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Randomized Control Trials

Impact of colonic fermentation on sterols after the intake of a plant sterol-enriched beverage: A randomized, double-blind crossover trial

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SUMMARY

Background: Cholesterol microbial transformation has been widely studied using *in vitro* fermentation assays, but less information is available on the biotransformation of plant sterols (PS). The excretion percentage of animal sterols (AS) (67–73%) is considerably greater than that of PS (27–33%) in feces from healthy humans following a Western diet. However, a lower content of AS in feces from subjects following a vegetarian, vegan or low-fat animal diet has been seen when compared to omnivorous subjects. Although only one human study has reported fecal sterol excretion after the consumption of PS-enriched food (8.6 g PS/day), it was found that the target group showed an increase in the excretion of cholesterol and a 57% decrease in its metabolites compared to the control group.

Objective: Evaluation of the impact of a PS-enriched milk based fruit beverage intake on fecal sterol excretion and the microbial conversion of sterols in postmenopausal women with mild hypercholesterolemia.

Methods: Forty postmenopausal women participated in a randomized, double-blind, crossover study with two beverages, with a PS-enriched (2 g PS/day) or without. The women were divided in two groups: 20 women consumed the PS-enriched beverage and the other 20 women consumed a placebo (without PS) beverage for 6 weeks. After a four-week washout period, the type of beverage was exchanged and consumed for another 6 weeks.

Feces were collected at the start (0 and 10 weeks) and end of each intervention period (6 and 16 weeks), and fecal sterols were determined by capillary gas chromatography with mass spectrometry.

Results: The intake of the PS-enriched beverage modified the fecal sterol excretion profile. A significant increase mainly in PS and their metabolites *versus* the placebo intervention period was observed. Although the same effect was not observed in the case of AS, a tendency towards increased cholesterol and decreased coprostanol (the main metabolite of cholesterol) was recorded after PS-enriched beverage intake *versus* placebo. Furthermore, the PS-enriched beverage also modified the microbial conversion of sterols. In this context, an important decrease in the conversion percentage of cholesterol in 16 women (between 11% and 50%) and of sitosterol in 24 women (between 15% and 61%) was observed.

Conclusions: The results obtained suggest that the microbiota could preferably use PS as a substrate, when present in a greater proportion compared with cholesterol. Besides, a lower sitosterol and cholesterol conversion trend would mean that intake of the PS-enriched beverage could modulate the metabolic activity of the gut microbiota. Therefore, further studies on the impact of PS-enriched foods upon gut microbiota modulation are needed.

Clinical Trial Registry Number: NCT 02065024 listed on the NIH website: ClinicalTrials.gov.

Abbreviations: PS, plant sterols; AS, animal sterols; TMSE, trimethylsilyl ethers; ROC, receiver operating characteristic.

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Clinical Trial Registry Name: Food Matrix and Genetic Variability as Determinants of Bioavailability and Biological Effects of Beta-cryptoxanthin and Phytosterols (foodmagenpol).
The full trial protocol is available upon request to the corresponding author.

1. Introduction

The daily dietary intake of plant sterols (PS) (160–400 mg/day) [1] does not reach the established levels to have a hypocholesterolemic effect (1.5–3.0 g/day) [2]. Therefore, several foods (yellow fat spreads, salad dressings, milk type products, fermented milk products, soya drinks, cheese type products, rye bread and rice drinks) may be enriched with PS for this purpose and can also be used in patients with mild hypercholesterolemia.

There is an association between postmenopausal women and higher serum levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C), which could increase the risk of cardiovascular disease [3,4]. In fact, in a previous clinical study (NCT01074723) of our group with postmenopausal women [5], β-critptoxanthin (β-Cx) improved the cholesterol lowering effect of PS when they were supplied simultaneously from a PS-enriched milk-based fruit beverage rich in β-Cx. This combination may also be beneficial in reducing the risk of osteoporosis, suggesting a synergistic effect. The effect observed, however, implies a moderate reduction of c-LDL and total cholesterol, on average 5–7%, which is in the range of other studies using different food matrices.

Fifty percent of dietary cholesterol is absorbed [6], although its absorption varies substantially between individuals (20–80%) [7]. However, the intestinal absorption rate of total PS is only 2–3% [8]. Non-absorbed sterols reach the colon and can be biotransformed by the gut microbiota. Although cholesterol biotransformation mediated by gut microbiota has been widely studied using *in vitro* assays (Wong, 2014) [9] and some studies have suggested that cholesterol metabolites could act as carcinogenic compounds [10–13], less information is available on the biotransformation of PS and its possible effects (in the case of high intake levels) upon cholesterol metabolization within the colon. Sterols and their corresponding microbial metabolites have been detected in feces from healthy humans following a Western diet [14–18] - the percentage of animal sterols (AS) (67–73%) being considerably greater than PS (27–33%). The AS excretion in subjects following a vegetarian [18–21], vegan [22] or low-animal fat diet [10,23,24] is lower (between 10 and 50%) than in omnivorous subjects. Besides, compared with healthy individuals, greater excretion of cholesterol and its metabolites has been found in feces from subjects with colon cancer (74–92%) [11,20,25] or with precancerous conditions such as adenomatous polyposis (80%) [11] or ulcerative colitis (83%) [26]. These authors attributed the increased excretion of cholesterol metabolites to diet and microbial conversion. However, other investigators have found no differences in AS excretion between patients with colon cancer [13] or familial polyposis [27,28] and healthy subjects.

As far as we know, only one human study has reported fecal sterol excretion after PS-enriched food intake (margarine, 8.6 g PS/day) [29] – the mentioned content exceeding the values established by the European Commission. Therefore, the aim of the present study was to evaluate the impact of the daily consumption of a PS-enriched milk based fruit beverage (2 g PS/day) on fecal sterol excretion and its microbial conversion in postmenopausal women with mild hypercholesterolemia through a clinical trial.

2. Material and methods

2.1. Clinical study

This clinical trial was a single and combined randomized, double-blind, crossover trial carried out with two beverages: a PS-enriched skimmed milk based fruit beverage containing β-Cx and 2 g of PS/250 mL (active beverage), and a skimmed milk based fruit beverage (placebo beverage), in postmenopausal women with mild hypercholesterolemia (ClinicalTrials.gov number NCT 02065024).

The inclusion criteria were: age 45–65 years, amenorrhea for over 12 months, and mild hypercholesterolemia (200–239 mg/dL) according to the guidelines of the American Heart Association [30]. Non-dieting and non-intake of vitamin D, calcium, ω-3 fatty acids and PS or vitamin-enriched foods, supplements or other dietary bioactive components were also considered as inclusion criteria.

Body mass index (BMI) > 35 kg/m^2, the use of vitamins, antibiotics, hormone replacement therapy, fibrates or statins, as well as acute inflammation, chronic medication and infection or intercurrent illness capable of affecting the bioavailability or status of the compounds of interest were regarded as exclusion criteria.

The nutritional composition of the two beverages (active and placebo) is shown in Table 1. It should be noted that although both beverages had a similar composition (50% skimmed milk/50% fruit juice), the placebo beverage was formulated with grape juice from concentrate, and banana puree, while the active beverage was elaborated mainly with mandarin juice from concentrate (as a β-Cx source) and a lower proportion of grape and banana. Free microcrystalline PS from tall oil in powder form (Lipohytol® 146 ME Dispersible, Lipofoods) was added only to the active beverage. The manufacturing conditions were similar for both beverages, which had the same appearance, but with different anonymous labeling (A or B). Plant sterols from the active beverage were characterized (sitosterol: 79%, sitostanol: 12%, campesterol: 7%, stigmasterol: 0.8% and campestanol: 1%) [31], and their stability along a 6-months period (intervention period) was confirmed.

