts on cholesterol bioaccessibility *in vitro* and the levels of serum cholesterol. Therefore, we formulated two major aims of our study: (1) to determine the effect of several saponin extracts on the *in vitro* bioaccessibility of cholesterol and saturated fatty acids (SFA), and (2) to check if the observed trends translate into real effects on serum cholesterol *in vivo*. We studied the bioaccessibility of SFA as well, because the latter are known to affect the cholesterol metabolism by stimulating its synthesis in the liver[24] and because there are no reports on the effect of saponin extracts on SFA bioaccessibility during *in vitro* digestion. The colloidal mechanisms behind the observed effects are also studied and discussed (section 4).

Most of the experiments were performed with the saponin extracts Quillaja Dry (QD), obtained from the *Quillaja saponaria* tree and Sapindin (SAP), from *Sapindus trifoliatus*. QD was chosen for two main reasons: (1) it is approved as a food additive in the USA (FEMA no. 2973) and EU (E999) and is used as an emulsifier and a foamer in several food technologies; (2) it is known to lower serum cholesterol in rats[10] and to induce cholesterol precipitation *in vitro*.[25] Therefore, the main purpose of the experiments with QD was to serve as a positive control, thus validating the used *in vitro* and *in vivo* methods. On the other hand, there are no reports of the effect of SAP on cholesterol and SFA, neither *in vitro* nor *in vivo*. Therefore, the observed cholesterol-lowering effect of SAP extract is reported for first time in the current paper. The other four saponin extracts did not show significant effects *in vitro* and, therefore, were not studied *in vivo*.

The *in vitro* part of our study is performed using a recently developed digestion model, which has the advantage of using sodium bicarbonate for buffering (as it is *in vivo*) and matches closely the pH-profile in the small intestine.[26] Filtration through a 200 nm cut-off filter is used to separate the clear aqueous phase containing small DMM that should be able to pass through the intestinal mucus layer. Gas chromatography is applied to determine the degree of fat digestion and the concentrations of solubilized cholesterol and SFA in the DMM. Bioaccessibility is defined as the fraction of cholesterol or SFA solubilized in the DMM, at the end of the *in vitro* digestion experiment.

In the *in vivo* part of our study, we fed male mice (7 days) with experimental diets enriched in saturated fat, cholesterol and/or saponin extract. After the end of the experiment, we collected blood from the animals and analysed it for serum lipids: total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides (TG). Good agreement was observed between the results obtained *in vivo* and *in vitro* for both QD and SAP extracts.

The article is organized as follows: the used materials and methods are described in section 2. Section 3.1 presents the results for the effect of saponins on the cholesterol and SFA bioaccessibility, obtained *in vitro*. Section 3.2 presents the results from the *in vivo* experiments with mice. The experimental results are discussed in section 4, and the main conclusions are summarized in section 5. Additional information about specific details in the methods used and some auxiliary results are provided in the ESI†.

2.   Materials and methods

2.1.   Materials

2.1.1.   Saponin extracts. Quillaja Dry 100 NP extract from the *Quillaja saponaria* tree was kindly donated by Desert King Company, Chile. It is obtained by spray-drying of non-purified extract of Quillaja bark and contains 26 wt% Quillaja saponins (according to HPLC analysis by the producer), 1.25 wt% calcium(II) ions (soluble form, determined by AAS, in our experiments), polyphenols, phenolic acids and polysaccharides.[27] The extract powder is coloured in brown, due to the presence of polyphenols (the purified saponins are colourless). The average molecular weight of QD saponins is *ca.* 1650 g mol^{-1}.[28,29]

The extract from *Sapindus trifoliatus*, SAP, was purchased from Sabinsa Corporation, USA, and contains 50 wt% saponins, according to the manufacturer. This extract is also in the form of dark-brown powder, indicating the presence of polyphenols. It has a low Ca^{2+} concentration of 0.04 wt%, determined by atomic absorption spectrometry. A molecular weight of 850 g mol^{-1} was used for the saponins in SAP.[30]

The following saponin extracts were studied in the *in vitro* digestion model only: Ayurvedic saponin concentrate (ASC) obtained from *Acacia concinna* (30% saponins, Ecological Surfactants LLC), Escin (ESC) obtained from *Aesculus hippocastanum* (95% saponins, Sigma), Ginsenosides (GS) obtained from *Panax ginseng* (80% saponins, Xianyang Hua Yue Biol. Engin. Co., Ltd) and Fenusterols (FEN) obtained from *Trigonella foenum-graecum* (50% saponins, Sabinsa Corp.).

2.1.2. Enzymes, lipids and inorganic salts. Pancreatin from porcine pancreas, 4× USP specification, was obtained from Sigma-Aldrich (Cat. N P1750). It contains pancreatic lipase and colipase at a molar ratio of 1 : 1[31,32] and a range of other enzymes, such as amylase, trypsin, ribonuclease and protease. The lot number of the used sample was 029K1095, with a lipase activity of at least 8 USP units, as declared by the manufacturer. One unit corresponds to the amount of pancreatin that liberates 1 μEq of fatty acid (FA) per minute, at pH = 9 and 37 °C, using an olive oil emulsion as a substrate.

As a bile salt source we used porcine bile extract, obtained from Sigma-Aldrich (Cat. No. B-8631), which contains 50 wt% bile acids, 6 wt% phosphatidylcholine and less than 0.06 wt% Ca^{2+}.[33] Gas chromatographic analysis showed that it contains also 1.24 ± 0.18 wt% cholesterol and 6.7 wt% FA.

Pepsin from porcine gastric mucosa (Fluka, Cat. No. 77160) with lot number 1 238 420 was used in the "stomach" stage of the *in vitro* model. The pepsin activity was 643 U mg^{-1}, as declared by the manufacturer. One unit here corresponds to the amount of enzyme which increases the light absorbance at 280 nm by 0.001 per minute at pH = 2.0 and 37 °C using hemoglobin as the substrate.

All aqueous solutions were prepared using deionized water from water-purification system Elix 3 (Millipore, USA). For preparation of electrolyte solutions we used NaCl, KCl (Merck), $CaCl_2$ (Fluka) and $NaHCO_3$ (Teokom), all of purity higher than 99%.

Cholesterol was purchased from Sigma (>95%, Cat. No. 26740).

Cocoa butter (Chemax Pharma Ltd) and sunflower oil (SFO, Papas oil Ltd) were purchased from local producers and used without purification.

