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Cholesterol solubilization: Interplay between phytosterols, saponins and lipid digestion products

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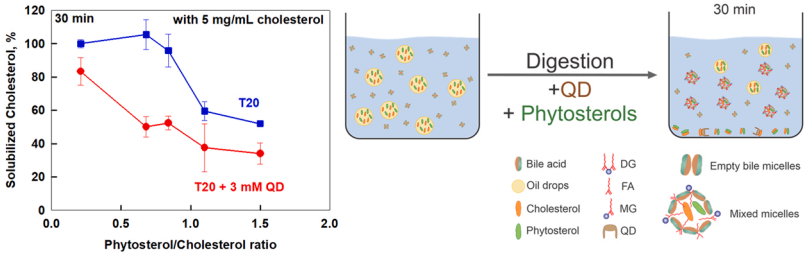
GRAPHICAL ABSTRACT



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ABSTRACT

High plasma concentrations of cholesterol are associated with cardio-vascular disease complications and high risk of myocardial infarction. The absorption of dietary cholesterol depends on its bioaccessibility, which is in turn influenced by phytosterols, saponins and lipid digestion products. Therefore, we explored the interplay between phytosterols, Quillaja Dry saponin extract (QD), their combinations and lipid digestion on cholesterol bioaccessibility via an in vitro.

1. Introduction

High blood cholesterol concentrations continue to be one of the major risks for patients with cardiovascular diseases [1–3]. As such conditions are usually chronic, a common treatment strategy includes dietary adjustments and intake of supplements which can reduce or at least prevent further increase of blood cholesterol [4–6]. One of the mechanisms by which some of these approaches manage to affect blood cholesterol is by interfering with its absorption after food intake [7].

Although cholesterol is a critical building block of the cell membrane [8], it is practically insoluble in water. At the same time, for a substance to be absorbed in the human intestine it has to be in a soluble form [9]: only in this way it can penetrate through the intestinal mucus layer and reach the vicinity of the enterocytes. Once the soluble form of cholesterol is near the enterocytes, uptake occurs via a combination of mechanisms that include passive diffusion and transport proteins (SR-BI, NPC1L1, CD36, P-glycoprotein etc.) which act as influx or efflux pumps [10]. Reaching the enterocytes in a soluble form is a common problem for poorly water-soluble drugs and is one of the challenges faced by the modern pharmaceutical industry [11].

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In the human gastrointestinal tract, cholesterol absorption is supported by the bile salts and phospholipids found in the small intestine [12]. These natural surfactants aid the digestion absorption of lipids and together with the lipid digestion products form the so-called dietary mixed micelles (DMM) [13]. One of the key mechanisms by which DMM facilitate cholesterol absorption is by solubilization in intestinal colloids [14,15]: cholesterol is included in the micelles and vesicles formed by bile salts, phospholipids and lipid digestion products. Hence, cholesterol solubilization was studied at biorelevant conditions in order to understand how it is influenced by the interactions between food, supplements and enzymatic digestion.

In respect to the effects of food, the digestion of lipids rich in unsaturated fatty acids was shown by our group to increase significantly cholesterol solubilization [16]. In addition, we also showed that this effect of unsaturated fatty acids can be overcome by the supplementation of calcium ions, which form insoluble calcium soaps with the fatty acids and prevent the increase of cholesterol solubilization [16,17]. In another series of papers, we demonstrated the cholesterol-lowering effect of saponins, which was found to be due to a combination of direct cholesterol precipitation and displacement of cholesterol from the DMM [18,19]. Another class of plant-based actives that can lower cholesterol absorption are the phytosterols [20–22]. Phytosterols are believed to act by precipitating cholesterol, thus decreasing its bioaccessibility and limiting its oral absorption [15].

However, most studies on phytosterol action so far have been performed in relatively simple solutions of bile salts. The possible impact of co-administration of dietary lipids, their enzymatic digestion and the interplay between phytosterols and lipid digestion products has not been considered yet. The interactions between phytosterols and saponins and their combined impact on cholesterol solubilization have not been investigated either.

Therefore, we aimed to study the possible synergistic effects of mixtures of phytosterols and saponins on decreasing cholesterol solubilization during digestion of model fat emulsions in an in vitro model of the human gastrointestinal tract. To facilitate the possible practical application of the results, we used an emulsifier (polysorbate 20) and Quillaja extract (Quillaja Dry 100) labeled as "Generally recognized as safe" (GRAS) and approved for use as food additives [23–26].

2. Materials and methods

2.1. Materials

Cholesterol was obtained from Sigma-Aldrich (95% ≥cat. no. 26740) and phytosterols were kindly donated by Unilever. According to literature data, the phytosterols were composed of three primary components: sitosterol (43–52%), campesterol (24–28%) and stigmasterol (14–24%) [27]. Sunflower oil (SFO) was purchased from a local supermarket and was used without further purification. Quillaja saponins extract Quillaja Dry 100 (QD) was kindly donated by Desert King Co, Chile. QD contains 38.3 wt% saponins (determined via HPLC by the producer), polyphenols, phenolic acids and polysaccharides [28].