The clinical study took place in the Vitamins Unit of the Department of Clinical Biochemistry of Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain).

Fifty postmenopausal women were contacted for participation and interviewed in order to confirm that they met the inclusion criteria (enrollment). A total of 40 apparently healthy postmenopausal women were finally included in the study and were sequentially numbered from 1 to 40. The sample size was calculated taking into account the results referred to total PS and cholesterol

Table 1
Energy and nutritional composition per 100 mL of active and placebo beverages used in the clinical trial intervention.

	Active	Placebo
Energy (Kcal)	65.3	64.0
Protein (g)	3.1	2.6
Carbohydrate (g)	8.9	10
Fat without PS (g)	1.6	1.5
Fiber (g)	1.5	1.5

Information provided by the manufacturer (Hero España, S.A).

obtained in a previous clinical trial (ClinicalTrials.gov number NCT01074723). Taken from previous assumption, we chose the more conservative option to ensure the detection of a 7% decrease in cholesterol levels in mildly hypercholesterolemic subjects (e.g., 15 mg/dL) with a type I error of 0.05 and a statistical power of 80%. Furthermore, taking into account that 45% of the Western population may present polymorphisms implicated in the cholesterol absorption process, and assuming a drop-out rate of 10%, the final required sample size was considered to comprise 40 subjects.

During the trial period, 40 women were selected and randomly distributed into two groups: 20 women consumed the active beverage (1 brick x 250 mL/day) and the other 20 women consumed the placebo beverage (1 brick x 250 mL/day) for 6 weeks. After a four-week washout period, the type of beverage was exchanged and consumed for another 6 weeks.

The volunteers were allocated to receive either intervention in random order by using a computer-generated pseudo-random numbers table. A member of the research team (not involved in subject selection) requested each subject to randomly select one of a series of opaque sealed envelopes containing identification of the type of beverage. After opening the selected envelope, the investigator recorded which type of beverage (active or placebo) should be assigned to each subject, and prepared a pack with enough tetra-bricks to cover the first experimental period (6 weeks). This investigator also ensured that each subject was assigned to the other study group (placebo or active) following the corresponding washout period. The details of group assignment were kept in a sealed envelope that was opened at the end of the complete experimental period. Neither the subject nor the rest of the research team knew about subject assignment during the experimental period.

The participants were provided with a list of foods and beverages rich in β-Cx that were to be avoided, and were asked not to change their usual diet or physical activity. They were also instructed to record any side effects during the study, and to complete a semi-quantitative Food Frequency Questionnaire (FFQ) (Supplementary Table 1) at the end of each intervention period, in which the women recorded the number of food portions per week. In addition, the subjects reported no differences in the organoleptic properties of the two beverages in the FFQ. However, the FFQ was not validated, constituting a limitation of the study.

Study compliance was assessed by means of an adherence questionnaire. Each participant completed this questionnaire after the active and placebo periods, reporting the number of non-ingested tetra-bricks.

Feces collection was performed before and after each 6-week treatment period. At this time, a centralized service assigned an identification number (7 digits) to each subject (following the usual practice for all hospital patients), and a member of the research team supervised that samples from each subject were collected in the sterile plastic containers and stored at −20 °C until analysis. In order to confirm mild hypercholesterolemia in the women, serum total cholesterol levels were measured by a routine quality controlled method using an Advia 2400 Clinical Chemistry system (Siemens Healthineers). Only phlebotomists or laboratory technicians knew the assigned number of the sample, and were unaware of which treatment was received by the patients.

The women of this study had a mean age of 55.7 ± 3.4 years (range 50–65), with a mean body mass index of 24.6 ± 4.7 kg/m^2 [32], and presented untreated mild hypercholesterolemia (220 ± 27.8 mg/dL). In addition, in Table 2, the serum cholesterol profile after each intervention period (placebo and active) is shown.

An overview of the clinical trial is provided in Fig. 1. The study protocol was approved by the Clinical Research Ethics Committee of Hospital Universitario Puerta de Hierro-Majadahonda (Madrid, Spain), and all subjects gave written consent to participate in the study.

Table 2
Serum cholesterol profile response upon regular consumption of the beverages (n = 36). Results are expressed as Mean ± SD.

mg/dL	Placebo beverage		PS-enriched beverage	
	Basal	Final	Basal	Final
Total cholesterol	219.7 ± 24.5^a	221.8 ± 25.4^a	219.2 ± 28.0^a	212.6 ± 25.5^b
LDL-cholesterol	129.6 ± 27.5^a	131.5 ± 23.6^a	128.6 ± 29.0^b	121.3 ± 24.4^b
HDL-cholesterol	71.9 ± 18.7^a	70.1 ± 17.3^a	71.9 ± 17.3^a	71.9 ± 20.4^a

Different superscript letters denote significant differences ($p < 0.05$) in the same type of beverage (with PS-enriched or placebo) among basal and final values (within lines) (a,b). Reference range (mg/dL): total cholesterol (150–200); LDL-cholesterol (70–160); HDL-cholesterol (35–75). The statistical analysis was done by applying a t-test for paired samples using the SPSS program.

2.2. Analyses performed

2.2.1. Fecal samples

Fresh fecal samples were collected before (V_1 and V_3) and after (V_2 and V_4) each intervention period (see Fig. 1). The samples were then stored at −20 °C and subsequently freeze-dried (Sentry 2.0, Virtis SP Scientific) and crushed in a glass mortar and stored at −20 °C until analysis.

2.2.2. Sterol analysis

Fecal sterols and their metabolites as secondary outcome within the clinical trial were determined according to Cuevas-Tena et al. (2017) [33]. Briefly, approximately 30 mg of freeze-dried feces were dispersed in 5 mL of Milli-Q water, sonicated (20 min) and allowed to stand for two hours at room temperature. The analysis was performed in triplicate using 5α-cholestane (20 μg) as internal standard in aliquots of 100 and 500 μL. The saponification step was carried out with 1 mL of ethanolic potassium hydroxide solution 0.71 M (65 °C/1 h) using a block heater. The unsaponifiable fraction was extracted with 0.5 mL of Milli-Q water and 2 mL of n-hexane (centrifuged at 18 °C/10 min/3600 rpm). The n-hexane extraction step was performed twice under the same conditions as described above. The organic extracts were evaporated to dryness under nitrogen. In order to obtain the trimethylsilyl ether (TMSE) derivatives, 200 μL of BSTFA + 1% TMCS:pyridine 10:3 (v/v) were added (65 °C/1 h). The TMSE derivatives obtained were dissolved with 3 mL of hexane, filtered (Millex-FH filter unit, 0.45 μm Millipore, Milford, MA, USA), evaporated under nitrogen, and dissolved in 40 μL of hexane. One μL of this solution was injected into a GC/MS system (Thermo Science Trace® GC-Ultra with ion trap ITQ 900, Waltham, MA, USA) with a CP-Sil8 CB low bleed/MS (50 m × 25 mm x 0.25 μm) column (Agilent Technologies®, CA, USA). Hydrogen was used as carrier gas, operating at a constant flow of 1 mL/min. The mass spectrometer operated at −70 eV, and a mass range from 50 to 650 m/z was scanned.