2.2. Preparation of emulsions for *in vitro* digestion

Oil-in-water emulsions were used as a source of TG in the *in vitro* digestion experiments. Emulsions from two types of fats were prepared: sunflower oil (SFO, containing mainly unsaturated fatty acids, UFA) and cocoa butter (primarily composed of SFA). The FA composition of these oils is described in detail in Table S1 in the ESI†.

The emulsion of cocoa butter was prepared in the following way: first, the cocoa butter was melted at 50 °C, then 30 mL of it were added to a 20 mL emulsifier solution, which was also thermostated at 50 °C. Then, emulsification was performed with a rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA-Labortechnik), operating at 13 500 rpm for 5 min at 50 °C. The emulsifier solution contained 1 wt% surfactant Tween 80 (product of Sigma), 10 mM NaCl and 0.1 g L^{-1} NaN$_3$ (as a preservative). The same protocol was followed for the SFO emulsion, however, at room temperature (20–25 °C).

The drop size distribution in the emulsion was determined by using video-enhanced optical microscopy.[34,35] The diameters of the recorded oil drops were measured using the custom-made image analysis software. For each sample, the diameters of at least 1000 drops were measured. The accuracy

of these optical measurements was found to be ±0.3 μm.[35] The mean drop size in the studied emulsions was characterized by the so-called volume–surface diameter, d_{32}, which was calculated from the relationship:

$$d_{32} = \sum N_i d_i^3 / \sum N_i d_i^2 \qquad (1)$$

where N_i is the number of drops with diameter d_i. Our emulsions had $d_{32} = 20 \pm 3$ μm for sunflower oil and $d_{32} = 13 \pm 2$ μm for cocoa butter.

The obtained stock emulsions were immediately used in lipolysis experiments: the required amount of the stock emulsion was taken by a pipette and diluted in the electrolyte–enzyme solutions, as explained in section 2.3.
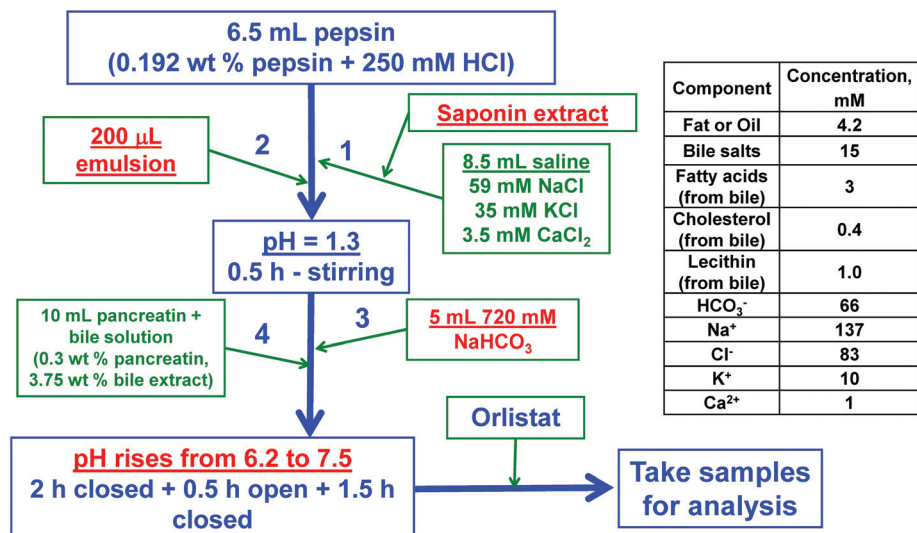
2.3. *In vitro* digestion model

The used *in vitro* model is described in detail by Vinarov *et al.*[26] Briefly, it consists of two stages which simulate the digestion in the stomach and the small intestine, respectively (see Fig. 1). In the "stomach" stage, the pH is acidic (pH = 1.3) and the protease pepsin is present. In the following "intestinal" stage, we introduce sodium bicarbonate to increase the pH to around 6 (as it is *in vivo*) and then we add a mixture of bile salts and pancreatin (containing pancreatic lipase, proteases and other digestive enzymes). Note that the reaction mixture contains lecithin, cholesterol and FA also, which originate from the used bile extract. The pH increases gradually from 6.2 to 7.5 for 4 h after addition of sodium bicarbonate in the "intestinal" stage of the experiments, mimicking the pH-profile observed *in vivo* (see Vinarov *et al.* for more details[26]).

Saponin extract was dissolved in saline solution containing 59 mM NaCl, 35 mM KCl and 3.5 mM $CaCl_2$ at final concentrations one day before the actual *in vitro* experiment. The pepsin, bile salts and pancreatin solutions were prepared directly at 37 °C, just before their use in the actual experiments.

After the end of the total reaction time of 4.5 h, we added the irreversible lipase inhibitor Orlistat (Xenical®, Roche). Afterwards the oil soluble components in the sample were extracted with chloroform and analyzed by GC (see ESI† methods) or the sample was filtered to obtain the aqueous phase for analysis of bioaccessibility.

2.4. Phase separation by filtration

To determine the cholesterol and SFA bioaccessibility at the end of the *in vitro* digestion experiment, we separated the DMM (containing bile salts, phospholipids, cholesterol and the lipolysis products) from the emulsion oil droplets and the solid precipitates by filtration. The reaction mixture was first filtered through a filter paper with a pore size of 2–3 μm and 84 g m^{-2} weight (BOECO, Germany). The filtration was carried out in a glass funnel and the filtrate was collected in a glass flask. Afterwards the obtained permeate was further filtered through a 200 nm nylon filter (Minisart NY25, Sartorius, Germany) using a syringe. The obtained permeate was clear and was then subjected to chloroform extraction and GC analy-

Fig. 1 Schematic presentation of the protocol for mixing the reagent solutions and performing the lipolysis reaction in the used *in vitro* digestion model. The inserted table shows the molar concentrations of the main components in the complete reaction mixture, obtained after mixing all solutions (in the "intestine" stage).

sis (see ESI† methods). All filtration operations were performed at 37 °C.