Porcine pepsin (P0103) and pancreatin (P0636) were purchased from TCI. As bile salts source we used porcine bile extract, purchased from Sigma-Aldrich (cat. no. B-8631) which contained 50 wt% bile salts, 6 wt% phosphatidylcholine and less than 0.06 wt% Ca^{2+} [29]. GC analysis showed that it contains also 1.32 wt% cholesterol. Tween 20 (polysorbate 20) surfactant (Sigma) was used as emulsifier and sodium benzoate (Teokom) was used as a preservative for the model food emulsions.

Chloroform (99.99%, Sigma), pyridine (99.8%, anhydrous, Sigma), BSTFA with 1% trimethylchlorosilane (derivatization grade, LiChropur, Supelco), hexadecanol (99%, Sigma) and isooctane (≥99%, Honeywell) were used for lipid extraction, derivatization and dilution for GC analysis.

All aqueous solutions were prepared with deionized water from water-purification system Elix 3 (Millipore, USA). For preparation of electrolyte solutions, we used NaCl (Merck), KCl (Merck), $CaCl_2$ (Fluka) and $NaHCO_3$ (Sigma), all with purity ≥ 97%.

2.2. Emulsion preparation

The oil in water emulsions were prepared by the following procedure. An aqueous solution containing: 1% Tween 20 or 1% QD, 2.5% sodium benzoate and 10 mM NaCl was added to the oil phase (SFO) to obtain crude emulsion with 60% oil volume. Then emulsification was performed via sonication by Syclon SKL-650 W with 1 s pulsations for 5 min at 350 W. For the emulsions with cholesterol and phytosterols, the latter were pre-dissolved in the SFO before emulsification by the same protocol. The studied cholesterol and phytosterol concentrations (calculated based on the volume of the SFO) were 5, 10, 15 and 30 mg/mL cholesterol and 5, 10 an 15 mg/mL phytosterols.

2.3. In vitro digestion model

The in vitro digestion model was previously developed by Vinarov et al. [17]. It consists of two stages that simulate the conditions in the stomach and the small intestine. The "stomach" phase is simulated using a mixture of hydrochloric acid and saline solution (59 mM NaCl, 35 mM KCl and 3.5 mM $CaCl_2$), pepsin is also present to further mimic the conditions in the stomach (pH = 1.3). The transition to the intestinal phase is accomplished by the addition of bicarbonate buffer, bile salts and pancreatic enzymes. The pH rises to around 6 after bicarbonate is introduced. Next bile salts and pancreatin solutions are added to complete the intestinal phase transition. Afterwards, the mixture was stirred for 30, 60 or 240 min and an irreversible lipase inhibitor (Orlistat) was added to stop fat lipolysis, see Fig. S1. To characterize the degree of lipid digestion, the oil-soluble components were directly extracted using chloroform. To determine cholesterol solubilization, separate samples were centrifuged and the cholesterol and lipid digestion products in the supernatant were also extracted by using chloroform. The obtained chloroform extracts of the whole samples and the supernatants after centrifugation were derivatized and analysed by gas chromatography (GC).

In the experiments where the effect of QD on the lipolysis and cholesterol solubilization was investigated, QD was pre-dissolved in the saline solution to obtain final concentrations of 1 and 3 mM at the intestinal stage.

2.4. Lipid extraction and analysis by GC

Triglycerides (TG) and their lipolysis products (fatty acids - FA, monoglycerides - MG and diglycerides - DG) were determined by liquid–liquid extraction, followed by derivatization and GC, as adapted from our previous study [16]. Briefly, the protocol consisted of the following:

The pH of the samples obtained at the end of the in vitro digestion study was decreased to ≈ 2 by adding HCl to lower the FA aqueous solubility. Afterwards, chloroform was added (at a ratio of 1.5:2.0 chloroform:aqueous phase) and the sample was sonicated in a standard ultrasound bath for 15 min. The sample was homogenized by shaking after each 5 min of sonication. The extraction was completed by centrifugation of the obtained turbid dispersion for 30 min at 3622g, leading to clear chloroform and aqueous phases.