3. Statistical analysis

To confirm the use of a nonparametric test, the normal distribution of neutral sterol content and net increment was evaluated using the Shapiro–Wilk test. The two-sample Wilcoxon test was used to detect significant differences in fecal sterol contents between the basal and final intervention period and in net increments between placebo and active beverage intake. Univariate correlations between excreted contents after active beverage or placebo intake were investigated using the Spearman coefficient. In all cases, $p < 0.05$ was used as the criterion for statistical significance. The statistical analyses were performed using the Statgraphics Centurion XVI.I statistical package. It should be noted that most excretion values for cholestanone were below the limit of

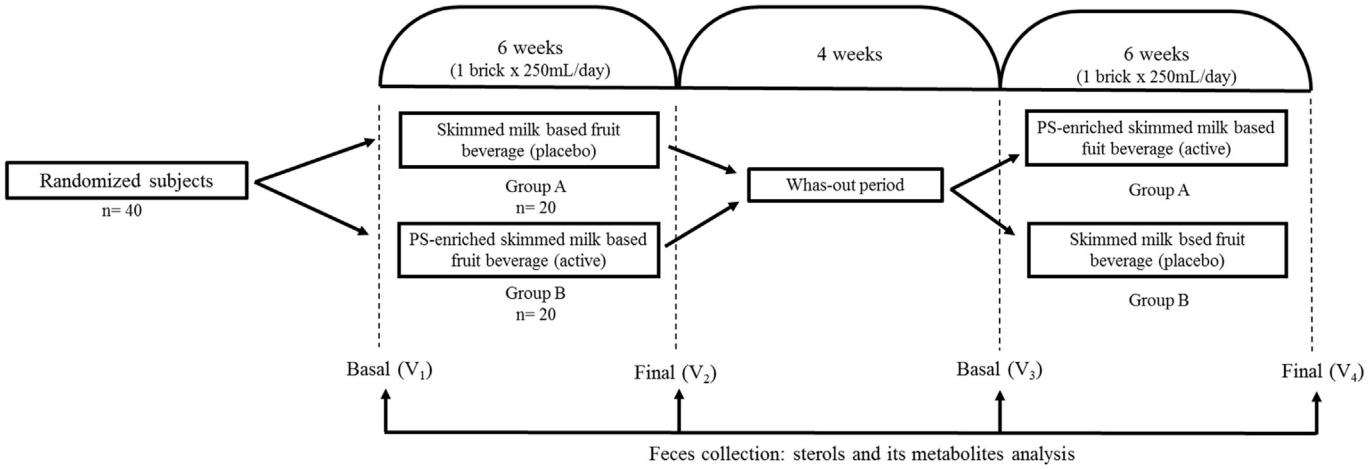

Fig. 1. Overview of the study.

quantification (0.0036 mg/g); limits of detection (0.0011 mg/g) were therefore used.

We used a binary logistic regression analysis for paired data to determine which fecal sterol grouping discriminates best between active or placebo beverage intake. Logistic regression for paired data was necessary due to the crossover design of the trial. These analyzes were performed using the statistical package R V3.2.2 (http://cran.r-project.org). The goodness of fit of the resulting models was tested according to the area under the curve (AUC) of the receiver operating characteristic (ROC).

4. Results

4.1. Participant enrollment

The participant flow of the randomized, double-blind crossover trial is shown in Fig. 2, starting in March 2014 and ending in August 2015. Fifty postmenopausal women with mild hypercholesterolemia (age range 50–65 years) were enrolled. Of these, 10 were excluded and 40 were randomized to participate in the intervention study (from April to July 2014). Thirty-six women finally completed the study.

4.2. Subject adherence

In both intervention periods, active and placebo (6 weeks), one tetra-brick beverage was ingested per day. Thus, the tetra-bricks consumed in each period totaled 42. According to the registry of non-ingested tetra-bricks, 35 women consumed ≥38 tetra-bricks of active beverage and ≥39 tetra-bricks of placebo beverage during the intervention periods, and only one woman consumed 35 or 36 tetra-bricks of active and placebo beverage, respectively.

Binary logistic regression analysis was used to diagnose active or placebo beverage intake. For this purpose, ROC curves were plotted to calculate the sensitivity and specificity of animal and plant fecal sterols as predictors (cut-off points). Figure 3 shows the ROC curves with several cut-off points corresponding to cholesterol and its metabolites (area under the curve [AUC]: 0.60, 95%CI: 0.46–0.73, Fig. 3A), which were not found to be of use in the study adherence determination. Some PS and their metabolites such as sitosterol (Fig. 3B), campesterol (Fig. 3C) and stigmasterol (Fig. 3D) showed high sensitivity and specificity (AUC: 0.92, 95%CI: 0.84–0.99; AUC: 0.87, 95%CI: 0.78–0.97; and AUC: 0.82, 95%CI: 0.71–0.93, respectively). In addition, neutral sterols such as cholesterol, sitosterol, campesterol and stigmasterol (Fig. 3E), and total AS together with total PS (Fig. 3F) were also used as cut-off points, and were seen to be highly sensitive and specific (AUC: 0.88, 95%CI: 0.79–0.97; and AUC: 0.91, 95%CI: 0.83–0.99, respectively) in diagnosing active or placebo beverage intake.

4.3. Excretion of fecal sterols

Figure 4 shows the net excretion increments for AS and PS after active and placebo beverage intake. Generally, the postmenopausal women showed a higher net increment in PS excretion after active beverage intake compared to placebo. After active beverage intake, fewer outliers in the net increment in excretion of neutral sterols (cholesterol, sitosterol, sitostanol, campesterol and stigmasterol) were observed (1, 1, 1, 1 and 0 outliers, respectively) compared to placebo (7, 8, 5, 8 and 2 outliers, respectively). Conversely, the outliers in the net increment in excretion of sterol metabolites such as coprostanol, coprostanone and methylcoprostanone proved more numerous after active beverage intake (3, 5 and 5 outliers, respectively) *versus* placebo (0, 3 and 3 outliers, respectively). However, the outliers of ethylcoprostanol were more numerous after placebo intake (2 outliers) *versus* active beverage (0 outliers). For the rest of the sterol metabolites, the number of outliers remained the same after both beverages.

Fecal AS contents at basal and after active and placebo beverage (final), and their net increments, are shown in Table 3. Due to the non-normal distribution of the data, the total AS contents, expressed as medians, were found to be 19.82 and 18.81 mg/g freeze-dried feces at basal and 21.58 and 18.64 mg/g freeze-dried feces after placebo and active beverage intake (final), respectively.

After placebo intake, only the cholesterol content showed a significant increase (34%). However, after active beverage intake, significant increases in cholesterol (65%), coprostanone (80%), cholestanol + methylcoprostanol (42%), and lathosterol (9%) were observed.

Cholestanol + methylcoprostanol increased significantly after active beverage intake compared to placebo. In the case of cholestanone, the statistically significant difference proved irrelevant, as the values obtained were below the detection or quantitation limits.

Fecal PS contents after the intervention and their net increments are shown in Table 4. A statistically significant increase in total and individual PS (except ethylcoprostenol and brassicasterol) was recorded after active beverage intake (final). Furthermore, statistically significant differences ($p < 0.05$) in net increment between placebo and active beverage intake were observed for all PS except for ethylcoprostenol and brassicasterol.