2.5. Design of the *in vivo* study

Male ICR mice (18–25 g) were obtained from Hristova's farm (Sofia, Bulgaria). The animals had access to water *ad libitum* and were maintained at 24 ± 2 °C with a 12 h/12 h light/dark cycle. The animals were divided in groups of five animals each. The animals were fed using standard rodent pellet food (18% protein, 12% fibre), supplemented with different additives: edible oil, oil + saponin, oil + cholesterol, oil + cholesterol + saponin, or no additive (control). To induce dietary hypercholesterolemia, we used a cholesterol concentration of ≈0.4 wt% in the food, for the high-cholesterol diets. As shown in the Experimental results section, the latter was sufficient to increase significantly the total serum cholesterol. The concentration of saponin extracts in the food was 4.1 wt% saponins for SAP and 3.6 wt% saponins for QD. The weight ratio of saponin to cholesterol in the diets is approx. 10 : 1, which corresponds roughly to their weight ratio in the *in vitro* digestion experiments, performed at 0.165 wt% saponin and 0.015 wt% cholesterol (corresponding to ≈0.4 mM cholesterol). Edible oil was added at a concentration of ≈4.3 wt% in the food, which is the fat content of typical rodent foods.

Feeding of the animals was carried out twice a day, at 12 h (light-correlated) interval – 4 g per animal in the morning and 6 g per animal in the evening. The body weight of the animals was measured daily. The animals were sacrificed on the 7th day, two hours after last morning feeding. Blood samples were collected (cardiac puncture) after anaesthesia.

This is purely an academic study, which has been neither requested nor supported by any means by national or international companies or contract research organisations. All experiments and procedures with animals were performed in the period of 01.10.2012 to 15.11.2012, in accordance with the Public Health Service (PHS) policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, and under the supervision of the Ethical Committee on Animal Experiments of the Bulgarian Academy of Sciences.

2.6. Serum TG and cholesterol analyses

Blood serum was used as a sample for analyses of serum TG, total cholesterol (Chol), and high-density lipoprotein cholesterol (HDL-C). The low-density lipoprotein cholesterol (LDL-C) concentration was calculated using Friedewald's formula: (LDL-C) = (Total cholesterol) − (HDL-C) − (TG)/2.2, where all concentrations are in mmol L^{-1}. The analyses were performed using the clinical chemistry analyzer Mindray BA – 88A (Shenzhen Mindray Bio-Medical Electronics, China) and kits from Sentinel Diagnostics (Italy).

2.7. Statistical analysis

Statistical analysis of the data from the *in vivo* experiments was performed by one-way ANOVA (GraphPad Prism software, California, USA), using Tukey's *post-hoc* analysis for multiple comparisons. The data are expressed as mean values ± SEM. The probability value of $p < 0.05$ was considered statistically significant.

3.   Experimental results

3.1. Effects of saponin extracts on the bioaccessibility of cholesterol and SFA: *in vitro* digestion experiments

In this section, we present the effect of the studied saponin extracts on the bioaccessibility of cholesterol and SFA. The results are obtained using the *in vitro* model described in

section 2.3, which mimics closely the composition and the pH profile in the human gastro-intestinal tract. Bioaccessibility is defined as the percentage of cholesterol or SFA solubilized in the DMM at the end of the digestion experiment. We used oil-in-water emulsions as a source of TG, which were hydrolysed to FA and monoglycerides (MG) by the pancreatic lipase added in the "intestinal" stage of the lipolysis model. The cholesterol in the reaction mixture originated from the bile extract.

3.1.1.   **Degree of TG lipolysis.** The solubilization of cholesterol in the simulated intestinal fluids depends significantly on the concentration of FA and MG,[26,36,37] which are the reaction products of the TG lipolysis. The saponin extracts could influence the speed and extent of TG digestion, resulting in different final concentrations of the reaction products. Therefore, we first investigated the effect of saponins on the degree of TG lipolysis for cocoa butter (rich in saturated fatty chains) and SFO (rich in unsaturated fatty chains).

The obtained results showed that the studied saponin extracts have no significant effect on the TG lipolysis of cocoa butter emulsions (see ESI† Fig. S2A): TGs were completely hydrolysed and the main reaction products were MG and FA for all saponin extracts and concentrations studied. Only a minor fraction of the TG was transformed to diglycerides or glycerol. Similar results were obtained with SFO emulsions in the presence of QD, see ESI† Fig. S2B.

The partial hydrolysis of TG to glycerol by the pancreatic lipase is most likely due to isomerization of the *sn*2-MG to *sn*1-MG, which is then hydrolysed by the lipase.[38] In our previous work, we found that up to 50% of the TG can be hydrolysed to glycerol during *in vitro* digestion at long reaction times.[39]

3.1.2.   **Cholesterol and SFA bioaccessibility.** We studied the effect of saponin extracts on cholesterol and SFA bioaccessibility by determining cholesterol solubilization in DMM at the end of the *in vitro* digestion experiment. As a fat source we used cocoa butter or SFO emulsions. We separated the micelle-containing aqueous phase by filtration of the reaction mixture (section 2.4). Bioaccessibility is presented as the ratio (in percent) of solubilized cholesterol/total cholesterol in the mixture.

The effect of the different saponin extracts on the bioaccessibility of cholesterol and SFA after lipolysis of cocoa butter emulsions is presented in Fig. 2. These series of experiments were performed at a constant concentration of saponin in the digestion experiment of 0.165 wt%. One sees that in the absence of saponins, *ca.* 78% of cholesterol is solubilized in the DMM and is thus, bioaccessible. Two of the studied saponin extracts decrease strongly the bioaccessibility of cholesterol, from 78% to 33% and 54% for QD and SAP, respectively (Fig. 2A). All other extracts studied (ASC, ESC, GS and FEN) have no significant effect.

In respect to the effect of the saponin extracts on SFA, QD was found to decrease significantly SFA bioaccessibility, from 69% to 33%, see Fig. 2B. There was no noticeable effect of any of the other extracts.

Further *in vitro* and *in vivo* experiments are performed with QD and SAP extracts only, as these two extracts showed significant effects on *in vitro* cholesterol bioaccessibility.