The obtained organic solvent extracts were derivatized in the following way: 400 μL of the chloroform extract was mixed with 200 μL anhydrous pyridine, 100 μL internal standard (18 mg/mL hexadecanol in chloroform) and 200 μL BSTFA. The final volume of the derivatization mixture was 900 μL and the concentrations of the components were 56 vol% chloroform, 22 vol% pyridine, 22 vol% BSTFA and 2 mg/mL hexadecanol. Then, the derivatization mixture was heated for 1 h at 60 °C. After cooling to room temperature, we diluted 75 μL of the

derivatized sample with 950 μL isooctane. The instrument used for GC analysis was Agilent 8890 (Agilent technologies, Santa Clara, CA, USA), which was connected to autosampler 7693 A. Agilent DB-5HT capillary column with the following specifications was used: (5%-Phenyl)-methylpolysiloxane, 30 m length, I.D. 0.32 mm, 0.1 μm film thickness. An injection volume of 1 μL and cold on-column injection was used. The oven was programmed in the following way: start at 60 °C, hold 1 min, the 1st ramp is to 180 °C at 10 °C/min, hold 0 min, 2nd ramp is to 375 °C at 30 °C/min, hold 10 min. Sample analysis time was 29.5 min. The flame ionization detector was operated at T = 380 °C. Helium at a constant flow of 2 mL/min was used as a carrier gas. Hydrogen, air and nitrogen (make-up gas) were used as detector gases. The concentrations of cholesterol, FA, MG, DG and TG were calculated from the internal standard hexadecanol, using correction factors determined from calibration curves with standard substances, see Fig. S2A.

Phytosterol shows 3 peaks on the chromatogram and the third peak coelutes with the bile salts, see Fig. S2B. The ratio between the first two peaks combined and the third peak is 1:1. In order to calculate phytosterol concentration in the samples the area of the first two peaks was multiplied by 2.

3. Experimental results

3.1. Extent of lipid digestion

The digestion of edible oils or fats by the pancreatic lipase occurs via consecutive reactions, where the TG is hydrolyzed to DG, MG and FA [30]. To quantify the extent of TG lipolysis in the end of the experiment, we used the total degree of TG lipolysis, α, defined as: $\frac{\alpha=C_{TG}^{INI}-C_{TG}(t)}{C_{TG}^{INI}}$. Here C_{TG}^{INI} is the initial molar concentration of TG, while $C_{TG}(t)$ is the molar concentration of the remaining, non-hydrolyzed TG, as determined by GC. The value of α accounts for the relative amount of TG that has been transformed into MG or DG.

We checked how lipid digestion varies with the different emulsifiers used (Tween 20 or QD) and when QD is added in the saline by analyzing samples without centrifugation (whole samples), see Fig. 1A. For all samples, the extent of lipid digestion increases with time and reaches up to 60–75% TG lipolysis at 240 min. Using QD as an emulsifier or the addition of low (1 mM) concentrations of QD via the saline solution increases slightly the extent of lipid digestion, although the obtained results are in the range of the experimental error. In contrast, the addition of 3 mM QD did not have any impact on TG lipolysis. The described trends in TG lipolysis are confirmed by the measured lipid digestion products concentrations, see Fig. 1B. In this case, the positive effect of low QD concentrations on lipid digestion was observed more clearly: significantly higher digestion products concentrations were measured for the QD-stabilized emulsion and at a QD concentration of 1 mM.

3.2. Cholesterol solubilization

The total cholesterol and phytosterols concentrations are the sum between the amounts introduced via the oily phase of the emulsion and the additional quantities of cholesterol coming from the porcine bile extract and the phytosterols originating from the SFO itself, see Table S1. The results for cholesterol solubilization described in the following sections are presented as the percentage of solubilized cholesterol in the permeate, as calculated from the ratio [CH in permeate/total CH]. The total cholesterol values were obtained from the reference samples obtained after digestion of the reference Tween 20-stabilized emulsion in absence of saponins.

3.2.1. Effect of phytosterols

In order to study the effect of phytosterol concentration on cholesterol solubilization we performed a series of experiments with different concentrations of cholesterol and phytosterol in the oily phase of the

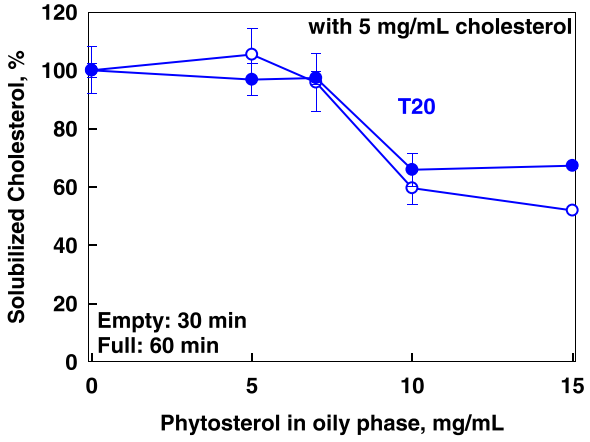

Fig. 2. Percentage of solubilized cholesterol in the permeate as a function of phytosterol concentration, after lipolysis of sunflower oil emulsions stabilized with T20 which contain 5 mg/mL added cholesterol in the oily phase. The empty symbols represent results after 30 min digestion (reaction time) and full after 60 min respectively. The results are average from (at least) two separate experiments. For some systems the scattering of the data is small and is represented by the size of the symbols.