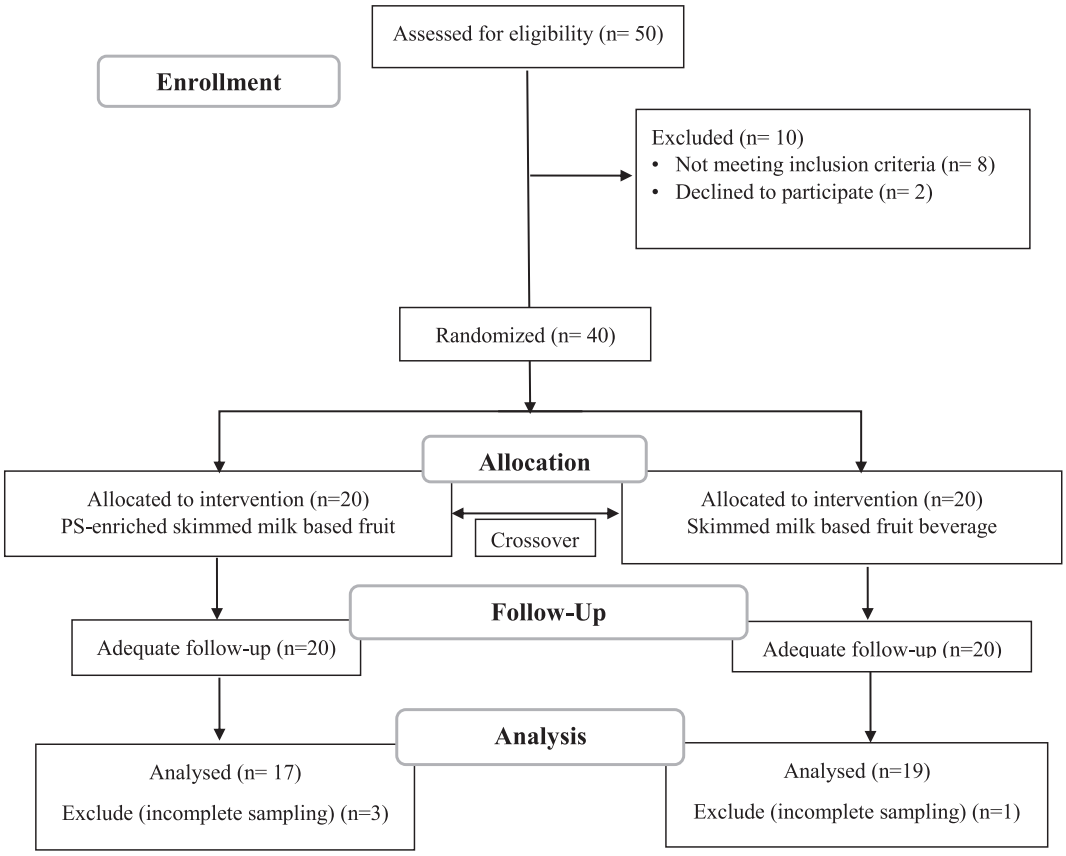

Fig. 2. Flow diagram of the progress through the phases of a randomized double-blind crossover trial (intervention allocation, follow-up, and data analysis).

4.4. Conversion of sterols

Figure 5 shows the conversion percentages of different neutral sterols (cholesterol, sitosterol, and stigmasterol) in all subjects after active and placebo beverage intake. To calculate this percentage, the following equation was used: [metabolites/(neutral sterol + metabolites)] x 100. In order to classify women as low or high converters, we considered that a low converter presents a sterol conversion rate of <50%, while a high converter presents a sterol conversion rate of ≥50% [34]. Hence, the number of women found to be high converters after placebo and active beverage intake were: cholesterol 33 and 29 (Fig. 5A); sitosterol 29 and 17 (Fig. 5B); and finally stigmasterol 18 and 27 (Fig. 5C), respectively. However, in the case of campesterol, all women were low converters after placebo and active beverage intake (data not shown).

We consider it interesting to note the effect of the active beverage upon the decrease in conversion percentage. Based on the conversion percentage frequency distribution used by Wilkins and Hackman (1974) [34], the ≥10% reduction in the conversion percentage of the sterols stands out (Fig. 5). In this context, active beverage intake produced a significant decrease (between 11% and 50%) in cholesterol conversion in 16 women (Fig. 5A, marked with arrows), although 12 of whom remained high converters and 4 became low converters. Regarding sitosterol, 24 women showed a significant decrease (between 15% and 61%) after active beverage intake (Fig. 5B, marked with arrows), and 7 of whom remained high converters and 4 low converters, while 13 women changed from high to low converters. However, in the case of stigmasterol, the intake of the active beverage only produced a significant decrease (between 14% and 67%) in 6 women (Fig. 5C, marked with arrows), of whom only 3 remained as high converters. In general, most women (n = 31) did not change their conversion percentage with regard to campesterol after active beverage intake.

4.5. Correlation of sterols

The Spearman correlation coefficient (ρ) was used to evaluate statistically significant associations referred to fecal AS, PS contents and their corresponding metabolites, after active or placebo beverage intake. In this sense, only after active beverage intake, was a statistically significant correlation found between fecal sitosterol (ρ: 0.632, p = 0.0002), campesterol (ρ: 0.428, p = 0.0112) and stigmasterol (ρ: 0.370, p = 0.0282) (sterols present in active beverage) and total fecal PS. In addition, a positive and strong correlation was observed between total fecal PS and total sterols after active beverage intake (ρ: 0.926, p = 0.0000), the correlation being weaker in the case of the placebo (ρ: 0.654, p = 0.0001). However, a weak correlation was observed between total fecal AS and total fecal sterols after active beverage intake (ρ: 0.643, p = 0.0001), with a strong correlation after placebo intake (ρ: 0.944, p = 0.0000).

It should be noted that the inverse relationship observed between fecal cholesterol and its metabolites after both active (ρ: −0.592, p = 0.0005) and placebo beverage intake (ρ: −0.335, p = 0.0471), was more intense and significant after consumption of the active beverage. Furthermore, negative and strong correlations were observed for fecal sitosterol, stigmasterol and campesterol with fecal cholesterol metabolites after active beverage intake (ρ: −0.519, p = 0.0021; ρ: −0.643, p = 0.0001; and ρ: −0.620, p = 0.0002, respectively) *versus* placebo (only for sitosterol ρ: −0.361, p = 0.0325; and stigmasterol ρ: −0.506, p = 0.0027).