The effect of saponin concentration on cholesterol bioaccessibility is presented in Fig. 3A. The increase of SAP concentration decreases significantly the cholesterol bioaccessibility from 78% to 45% at the highest SAP concentration of 0.35 wt% saponins. To check whether the "missing" cholesterol is precipitated, we performed complementary experiments in which we centrifuged the reaction mixture and analyzed the sediment. This analysis confirmed that the non-solubilized cholesterol is captured in the sediment (45% of the cholesterol were solubilized in the DMM and 55% were found in the sediment).
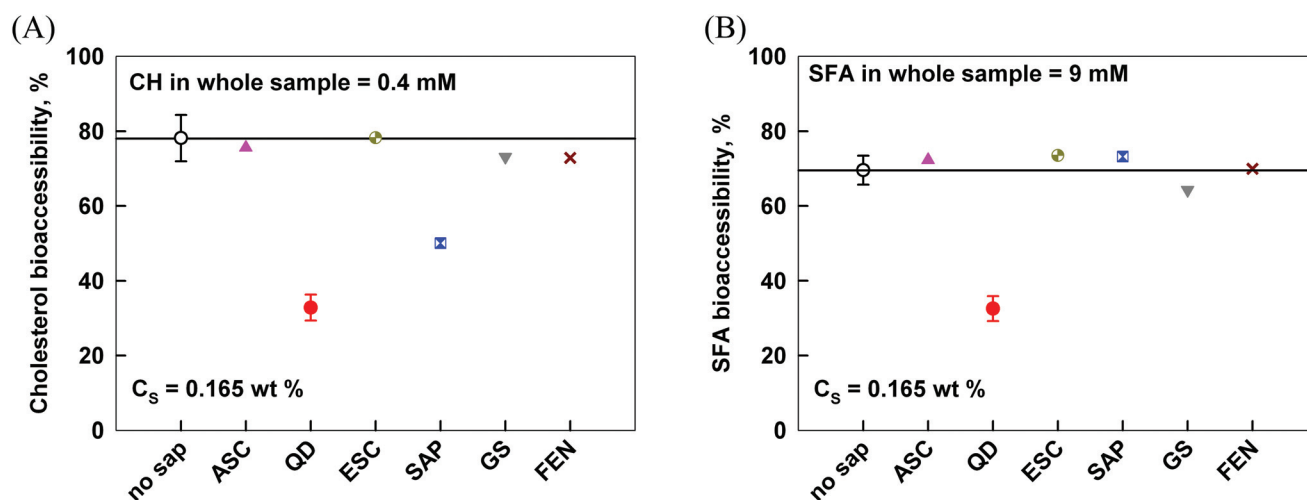

Fig. 2 Bioaccessibility of (A) cholesterol and (B) saturated fatty acids (SFA), as a function of saponin type at a constant saponin concentration of 0.165 wt% in the *in vitro* digestion experiment. The experiments are performed with cocoa butter emulsions. The solid horizontal line shows the bioaccessibility of cholesterol and SFA in the absence of saponins. The data for QD and that in the absence of saponins are averaged over at least 2 independent experiments (see Fig. 3 for other concentrations of QD and SAP). The other data are from single experiments.
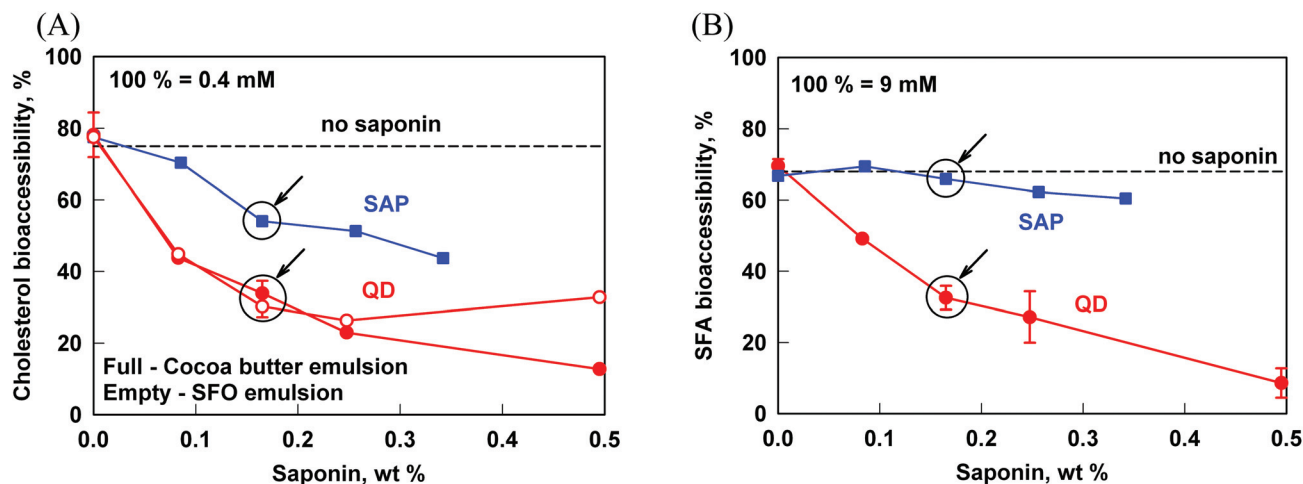
Fig. 3 Bioaccessibility of (A) cholesterol and (B) saturated fatty acids (SFA), as a function of weight concentration of saponin for QD (red circles) and SAP (blue squares). The emulsions used in the *in vitro* digestion experiment were prepared from either cocoa butter (full symbols) or sunflower oil (empty symbols). The circles indicate the results for the saponin concentration used in the *in vivo* experiments. The dashed horizontal lines show the bioaccessibility of cholesterol and SFA in the absence of saponins. The data for QD and cocoa butter emulsions are averaged over at least 2 independent experiments. The other data are from single experiments.

The QD extract has even stronger influence on cholesterol bioaccessibility: the solubilization of cholesterol is decreased to only 13% of the total cholesterol, at a QD concentration of 0.5 wt% saponins. In previous studies, we found that QD also acts by inducing cholesterol precipitation.[25] Comparing the effects of the two extracts at similar saponin weight concentrations, one sees that QD is more efficient than SAP in decreasing cholesterol bioaccessibility. The mechanisms that govern the effect of saponins on the solubilization of cholesterol in DMM are discussed in section 4.1.

To check whether the type of fat substrate (SFO or cocoa butter) can also impact cholesterol bioaccessibility, we performed additional experiments with the QD extract. One sees that the type of fat has no significant effect on the bioaccessibility of the cholesterol (Fig. 3A). An exception is the highest concentration of QD, where cholesterol bioaccessibility is higher when SFO emulsion is used. This is probably due to the higher concentration of solubilized FA after lipolysis of SFO, which was shown to enhance the cholesterol solubilization in the DMM.[39]

The effect of QD and SAP on the bioaccessibility of SFA is presented in Fig. 3B. QD decreases SFA bioaccessibility from 69% to 9%, whereas SAP has no effect. In our previous work, we found that the effect of QD is due to its high Ca^{2+} content (1.25 wt%), which leads to formation of insoluble Ca-SFA precipitates.[25] On the other hand, SAP extract contains only $\approx$0.04 wt% Ca^{2+}, which explains the lack of effect on SFA for this extract. The data for the effect of QD on the bioaccessibility of SFA after lipolysis of SFO are not shown, because the measured concentration of SFA is very low (SFO contains less than 13% SFA).