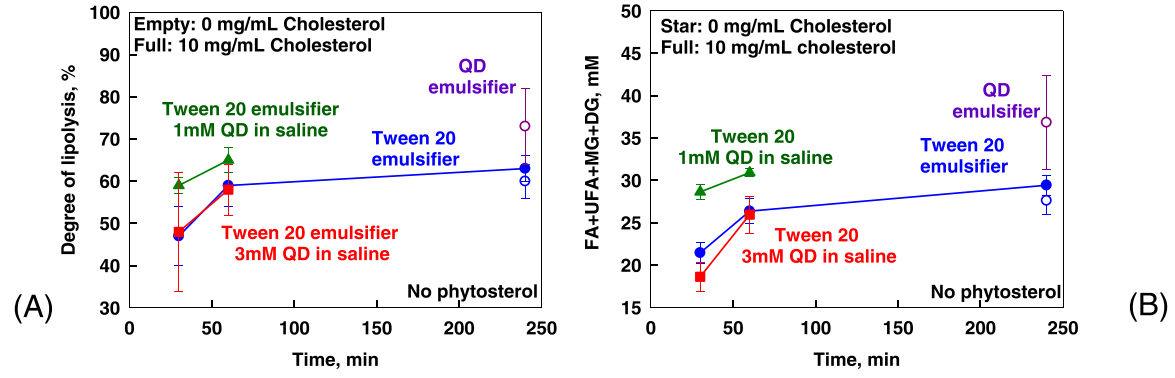

Fig. 1. Extent of lipid digestion (A) Overall degree of TG lipolysis, α, for sunflower oil in different studied conditions as a function of time of digestion and (B) sum of the reaction products as a function of time of digestion. Blue symbols-emulsion stabilized with 1 wt% T20; Purple symbols: emulsion stabilized with 1 wt% QD; Green symbol: emulsion stabilized with 1 wt% T20 and addition of 1 mM QD in saline during digestion; Red symbol: emulsion stabilized with 1 wt% T20 and addition of 3 mM QD in saline during digestion. Empty symbols represents the systems without additional Cholesterol and full symbols emulsions with 10 mg/mL Cholesterol (respect to oily phase) added in the oily phase. The results are for emulsions without additional Phytosterols added in the oily phase.

emulsions. In Fig. 2, we present the results for emulsions stabilized with T20. In the case of emulsions with 5 mg/mL added cholesterol we found that up to 7 mg/mL phytosterol additionally added in the oily phase we do not observe any effect on the cholesterol solubilization. However, with a further increase in added phytosterol, a significant decrease in solubilized cholesterol was observed. Here is important to note that the decrease with 40% of solubilized cholesterol is measured in the system with 10 mg/mL added phytosterol or higher.

At cholesterol concentrations in the oil phase of 10 mg/mL or higher, we were unable to conduct experiments at all phytosterol concentrations, due to precipitation during preparation of the oily phase of the emulsion. Hence, cholesterol solubilization remained constant at the lower phytosterol concentrations that we were able to study, see Fig. S3. Similar results were obtained also for emulsions stabilized with QD (see Fig. S4 in the Supporting Information).

3.2.2. *Effect of phytosterols and QD saponins*

In our previous study [19], we showed that the presence of QD extract in the system leads to a decrease in solubilized cholesterol. To check if the combination of QD and phytosterols would result in a synergistic effect, triggering even bigger decreases in cholesterol solubilization, we conducted a series of experiments in which QD was added at the stomach stage of the digestion model. We studied two different concentrations of QD in the final reaction mixture (intestinal conditions) of 1 mM and 3 mM, which correspond to the concentrations at which significant decrease in cholesterol solubilization was observed in our previous study [19]. The presented experimental results were obtained at cholesterol concentration in the oily phase of 5 mg/mL, due to the precipitation observed at higher cholesterol + phytosterol concentrations (see Fig. S5).

The results for both studied digestion times 30 min and 60 min are shown in Fig. 3. At short reaction times (30 min), the addition of 1 mM QD decreased cholesterol solubilization at lower phytosterol levels ($\geq$ 5 mg/mL), compared to the reference system in absence of saponins (phytosterol levels $\geq$ 10 mg/mL). The higher QD concentration of 3 mM QD had a more pronounced effect and decreased cholesterol solubilization even in the reference system without added phytosterol. At higher phytosterol concentrations, the values for solubilized cholesterol decreased further in presence of QD in the saline. The lowest cholesterol solubilization ($\approx$ 34%) was measured at 1 mM and 3 mM QD in presence of the highest phytosterol concentration of 15 mg/mL.

At the longer digestion times (60 min) the measured cholesterol solubilization was higher, but followed similar trends as at the short time scale, Fig. 3B. Again, the addition of QD saponins triggered a decrease in cholesterol solubilization at lower phytosterol concentrations (5 and 0 mg/mL phytosterols for 1 and 3 mM QD, respectively), compared to the reference system for which a decrease in solubilized cholesterol was observed only at higher phytosterol levels ($\geq$ 10 mg/mL). As the concentration of phytosterol increased, cholesterol solubilization was reduced for the reference system ($\geq$ 10 mg/mL phytosterols) and in presence of 1 mM QD ($\geq$ 5 mg/mL phytosterols), whereas cholesterol solubilization did not change significantly with phytosterol concentration at 3 mM QD.