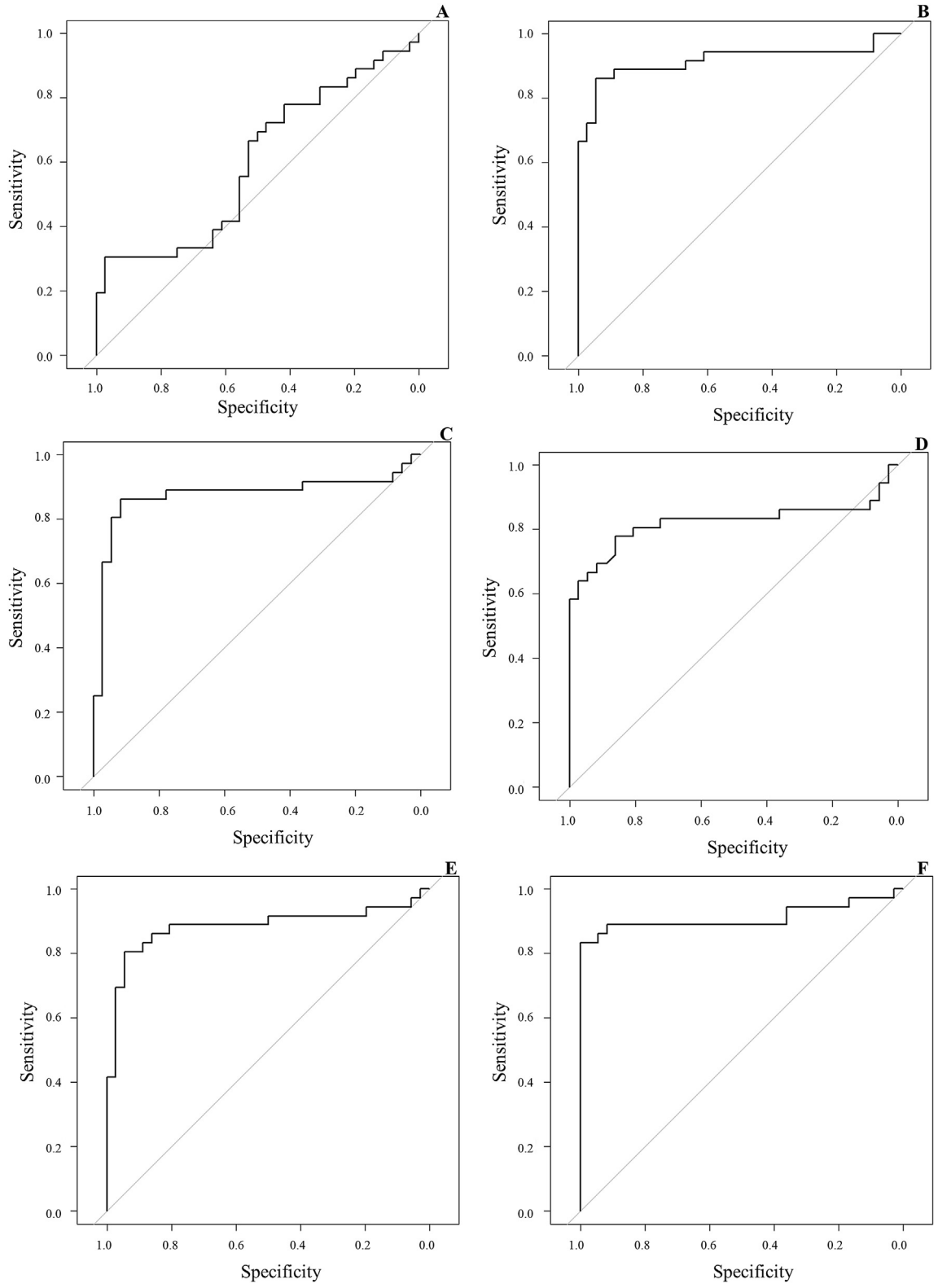

Fig. 3. Receiver Operating Characteristic (ROC) curves to predict beverage intake (active or placebo) in the intervention clinical trial. Active: PS-enriched skimmed milk based fruit beverage. Placebo: skimmed milk based fruit beverage. Group: A: cholesterol + coprostanol + coprostanone; B: sitosterol + sitostanol + ethylcoprostanol + ethylcoprostanone; C: campesterol + campestanol + methylcoprostanone; D: stigmasterol + stigmastenol + ethylcoprostenol; E: cholesterol + sitosterol + campesterol + stigmasterol; F: total animal sterols + total plant sterols.

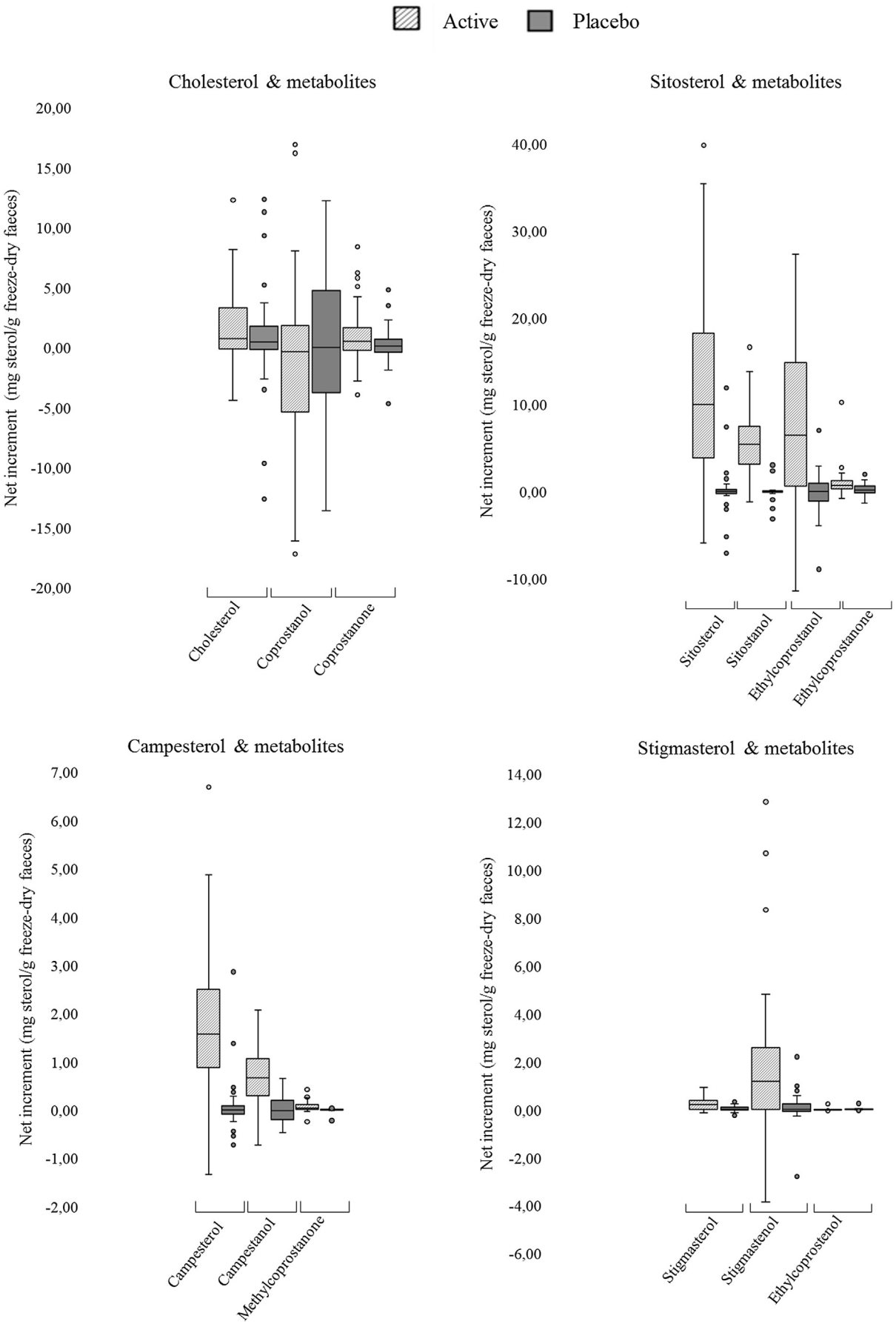

Fig. 4. Sterols and their metabolites response in feces upon regular consumption of active and placebo beverages (n = 36). Active: PS-enriched skimmed milk based fruit beverage. Placebo: skimmed milk based fruit beverage. Boxes represent the mean of the net increment: final − basal (n = 36). Points in each box represent outlier values.