Summarizing, *in vitro* lipolysis experiments showed that QD and SAP reduce significantly the cholesterol bioaccessibility (QD having a bigger effect) and QD decreases SFA bioaccessi-

bility, whereas the other extracts did not demonstrate such effects. Therefore, we continued our investigation by studying *in vivo* the effects of SAP and QD with mice.

3.2. *In vivo* experiments with mice

The animals were divided in groups and fed with standard rodent food, containing different additives: triglyceride oil, oil + saponin, oil + cholesterol, or oil + cholesterol + saponin (section 2.5). All experimental foods were formulated at a weight ratio of around 10 : 1 saponin : cholesterol, corresponding to a saponin concentration of 0.165 wt% in the *in vitro* digestion experiments (the *in vitro* cholesterol concentration was 0.4 mM = 0.015 wt%).

3.2.1. Weight of mice during the experiment. To check whether the caloric content of the experimental diets affects the weight of the mice, we plotted the average mouse weight in each group during the course of the experiment (ESI† Fig. S3). No significant difference between the weights of the animals in the different groups was observed. Therefore, the addition of fat, cholesterol and/or saponin extract to the standard rodent food had no significant effect on the weight of the mice for the studied 7-day period.

3.2.2. Effects of saponin extracts on serum lipids. After feeding the animals for seven days with the experimental diets, we analysed the concentration of TG, total cholesterol, LDL- and HDL-cholesterol in their blood serum. All results are presented in percent, relative to the control group which is fed only with the standard rodent food. The results for each group are averaged over all five animals and the respective standard deviations are presented. Only cocoa butter, rich in saturated FA (approx. 65 molar%), was used in the experiments described in the current section 3.2.2.

(A) Serum cholesterol. The results for the total and LDL-cholesterol (LDL-C) are presented as a function of the type of
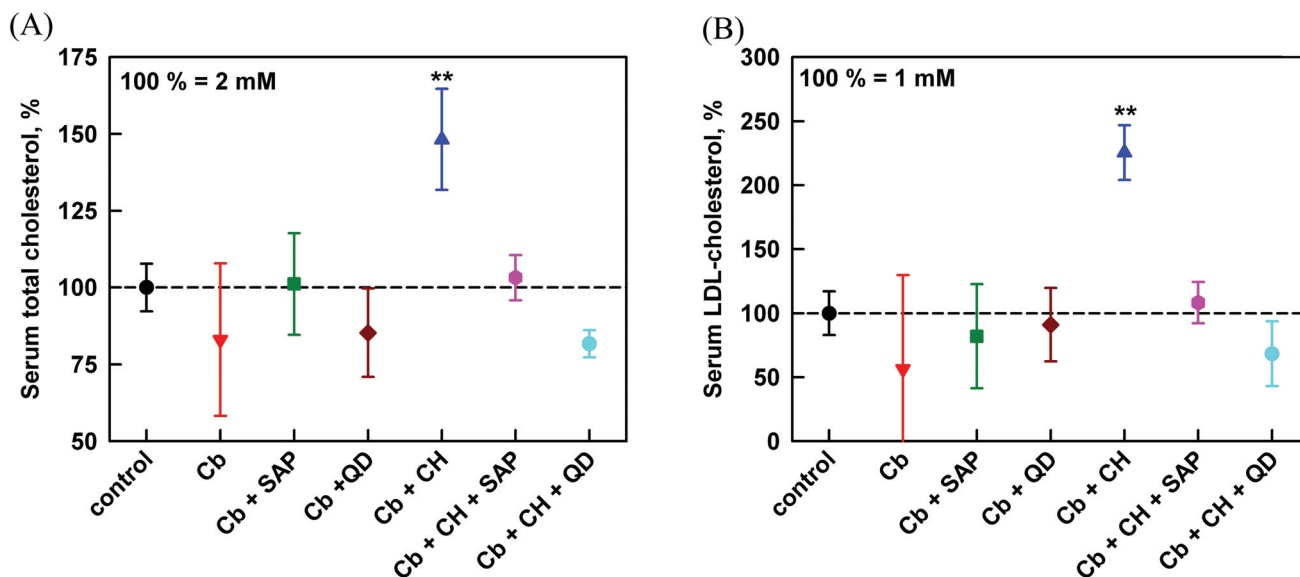
Fig. 4 (A) Total cholesterol and (B) LDL-cholesterol in the blood serum, as a function of the type of experimental diet: control group of mice, fed with the standard rodent food (black circles) and groups fed with food, supplemented with: cocoa butter (red triangles down), cocoa butter + SAP (green squares), cocoa butter + QD (brown diamonds), cocoa butter + cholesterol (blue triangles up), cocoa butter + cholesterol + SAP (pink hexagons) and cocoa butter + cholesterol + QD (cyan circles). Very significant difference for samples with $p < 0.01$ is denoted by ** sign, as determined by one-way ANOVA statistical analysis.

experimental diet in Fig. 4. One sees that the addition of cocoa butter or cocoa butter + SAP to the standard rodent food had no noticeable effect on total cholesterol (Fig. 4A): the latter remains the same as for the control group. In contrast, the cholesterol-enriched diet led to a very significant ($p < 0.01$) increase in serum total cholesterol levels to around 150%. However, the addition of SAP or QD to the cholesterol-enriched diet successfully prevented the increase of total serum cholesterol. For the group fed with cholesterol + QD there is a trend of decrease of serum cholesterol even below the values of the control group; however, the observed difference was not statistically significant.

If we compare the serum cholesterol level of the groups fed with additional cholesterol to those fed with cholesterol + saponins, we can calculate the decrease of serum cholesterol by the saponin extracts: 45% for QD and 30% for SAP. The value obtained for QD is in good agreement with the study of Oakenfull et al.,[10] where a similar calculation gives 38%.

The results for LDL-C follow closely the trends described above for the total cholesterol (Fig. 4B): cocoa butter and cocoa butter + SAP have no effect; the addition of cholesterol to the food increases very significantly the LDL-C (to 225%, $p < 0.01$) and both SAP and QD manage to prevent the hypercholesterolemic effect of the cholesterol-enriched food. Similarly to the results for total cholesterol, QD tends to decrease LDL-cholesterol below the control group values, but again the difference was not statistically significant.