3.2.3. *Effect of phytosterol:cholesterol ratio and precipitation of cholesterol by phytosterols*

To understand better the mechanism underpinning the observed decrease of cholesterol solubilization in presence of phytosterols and saponins, the results were plotted as a function of the ratio between the total phytosterols and cholesterol, see Fig. 4. It can be seen that at both reaction times of 30 and 60 min, the threshold ratio at which a decrease in cholesterol solubilization was observed for the reference system (in absence of QD) is around 1. Addition of saponins shifts the phytosterol-to-cholesterol ratio to lower values of $\approx$ 0.75 and 0.20 for 1 mM and 3 mM QD, respectively.

To further study the mechanisms of decreased cholesterol solubilization in presence of phytosterols, we calculated the insoluble cholesterol from the mass balance: insoluble cholesterol = total cholesterol – soluble cholesterol, where the total cholesterol is controlled by the initial experimental conditions (e.g. by the concentration of added cholesterol in the oily phase) and the soluble (or solubilized) cholesterol was measured analytically. The obtained results were displayed as the percentage of insoluble cholesterol from the total cholesterol, see Fig. 5. Good correlation between the increasing concentrations of insoluble cholesterol and insoluble phytosterols was observed, indicating that direct precipitation of cholesterols by the phytosterols may be the main mechanism of the observed effects (this hypothesis is discussed in detail in Section 4.2).

3.2.4. *Effect of lipid digestion products*

In order to study the effect of lipolysis products on cholesterol solubilization we determined the aqueous concentrations of saturated fatty acids (FA), unsaturated fatty acids (UFA), monoglycerides (MG) and diglycerides (DG) by GC. To check if cholesterol is co-solubilized with the lipid digestion products [16,17,19], we prepared plots for the solubilized cholesterol vs. the concentrations of unsaturated and saturated FA and MG and DG (Fig. 6A). It can be seen that very good correlations were obtained, as each of the concentrations of added cholesterol can be described by a separate straight line. The slopes of the fits increase with the concentration of added cholesterol. This is an indication that the maximum solubilization capacity of the dietary mixed micelles (DMM) has not been reached. It is clearly seen that there is a correlation

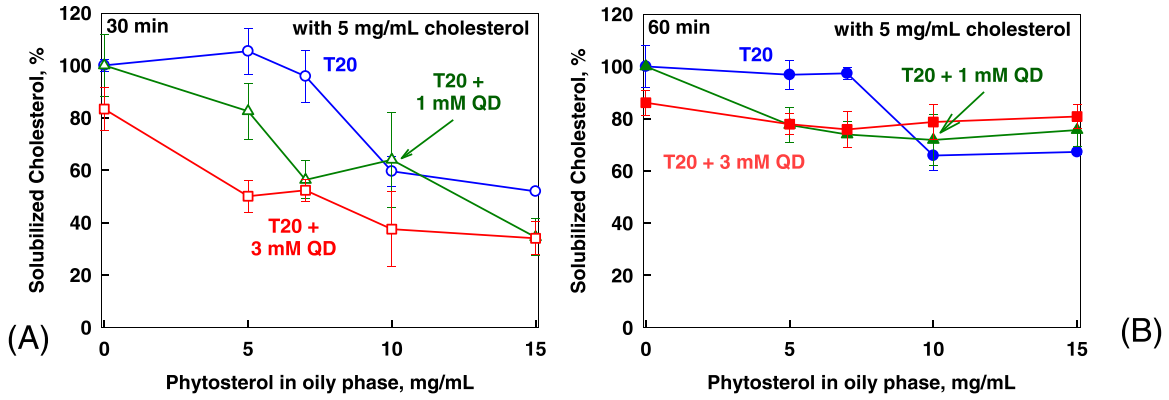

Fig. 3. Percentage of solubilized cholesterol in the permeate as a function of phytosterol concentration, after lipolysis of sunflower oil emulsions stabilized with T20 without QD in saline (blue symbols), T20 with 1 mM QD in saline (green symbols), T20 with 3 mM QD in saline (red symbols) which contain 5 mg/mL added cholesterol in the oily phase. The digestion (reaction time) is (A) 30 min and (B) 60 min. The results are average from (at least) two separate experiments. The error bars can be smaller than the symbols.