Table 3
Fecal animal sterols contents (mg/g freeze-dry feces) after placebo and active beverages intake.

Sterol	Basal	Final	p value	Net increment	p value
Placebo					
Cholesterol	1.51[a] (0.79; 3.02)	2.02[b] (1.12; 4.25)	0.03	0.43 (−0.14, 1.71)	
Coprostanol	12.74[a] (7.77, 22.02)	12.50[a] (9.51, 21.21)	1.00	0.00 (−3.72, 4.74)	
Coprostanone	1.40[a] (0.55, 2.85)	1.23[a] (0.60, 3.04)	0.49	0.10 (−0.38, 0.66)	
Cholestanol + methylcoprostanol[c]	0.55[a] (0.46, 0.81)	0.58[a] (0.40, 0.80)	0.61	−0.01 (−0.16, 0.10)	
Cholestanone[d]	0.0011[a] (0.0011, 0.095)	0.0011[a] (0.0011, 0.10)	0.93	0.00 (0.00, 0.02)	
Lathosterol	0.11[a] (0.09, 0.15)	0.12[a] (0.09, 0.16)	1.00	0.00 (−0.02, 0.03)	
Total animal sterols	19.82[a] (13.89, 28.50)	21.58[a] (14.47, 30.10)	0.61	−1.10 (−3.34, 6.45)	
Active					
Cholesterol	2.30[a] (1.70, 4.20)	3.80[b] (1.87, 7.24)	0.01	0.76 (−0.12, 3.20)	0.16
Coprostanol	12.87[a] (7.03, 19.05)	9.90[a] (6.39, 16.26)	0.13	−0.35 (−5.30, 1.52)	0.11
Coprostanone	1.24[a] (0.61, 3.00)	2.23[b] (0.83, 4.54)	0.04	0.52 (−0.26, 1.65)	0.14
Cholestanol + methylcoprostanol[c]	0.67[a] (0.44, 0.85)	0.95[b] (0.62, 1.53)	5×10^{-4}	0.36* (0.005, 0.71)	6×10^{-4}
Cholestanone[d]	0.0011[a] (0.0011, 0.090)	0.0011[b] (0.0011, 0.22)	0.01	0.00* (0.00, 0.10)	6×10^{-4}
Lathosterol	0.11[a] (0.09, 0.17)	0.12[b] (0.09, 0.17)	0.04	0.01 (−0.01, 0.05)	0.32
Total animal sterols	18.81[a] (14.18; 25.20)	18.64[a] (15.92; 28.51)	0.24	1.44 (−4.76; 7.38)	1.00

Placebo: skimmed milk based fruit beverage intake. Active: PS-enriched skimmed milk based fruit beverage intake. Net increment: final − basal. Values are expressed as median (n = 36). Percentile: 25–75% is indicated between parentheses.
Different lowercase letters (a, b) indicate statistically significant differences ($p < 0.05$) in the excretion to each sterol (mg sterol/g freeze-dry feces) between basal and final samples for each type of period (active or placebo).
*Indicate statistically significant differences ($p < 0.05$) in the net increment excretion of each sterol (mg sterol/g freeze-dry feces) between placebo and active period.
[c] The applied method does not allow the separation of these compounds.
[d] In those women who had cholestanone contents lower than the limit of detection (0.0011 mg/g) or quantitation (0.0036 mg/g); the limit of detection were used for statistical treatment.

However, negative correlations between fecal cholesterol and PS metabolites were similar after active (ρ: −0.397, p = 0.0187) and placebo beverage intake (ρ: −0.442, p = 0.0089). In addition, similar positive correlations between fecal cholesterol metabolites and PS metabolites after active (ρ: 0.702, p = 0.0000) and placebo beverage intake (ρ: 0.711, p = 0.0000) were observed. Finally, the positive correlation observed between fecal PS metabolites and total PS was greater after placebo (ρ: 0.873, p = 0.0000) than after active beverage intake (ρ: 0.589, p = 0.0005).

5. Discussion

This clinical study was carried out to evaluate the impact of high PS intake on excreted fecal sterols and their microbial conversion in postmenopausal women with mild hypercholesterolemia. Fecal sitosterol together with its metabolites (Fig. 3B) and fecal AS with PS (Fig. 3F) were identified as the most adequate predictors in the binary logistic regression analysis (ROC curves) for establishing subject adherence in the trial. In addition, individual fecal sterol PS

Table 4
Fecal plant sterols contents (mg/g freeze-dry feces) after placebo and active beverages intake.

Plant sterol	Basal	Final	p value	Net increment	p value
Placebo					
Sitosterol	1.21[a] (0.93; 2.19)	1.63[a] (1.08; 2.34)	1.00	−0.03 (−0.26; 0.21)	
Sitostanol	0.14[a] (0.07; 0.24)	0.13[a] (0.03; 0.21)	0.71	0.00 (−0.09; 0.05)	
Ethylcoprostanol	4.00[a] (3.06; 5.85)	4.42[a] (3.05; 5.22)	0.73	−0.02 (−1.14; 0.92)	
Ethylcoprostanone	0.96[a] (0.57; 1.45)	1.13[a] (0.75; 1.66)	0.49	0.15 (−0.20; 0.62)	
Campesterol	0.22[a] (0.04; 0.36)	0.24[a] (0.12; 0.33)	0.85	0.00 (−0.085; 0.07)	
Campestanol	0.37[a] (0.20; 0.58)	0.41[a] (0.13; 0.55)	0.47	−0.02 (−0.20; 0.19)	
Methylcoprostanone	0.01[a] (0.004; 0.03)	0.01[a] (0.004; 0.03)	0.90	0.00 (−0.01; 0.01)	
Stigmasterol	0.17[a] (0.09; 0.24)	0.18[a] (0.13; 0.26)	0.15	0.01 (−0.03; 0.08)	
Stigmastenol	0.21[a] (0.0003; 0.51)	0.23[a] (0.0003; 0.74)	0.23	0.00 (0.00; 0.01)	
Ethylcoprostenol	0.06[a] (0.04; 0.06)	0.06[a] (0.05; 0.07)	0.26	0.00 (0.00; 0.01)	
Brassicasterol	0.25[a] (0.15; 0.34)	0.22[a] (0.15; 0.34)	1.00	0.00 (−0.08; 0.05)	
Total plant sterols	8.73[a] (6.50; 10.94)	9.20[a] (7.11; 10.28)	0.86	0.10 (−1.33; 1.67)	
Active					
Sitosterol	1.83[a] (1.06; 2.68)	11.82[b] (5.87; 21.03)	4×10^{-6}	10.00* (4.12; 17.73)	7×10^{-7}
Sitostanol	0.11[a] (0.0002; 0.35)	5.75[b] (4.31; 7.89)	6×10^{-7}	5.42* (3.34; 7.38)	4×10^{-7}
Ethylcoprostanol	4.62[a] (2.64; 5.36)	11.42[b] (6.74; 20.63)	2×10^{-5}	6.40* (0.77; 14.72)	2×10^{-5}
Ethylcoprostanone	1.03[a] (0.73; 1.46)	1.85[b] (1.40; 2.23)	1×10^{-5}	0.66* (0.27; 1.20)	2×10^{-3}
Campesterol	0.31[a] (0.17; 0.50)	1.88[b] (1.06; 3.35)	2×10^{-6}	1.57* (0.91; 2.44)	5×10^{-6}
Campestanol	0.32[a] (0.15; 0.63)	1.01[b] (0.74; 1.40)	5×10^{-6}	0.67* (0.31; 1.06)	5×10^{-6}
Methylcoprostanone	0.01[a] (0.0004; 0.04)	0.05[b] (0.02; 0.13)	2×10^{-4}	0.04* (0.02; 0.10)	5×10^{-4}
Stigmasterol	0.18[a] (0.10; 0.28)	0.39[b] (0.22; 0.60)	3×10^{-5}	0.20* (−0.005; 0.36)	7×10^{-4}
Stigmastenol	0.10[a] (0.0003; 0.46)	1.58[b] (0.38; 3.57)	4×10^{-5}	1.17* (0.00; 2.53)	6×10^{-5}
Ethylcoprostenol	0.06[a] (0.05; 0.06)	0.06[a] (0.05; 0.07)	0.10	0.00 (0.00; 0.01)	0.52
Brassicasterol	0.25[a] (0.18; 0.38)	0.25[a] (0.18; 0.35)	0.88	0.00 (−0.07; 0.07)	1.00
Total plant sterols	8.58[a] (6.75; 11.64)	43.86[b] (33.51; 55.72)	1×10^{-6}	33.29* (22.19; 47.62)	2×10^{-6}