The results for HDL-cholesterol are summarized in Fig. 5. One sees that HDL-cholesterol decreases slightly for the groups fed with cocoa butter + cholesterol + saponin, both for
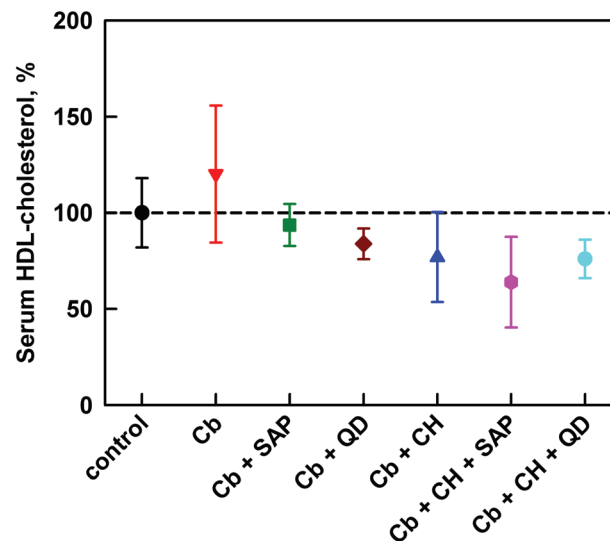

Fig. 5 Serum HDL-cholesterol, as a function of the type of experimental diet: control group, fed with the standard rodent food (black circles) and groups fed with food, supplemented with: cocoa butter (red triangles down), cocoa butter + SAP (green squares), cocoa butter + QD (brown diamonds), cocoa butter + cholesterol (blue triangles up), cocoa butter + cholesterol + SAP (pink hexagons) and cocoa butter + cholesterol + QD (cyan circles).

QD and SAP. However, the difference was statistically significant only for SAP ($p < 0.05$). In other words, none of the experimental diets affected the HDL-cholesterol, except for SAP + cholesterol, where a slight decrease in HDL-cholesterol was observed.

The obtained results can be summarized as follows: (1) the high cholesterol diet (no saponin) leads to significantly elevated total cholesterol concentrations in the serum, (2) this increase is due primarily to the increase of the LDL-cholesterol level, (3) both saponin extracts are able to prevent this increase for the total and the LDL-cholesterol. These results evidence that the used *in vitro* model is sensitive to the studied effects and allows one to detect effects known from the literature, like those with Quillaja saponins, or new effects (those with SAP) and to make a comparison with the phenomena studied *in vivo* with the same substances.

(B) Serum TG. The effect of different experimental diets on serum TG is presented in Fig. 6. One sees that none of the studied diets has a statistically significant effect on the serum TG. This result is somewhat surprising, as all groups of animals were supplemented with saturated fat (cocoa butter) and one could expect the TG to increase.[24] There are two possible explanations that can account for the lack of diet effect on serum TG: (1) the fat content of the meal was low ($\approx$5 wt%) and (2) the time-frame of the study (7 days) was too short for such metabolic effects to appear.

3.2.3. Effects of saturated and unsaturated fat on serum lipids. To check whether the effect of saponins on cholesterol depends on the degree of saturation of the dietary TG, we added different oils to the standard rodent food: SFO, containing around 87% unsaturated FA, or cocoa butter composed of 65% saturated FA.

The results for the concentration of serum total cholesterol are presented in Fig. 7A. In accordance with the results in the previous section, the increase in total cholesterol levels
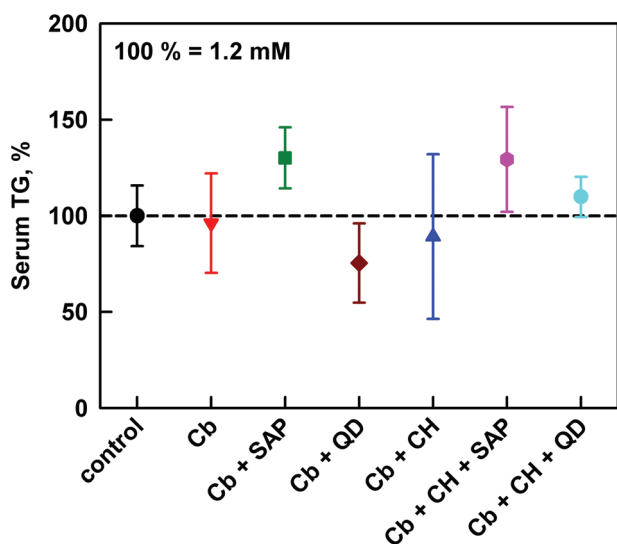

Fig. 6 Serum triglycerides, as a function of the type of experimental diet: control group, fed with the standard rodent food (black circles) and groups fed with food, supplemented with: cocoa butter (red triangles down), cocoa butter + SAP (green squares), cocoa butter + QD (brown diamonds), cocoa butter + cholesterol (blue triangles up), cocoa butter + cholesterol + SAP (pink hexagons) and cocoa butter + cholesterol + QD (cyan circles).

induced by the cholesterol-enriched diet is prevented by the addition of the QD extract. However, in contrast to cocoa butter, which had no effect on the cholesterol level, the presence of SFO alone increases significantly the total cholesterol in the serum.

The cholesterol-increasing effect of SFO is in agreement with our previous *in vitro* studies which showed that unsaturated FA and MG increase significantly the cholesterol solubilization in DMM.[39] On the other hand, it is accepted in the literature that the substitution of saturated fats with linoleic acid-rich oils (such as SFO) reduces the serum cholesterol.[40,41] The most probable reason for this discrepancy is the short duration of our study (7 days), which means that our experiment is representative of the effects taking place in the intestine during the absorption of cholesterol, while the same experiment is not representative of other possible effects on the lipid metabolism or regulatory mechanisms, appearing after longer time of exposure to a specific diet. For example, the study of Bravo *et al.*[41] had a duration of 21 days and showed an overall decrease of serum cholesterol when dietary SFA are replaced by linoleic acid, which could be explained by slower changes in lipid metabolism, appearing only after prolonged exposure to food enriched in linoleic acid.

The results for LDL-cholesterol show trends similar to those for the total cholesterol, Fig. 7B. However, in this case, the standard error is larger (because of the lower concentration of LDL-cholesterol) and the only significant increase of LDL-cholesterol is noted for the cholesterol-rich diet. Once again, the addition of QD extracts successfully prevents this increase, for both cocoa butter and SFO supplemented diets. No significant changes were observed for HDL-cholesterol (Fig. 7C).

The concentrations of serum TG for diets enriched with cocoa butter or SFO are presented in Fig. 7D. One sees that the addition of any of the studied fats to the diet has no effect on the serum TG, both in the presence or in the absence of QD. However, the animals fed with additional SFO + cholesterol showed an increase in the serum TG concentration, again regardless of the presence of QD. Such an effect was not observed for cocoa butter + cholesterol.