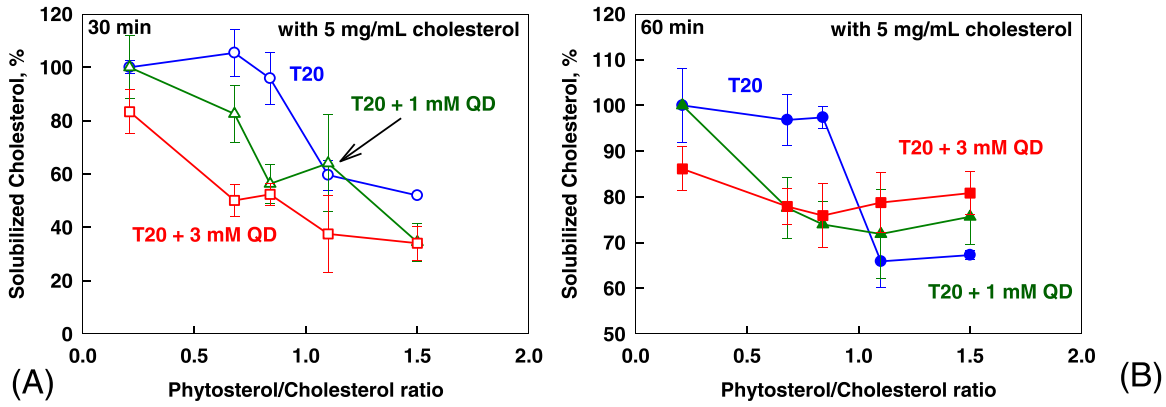

Fig. 4. Solubilized cholesterol as a function of the total phytosterol-to-total cholesterol ratio for Tween 20-stabilized emulsions of SFO containing 5 mg/mL cholesterol in the oily phase in absence (blue circles) or in presence of 1 mM (green triangles) or 3 mM (red squares) saponins from QD. The digestion (reaction time) is (A) 30 min and (B) 60 min. The results are average from (at least) two separate experiments. The error bars can be smaller than the symbols.

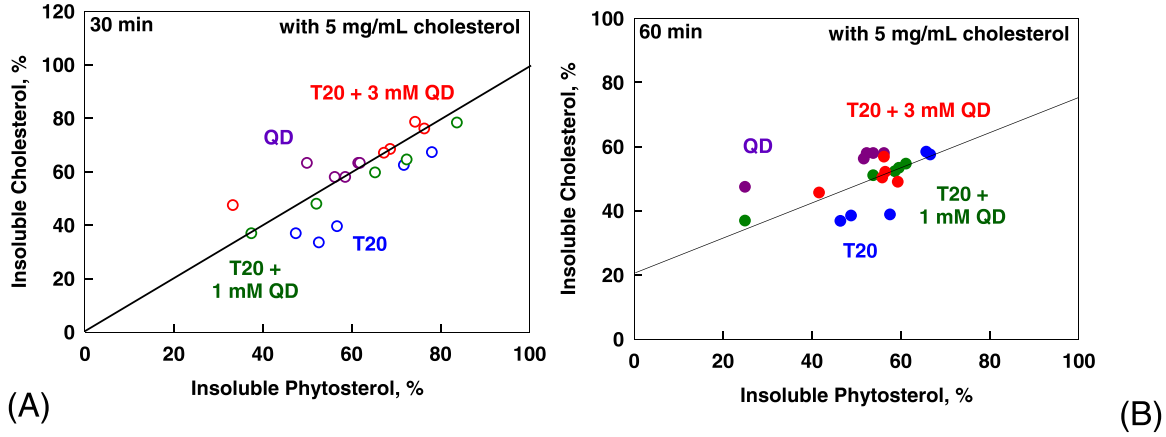

Fig. 5. Insoluble cholesterol as a function of the insoluble phytosterols for Tween 20-stabilized emulsions of SFO containing 5 mg/mL cholesterol in the oily phase in absence (blue circles) or in presence of 1 mM (green circles) or 3 mM (purple circles) saponins from QD or QD stabilized emulsion (purple circles). The digestion (reaction time) is (A) 30 min and (B) 60 min.