Placebo: skimmed milk based fruit beverage intake. Active: PS-enriched skimmed milk based fruit beverage intake. Net increment: final − basal. Values are expressed as median (n = 36). Percentile: 25–75% is indicated between parentheses.
Different lowercase letters (a, b) indicate statistically significant differences ($p < 0.05$) in the excretion to each sterol (mg sterol/g freeze-dry feces) between basal and final samples for each type of period (active or placebo).
*Indicate statistically significant differences ($p < 0.05$) in the net increment excretion of each sterol (mg sterol/g freeze-dry feces) between placebo and active period.

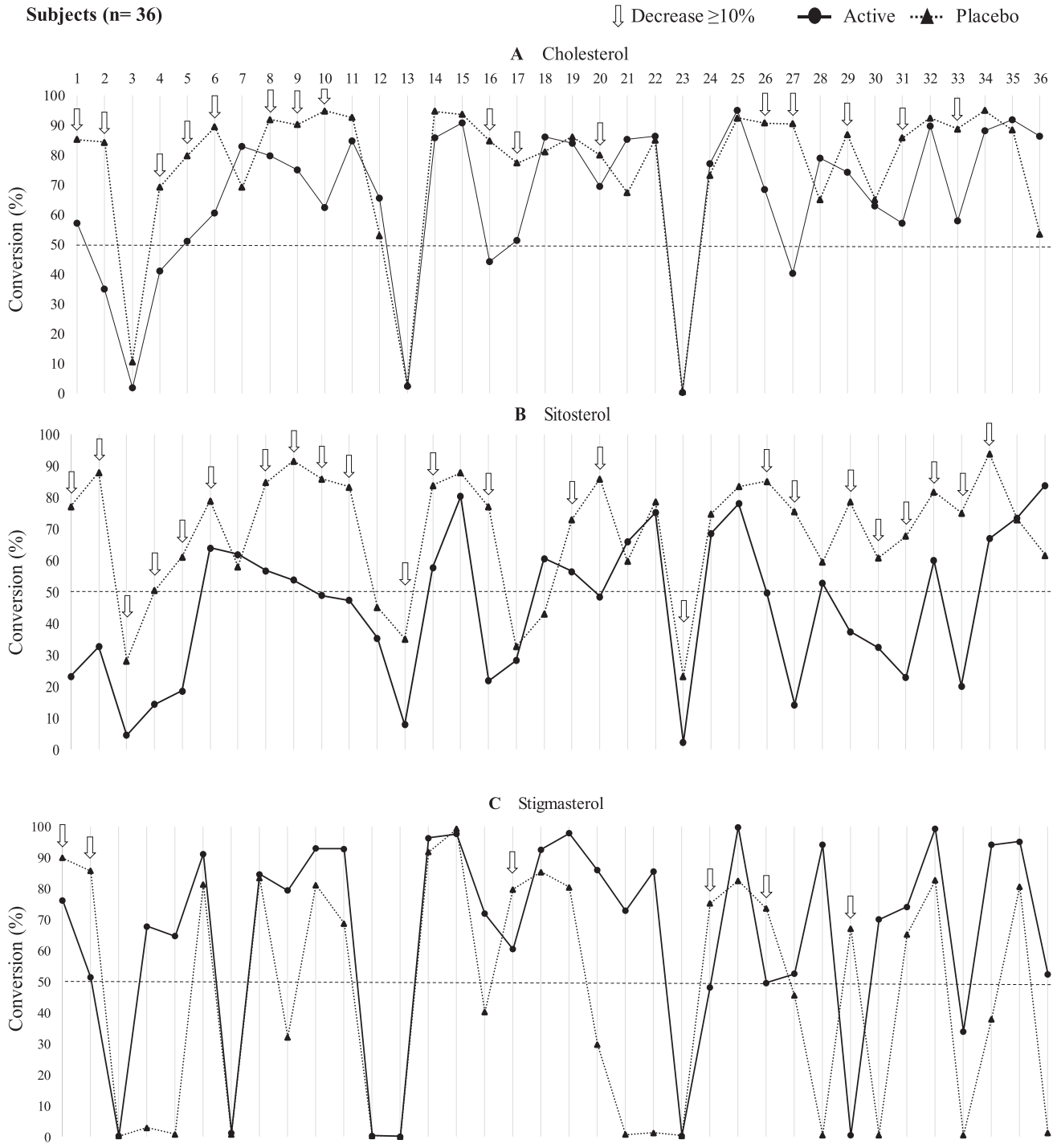

Fig. 5. Conversion percentages of cholesterol, sitosterol and stigmasterol from intervention clinical trial with post-menopausal women after active and placebo beverage intake (n = 36). Abscissa axis represents the 36 post-menopausal women who participated in the clinical study; Ordinate axis shows the sterol conversion percentage, which was calculated according to the following equation: [metabolites/(neutral sterols + metabolites)] x 100. A: cholesterol conversión [coprostanol + coprostanone/(cholesterol + comprostanol + coprostanone)]; B: sitosterol conversion [ethylcoprostanol + ethylcoprostanone/(sitosterol + sitotanol + ethylcoprostanol + ethylcoprostanone)]; C: stigmasterol conversion [stigmastenol + ethylcoprostenol/(stigmasterol + stigmastenol + ethylcoprostenol)]. Solid line with a circle as marker: conversion after active beverage; Dashed line with a triangle as marker: conversion after placebo. The dashed line located on each graph shows the threshold at 50% to define the low and high converters according to Wilkins & Hackman, (1974) [27]. The arrow indicates the significant decrease (≥10%) after active beverage intake compared to placebo.