The observed increase of serum TG in the blood of mice on a diet rich in SFO + cholesterol could be due to interactions between cholesterol, UFA and BS in the gut lumen, which results in increased UFA uptake. In support of this hypothesis, UFA and cholesterol are known to co-solubilize in the DMM,[26,36,37] whereas cholesterol does not interact with SFA in model SFA–cholesterol–BS mixtures.[39] Further experiments are needed to clarify the mechanism of this effect.

4. Discussion

4.1. Mechanisms of saponin effects on cholesterol bioaccessibility

In section 3.1.2 we demonstrated that SAP and QD extracts decrease significantly the solubilization of cholesterol in DMM, see Fig. 3A. The "missing" cholesterol was found in the
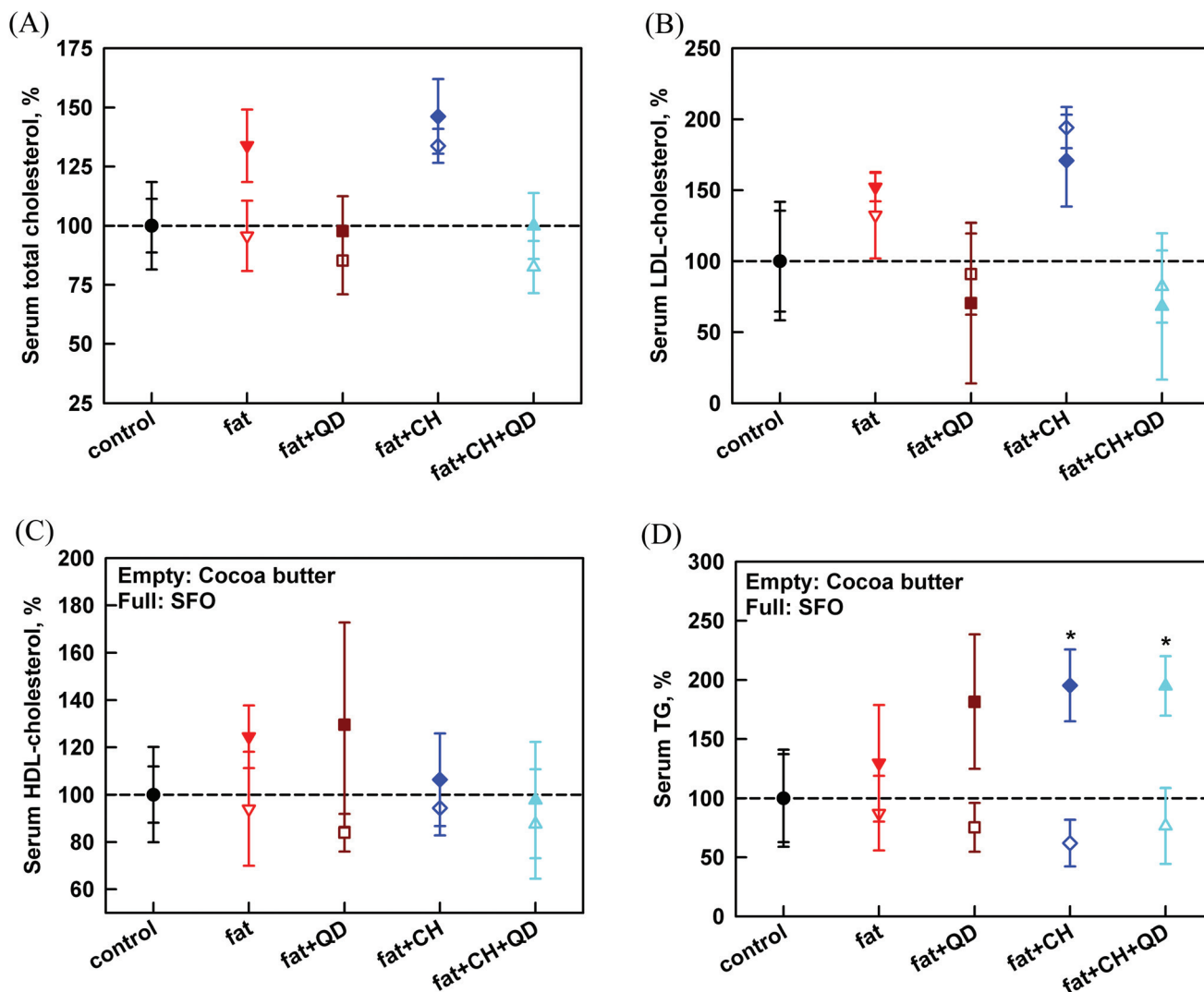
Fig. 7 Serum (A) total cholesterol, (B) LDL-cholesterol, (C) HDL-cholesterol and (D) triglycerides, as a function of the type of experimental diet, enriched with cocoa butter (empty symbols) or sunflower oil (full symbols): control group, fed with the standard rodent food (black circles) and groups fed with food, supplemented with: fat (red triangles down), fat + QD (brown squares), fat + cholesterol (blue diamonds), fat + cholesterol + QD (cyan triangles up). Significant difference for samples with $p < 0.05$ is denoted by * sign, as determined by one-way ANOVA statistical analysis.

precipitates which are too big in size to pass the intestinal mucus layer.

Three main mechanisms could be proposed to explain the precipitation of cholesterol in the presence of saponin solutions: (1) precipitation of BS by saponins, leading to a decreased concentration of the DMM, which results in a lower solubilization capacity of the digestion mixture with subsequent cholesterol precipitation (saponins and BS may form very large aggregates as shown by Sidhu & Oakenfull[9]); (2) direct formation of insoluble cholesterol–saponin complexes due to very strong affinity between these two components of the reaction mixture;[5,14] and (3) displacement of the cholesterol from the DMM by the saponin extract actives, thus causing an excess of cholesterol in the aqueous environment and its precipitation.[25]

In our previous study,[25] we showed that the main mechanism by which the QD extract induces cholesterol precipitation is by displacing cholesterol from the DMM, *viz.* mechanism 3. Additionally, QD was found to decrease even further the cholesterol solubilization capacity of the DMM by precipitating the solubilized FA (because of its high calcium content).[25] With respect to SAP, no data are available in the literature about its effect on cholesterol bioaccessibility and its mechanism of action.