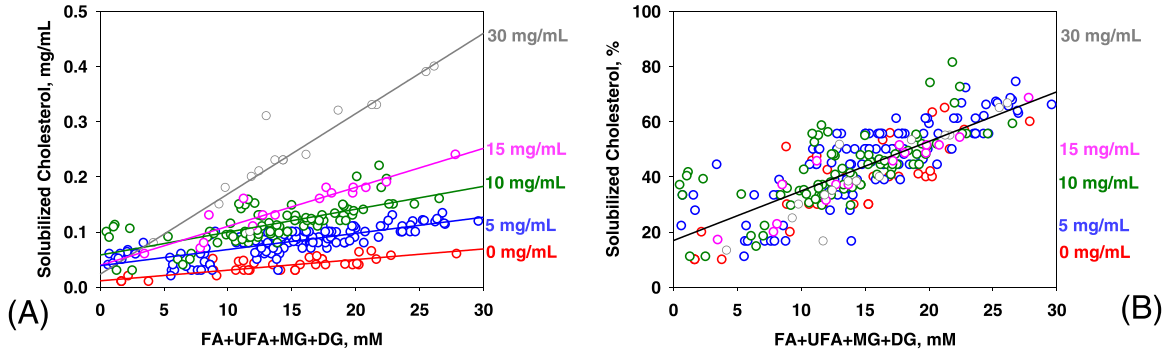

Fig. 6. Solubilized cholesterol in the permeate: (A) as a function of the sum [FA+UFA+MG+DG] after lipolysis; (C) solubilized cholesterol scaled with the total amount of cholesterol as a function of the sum [FA+UFA+MG+DG] after lipolysis of SFO emulsions without addition of cholesterol in oily phase (red symbols), with 5 mg/mL (blue symbols), 10 mg/mL (green symbols), 15 mg/mL (pink symbols) and 30 mg/mL (gray symbols).

between the solubilized cholesterol and the cholesterol added in the oil phase.

Finally, to check whether the obtained experimental data can be described by a universal curve, we scaled the solubilized cholesterol with the total cholesterol concentration for each system. Fig. 6B presents the scaled results and it is clearly seen that all the data fall into the same line and can be described by one universal curve, illustrating the strong co-solubilizing effect of lipid digestion products on cholesterol, see Fig. 7.

4. Factors controlling cholesterol solubilization at intestinal conditions

4.1. Co-solubilization of cholesterol by lipid digestion products

The solubilization of cholesterol at biorelevant conditions was

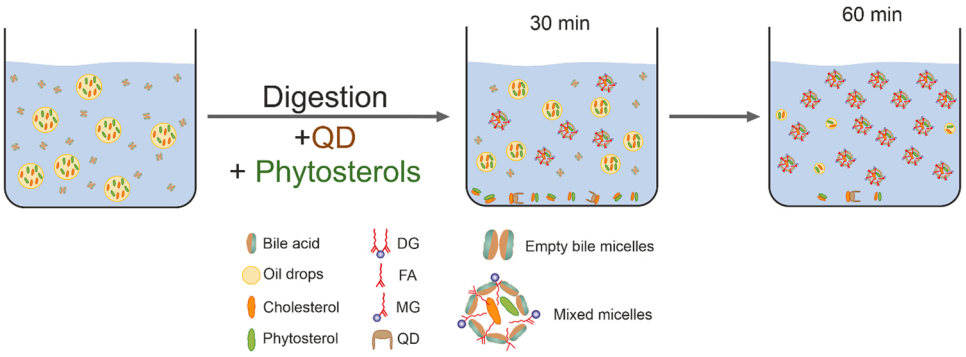

Fig. 7. Schematic presentation of the main mechanisms which controls the cholesterol solubility. From left to right: (1) starting mixture containing oil drops rich in cholesterol and phytosterol and micelles of bile salts. (2) At short reaction time (30 min) – the active components in QD and phytosterols have very strong affinity to cholesterol which leads to formation of precipitates. (3) At long reaction time (60 min) – extensive emulsion digestion leads to significantly lower ratios of QD to cholesterol and QD to lipid digestion products.

studied systematically in the context of gallstone formation in the pioneering works of Carey and Small [14,31,32]. The authors focused on the mechanisms of cholesterol solubilization in mixed solutions of bile salts and phospholipids and were able to determine the impact of bile salt structure [32] and to construct phase diagrams of ternary bile salt-phospholipid-cholesterol systems which included the effects of ionic strength [14,31].

Simmonds et al. were among the first to show that monoolein and oleic acid can increase significantly cholesterol solubilization in bile salt solutions in a concentration-dependent manner [33]. The findings were corroborated by Montet et al., who studied cholesterol solubilization in mixtures of different bile salts, monoolein and oleic acid at varying pH and observed similar effects [34].

Studies from our group on the impact of enzymatic lipid digestion of various edible fats and oils on cholesterol solubilization showed that the impact of the generated lipid digestion products (mono- and diglycerides, fatty acids) depends strongly on the fatty acid profile [16,17]: long-chain saturated species (e.g. stearic acid, monostearin) did not increase cholesterol solubilization, in contrast to the unsaturated oleic acid derivatives. In addition, a general dependence between the solubilized lipid digestion products and cholesterol solubilization was established, which evidenced for co-solubilization of these poorly-water soluble components in the DMM [16,17]. The findings in the current study are in excellent agreement with these observations (Fig. 6). Furthermore, the linear correlation between the solubilized lipid digestion products and cholesterol solubilization even in presence of phytosterols and saponins show that the co-solubilization effect is not hindered by cholesterol sequestering agents.