To check which of these three mechanisms is operative for SAP extracts, we used the methodology proposed by Vinarova *et al.*[25]

First, we studied the effect of SAP concentration on BS solubility and found that ≈100% of the BS remain soluble for all SAP concentrations studied (see Fig. S4 in the ESI†). As the BS are not precipitated, we can disregard mechanism 1.
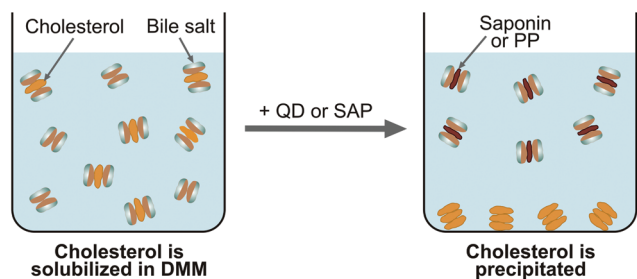
Fig. 8 Schematic presentation of the main mechanism of reducing the cholesterol bioaccessibility, as evidenced by our experiments. The active components in QD and SAP extracts (polyphenol and/or saponin fractions) have higher affinity to the dietary mixed micelles (DMM) than cholesterol. As a result, these components displace the cholesterol from the DMM, without affecting the concentration of the bile salts incorporated in DMM. The displaced cholesterol precipitates into large aggregates which are unable to pass the mucus layer in the intestinal walls.

To distinguish between mechanisms 2 and 3, we compared the effect of 0.26 wt% SAP saponins on cholesterol solubilisation, at two different BS concentrations (15 and 30 mM), while maintaining the total cholesterol concentration at 0.66 mM. The results showed that the doubling of BS concentration increased significantly the solubilized cholesterol from 0.28 to 0.49 mM. The latter result allows us to conclude that mechanism 3 is operative for SAP extract. Indeed, if SAP directly precipitates cholesterol *via* formation of insoluble cholesterol : saponin complexes (mechanism 2), cholesterol precipitation and solubilization should depend on the total concentrations of SAP and cholesterol only, while the BS concentration should have a minor effect (which contradicts the experimental result). In contrast, if cholesterol precipitates because it is displaced from the DMM by SAP constituents (mechanism 3), the cholesterol solubilization should increase with the BS concentration, just as observed experimentally.

We conclude that the main mechanism of action of SAP and QD extracts is the same: the cholesterol molecules are displaced from the DMM by the active extract components, thus leading to cholesterol precipitation and lower cholesterol bioaccessibility – see Fig. 8 for a schematic presentation of this mechanism.

Let us note that in ref. 25 (Vinarova *et al.*) we showed that the active cholesterol-lowering components in QD extract are the polyphenols (not saponins). No such investigation has been made for the active components in SAP extract. Therefore, the question about the chemical nature of the cholesterol-lowering components in SAP extract (saponins or polyphenols) remains open.

4.2. Comparison of the *in vitro* and *in vivo* experiments

The absorption of cholesterol through the enterocyte membrane is believed to occur both by passive diffusion and receptor-mediated transport.[23,42] To reach the membrane of the enterocytes, cholesterol is first solubilized in the DMM, composed of BS and phospholipids.[22] These aggregates facilitate the delivery of the lipophilic cholesterol from the lumen of the

intestine, through the intestinal mucus layer, to the vicinity of the enterocytes. Therefore, the observed correlation between the decreased cholesterol bioaccessibility in the presence of saponin extracts, which is controlled by the cholesterol solubilised in DMM (section 3.1), and the observed lower levels of serum cholesterol (section 3.2) validates very well the used *in vitro* and *in vivo* methods.

To quantify this *in vitro–in vivo* correlation, we compared the decrease of cholesterol bioaccessibility with the decrease of serum cholesterol levels, measured in sections 3.1 and 3.2. The first is calculated relative to the bioaccessibility of cholesterol in the absence of saponins. The second is calculated with respect to the serum cholesterol concentration of the group fed with a cholesterol-enriched diet (wherein the total cholesterol was increased).

As reported in sections 3.1 and 3.2, QD decreases bioaccessibility by 58% and serum cholesterol by ≈45%. For SAP the respective values are 31% and 30%. Therefore, we have a very good semi-quantitative agreement between the decrease of bioaccessibility of cholesterol, measured *in vitro* and the lowering of serum cholesterol *in vivo*. The results obtained with QD are also in very good agreement with those obtained by Oakenfull *et al.* who reported similar *in vivo* results in rats.[10]

5. Conclusions

Summarizing, we studied the effect of six saponin extracts on the *in vitro* bioaccessibility of cholesterol and SFA. From those extracts, noticeable effects were observed with QD and SAP extracts only (Fig. 2). For these two extracts, additional *in vivo* measurements were performed for the serum lipids in mice. The main conclusions are:

(1) both QD and SAP decrease significantly the bioaccessibility of cholesterol in the *in vitro* tests (Fig. 3). The main mechanism of lowering the cholesterol bioaccessibility is *via* displacement of cholesterol from the DMM, leading to formation of cholesterol precipitates, which cannot pass through the mucus layer in the intestine (Fig. 8). The concentration of bile salts in the DMM is affected very weakly by the addition of QD and SAP extracts.

(2) QD reduces the bioaccessibility of SFA as well (Fig. 2 and 3). This effect is explained by the precipitating effect of Ca^{2+} ions present in the QD extracts. SAP does not affect the SFA bioaccessibility, because no Ca^{2+} ions are present in this extract.

(3) In the absence of saponins, the cholesterol-enriched diet increases significantly both the total and LDL-cholesterol in the blood serum of mice. The addition of SAP or QD to the same cholesterol-rich diet prevents the increase of serum cholesterol (Fig. 4–7).

(4) There is good semi-quantitative agreement between the cholesterol bioaccessibility, measured *in vitro*, and the serum cholesterol levels determined *in vivo*.

This work confirms that *in vitro* digestion models are a very appropriate method for the study of the bioaccessibility of

poorly water-soluble components such as cholesterol and SFA. Therefore, these models can be successfully used for the discovery of new substances with cholesterol-lowering activity. An example from this study is the discovery of the cholesterol-lowering effect of the Sapindin extract, which has not been reported in the literature so far.

Abbreviations

ASC	Ayurvedic saponin concentrate
BS	Bile salts
DMM	Dietary mixed micelles
ESC	Escin
FA	Fatty acids (saturated and unsaturated)
FEN	Fenusterols
GS	Ginsenosides
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
MG	Monoglycerides
QD	Quillaja dry
SAP	Sapindin
SFA	Saturated fatty acids
SFO	Sunflower oil
TG	Triglycerides
UFA	Unsaturated fatty acids

Acknowledgements

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