4.2. *Precipitation of cholesterol in phytosterols and Quillaja saponins solutions*

The ability of phytosterols to reduce aqueous cholesterol concentrations has been documented in several reports [35–37]. Experiments with mixtures of cholesterol and phytosterols in solvents, in oil and in melt have demonstrated a direct precipitation mechanism [35,36], whereas at biorelevant conditions it was suggested that the precipitation is induced by a dynamic competition mechanism (the phytosterols displace cholesterol from the DMM) [37]. On the other hand, the results presented in Fig. 4 show that at the conditions of in vitro lipid digestion a ratio of at least 1:1 phytosterols to cholesterol is required to reduce cholesterol solubilization. The fact that cholesterol solubilization is not affected by lower phytosterol concentrations questions the dynamic displacement mechanism [37], while suggesting that direct precipitation of cholesterol by phytosterols might occur in the intestine in vivo. The different outcome of the two studies might be due to experimental differences: the work of Mel'nikov et al. [37] was performed in model mixtures of bile salts, phospholipids and lipid digestion products at constant concentrations, whereas in the current study we started from a triglyceride emulsion, which was continuously hydrolyzed by the pancreatic lipase to trigger the release of cholesterol and phytosterols (which were pre-dissolved in the oil), mimicking the process in vivo.

Related to the action of saponins, we have previously shown that the QD extract decreases cholesterol solubilization at in vitro digestion conditions by several mechanisms, which include direct precipitation and also displacement of cholesterol from the DMM [19]. The addition of the QD extract to a digestion medium containing phytosterols shows a synergistic effect and results in a significant decrease of the threshold phytosterol-to-cholesterol ratio required for cholesterol precipitation (see Fig. 4): from 1 in absence of saponins, to 0.2 in presence of 3 mM QD. Therefore, the mixtures of QD and phytosterols appear to be a promising avenue for the future development of cholesterol-lowering combined nutraceutical formulations.

On the molecular level, the active substance in the QD extract should be considered in order to explain the synergistic effect of the combination of QD extract and phytosterols on cholesterol precipitation. Besides from the saponins, polyphenols with large molecular weight may also interact with the phytosterols, thus causing the significant precipitation of cholesterol [19]. Further studies are required to identify the active compound in the QD extract which drives the effects observed in the current study.

4.3. *Interplay between digestion kinetics and cholesterol solubilization*

The results presented in Fig. 3 reveal an unexpected behavior of the solubilized cholesterol: the concentrations increase significantly 60 min after digestion, compared to 30 min, for the systems in which the combination of phytosterols + QD was investigated. The latter may be rationalized in the following way.

In the beginning, the intestinal mixture contains bile salt/phospholipid aggregates, oil droplets rich in pre-dissolved cholesterol and phytosterols, and QD dissolved in the aqueous phase. At short reaction times (t = 30 min), cholesterol and phytosterol are released from the emulsion droplets (due to the digestion) and FA, MG and DG are generated, forming the DMM. At these conditions, when the lipid digestion products concentrations are still low, QD and the phytosterols act as synergistic precipitating agents and reduce the solubilized cholesterol.

At long reaction times (t = 60 min), the degree of lipid digestion has increased considerably (see Fig. 1), leading to the release of more cholesterol and phytosterols from the emulsion droplets and more lipid digestion products. While the ratio of phytosterols-to-cholesterol remains the same, this is not the case with respect to QD: as its concentration is constant, its ratio towards cholesterol and the lipid digestion products decreases significantly. Hence, the synergistic effect of QD + phytosterols is suppressed, and cholesterol solubilization is only slightly different than the reference phytosterol-only system (see Fig. 3).

5. Main results and conclusions

We studied the combined action of phytosterols and QD saponin extract on the solubilization of cholesterol during in vitro digestion of model emulsions enriched with cholesterol and phytosterols. The

experimental results clearly showed that cholesterol solubilization was governed by three major factors: (1) the ratio between phytosterols and cholesterol, (2) the presence of QD extract and (3) the concentrations of solubilized lipid digestion products. The threshold phytosterol-to-cholesterol ratio required to induce cholesterol precipitation decreased from 1 to 0.2 upon the addition of 3 mM QD extract. There is a synergy between phytosterols and saponins in decreasing the cholesterol solubilization after 30 min and the lowest cholesterol solubilization (≈ 34%) was measured at 1 mM and 3 mM QD in presence of the highest phytosterol concentration of 15 mg/mL. However, the generation of lipid digestion products in the course of emulsion lipolysis was found to significantly increase the solubilized cholesterol by a co-solubilization effect, partially counteracting the synergism of QD and phytosterols at long digestion time (t = 60 min). The obtained results illustrate the complex processes governing cholesterol solubilization at simulated intestinal conditions and suggest the combination of QD and phytosterols as a possible avenue for development of new cholesterol-lowering nutraceuticals.

CRediT authorship contribution statement

Sonya Tsibranska: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft. **Vladimir Petkov:** Investigation, Formal analysis. **Vladimir Katev:** Investigation, Formal analysis. **Delyan Krastev:** Investigation, Formal analysis. **Zahari Vinarov:** Conceptualization, Methodology, Formal analysis, Funding acquisition, Writing – review & editing. **Slavka Tcholakova:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfa.2023.131052.

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