(sitosterol, campesterol and stigmasterol present in the active beverage) were directly and strongly correlated to total fecal PS after active beverage intake.

Furthermore, the net increment of excretion of neutral sterols (those present in the active beverage) showed fewer outliers after active beverage intake (Fig. 4). This suggests that both groups, A and B (Fig. 1), responded homogeneously to active beverage intake, independently of the timing of ingestion. However, the net increment in ethylcoprostanol excretion showed a more homogeneous response after active beverage intake, perhaps due to the high

sitosterol content present in the latter (Fig. 4B). The heterogeneous response referred to the net increment excretion of neutral sterols after placebo beverage intake was possibly the result of variability in dietary habits among the women [10,21,22,24], which was not controlled and has emerged as an instrumental factor in the configuration of the gut microbiota [22,24,35,36]. In this context, the response of fecal sterol metabolites after active beverage intake was more heterogeneous, especially with regard to AS (Fig. 4A) and methylcoprostanone (Fig. 4C), due to interindividual microbial variability. In fact, each individual human is known to harbor specific bacteria [37], and the long-term intake of high-animal fat diets is associated to changes in the gut microbiota [38,39].

The total fecal AS contents found in our study (13.9–30.10 mg/g freeze-dried feces) (Table 3) were similar to those reported by other authors after diets not enriched with PS (19.4–28.5 mg/g freeze-dried feces) [12,16,19,29,33,34,40]. In humans with PS-enriched margarine intake (8.6 g PS/day) [29], an increase has been evidenced in fecal cholesterol content (net cholesterol excretion 20.7 mg/g freeze-dried feces). However, this fact was not observed in our study, probably due to a lower PS intake (2.0 g/day).

The fecal contents in PS and their metabolites (6.50–11.64 mg/g freeze-dried feces) were similar in the two basal periods and in the final period for placebo, in concordance with other authors (5.50–10.20 mg/g freeze-dried feces) [18,33,34,40]. However, active beverage intake produced greater (nearly 5-fold) PS excretion with respect to placebo (Table 4), in agreement with one of the aforementioned studies after PS-enriched margarine consumption (nearly 17-fold) [29]. We also found a significant increase (nearly 3-fold) in ethylcoprostanol excretion after active beverage intake. These results suggest that the gut microbiota preferentially uses PS as substrate, as they were present in a greater proportion with respect to cholesterol. In addition, the relative percentages of fecal AS and PS with respect to total fecal sterols in the two basal periods and in the final period for placebo were consistent with the results of other authors (~70 and ~30%, respectively) [15,18,29,33,34,40]. However, these relative percentages were reversed (~30 and ~70%, respectively) after active beverage intake. Similarly, while the positive correlation between total fecal PS/total sterols was greater after active beverage intake, the correlation between total fecal AS/total sterols was greater after placebo.

An inverse correlation between fecal cholesterol and its metabolites was observed that proved greater after active beverage intake than after placebo consumption. In this context, a significant net increment in the excretion of coprostanone, and a slight and nonsignificant decrease in the excretion of coprostanol, were recorded after active beverage intake. However, other authors reported a decrease in the excretion not only of coprostanol but also of coprostanone (9 and 1.5 mg/g freeze-dried feces, respectively) after PS-enriched food intake [29]. Besides, epidemiological studies with subjects following vegetarian and low-animal fat diets have reported a decrease in coprostanol (from 12.2 to 2.3 mg/g freeze-dried feces) and coprostanone (between 2.3 and 0.3 mg/g freeze-dried feces), compared with high-animal fat (Western) diets [10,19–24]. In our study, this fact could be due to the diet effect in the women, since the real sterol intake had not been controlled – this constituting a limitation of the study. Besides, the significant increase in coprostanone after active beverage intake could also suggest that the gut microbiota metabolized cholesterol through an indirect pathway, which was interrupted during this step, causing a lower production of coprostanol. This also suggests that the capacity of the gut microbiota was not sufficient to transform AS in the usual manner, due to the large amounts of PS present [41]. In fact, it has been suggested that the efficiency of cholesterol conversion is related to microbial density. In this regard, a coprostanol/cholesterol ratio of $\geq$15 has been associated to high levels of coprostanoligenic bacteria (10^8 cells/g) and to nearly complete cholesterol conversion [42]. In this context, we found lower coprostanol/cholesterol ratios in postmenopausal women after active beverage intake compared with placebo (3.2 *versus* 6.2). In coincidence with our findings, other authors have recorded lower ratios after the intake of PS-enriched margarine compared to the controls (0.35 *versus* 2.6) [29], in vegetarian subjects compared to omnivorous individuals (1.62 *versus* 4.38) [19], and in low converters *versus* high converters (0.13 *versus* 7.6) [34]. In concordance with other authors [19,34,43], we found a low coprostanol/cholesterol ratio (<0.9) after active beverage intake *versus* placebo (8%) in 20% of the postmenopausal women. Besides, it has also been suggested that low cholesterol conversion could be due to a lack of mucosal receptors for coprostanoligenic bacteria [43] or to the inhibition of these bacteria by other components of the gut microbiota [44].

As far as we know, this is the first time that microbial conversion percentages corresponding to cholesterol, sitosterol and stigmasterol are reported after a PS-enriched food intervention in postmenopausal women with mild hypercholesterolemia, describing the effect of high PS intake upon their conversion percentage rates. Most subjects in our study were high cholesterol converters. They showed an average conversion percentage of 82.3 ± 11.7 after placebo *versus* 75.4 ± 13.3 after active beverage intake, in the same way as in subjects following a Western diet (75–89%) [12,19,34,45] and in vegetarians (66%) [19]. Low converters showed percentages of 23.5 ± 20.9 and 4.5 ± 5.2 after active and placebo intake, respectively, according to the values reported in other studies (1.0–43.0) among subjects following a Western diet [12,34].

However, there are no consensus-based data regarding the thresholds that classify high and low converters. While Wilkins and Hackman (1974) [34] reported that high converters present a sterol conversion percentage of $\geq$50%, Midtvedt et al. (1990) [43] reported a value of $\geq$40%. Most authors consider the threshold to be $\geq$ 50% [12,13,16,19].

On the other hand, in 16 subjects, active beverage intake produced a significant decrease in the fecal cholesterol conversion percentage compared to placebo – a condition that has been reported in humans after PS-enriched margarine intake [29]. However, this decrease has not been observed in vegetarian *versus* omnivorous diets (66.3 ± 7.9 *versus* 75.3 ± 6.3) [19]. In this sense, it has been suggested that an increase in PS intake could reduce or block fecal cholesterol conversion, thereby resulting in a lower production of cholesterol metabolites, which are associated to procarcinogenic action and could increase the risk of colon cancer [11,29,46]. Furthermore, a previous study by our group found PS at human colonic concentrations to exhibit antiproliferative effects against colon cancer cells (Caco-2 cells) [47].

Most subjects were high sitosterol converters after placebo, in agreement with the only study that reports data in this regard (23 high and 8 low converters) [34], although fewer than half of whom were high converters after active beverage intake. The high converters showed an average in fecal sitosterol conversion percentage of 52.7 ± 9.6 after active beverage *versus* 75.9 ± 11.3 after placebo intake, while the values for the low converters were 26.9 ± 15.1 and 34.5 ± 8.5, respectively. After active beverage intake, the fecal sitosterol conversion percentage decreased in 24 subjects. Thus, the sitosterol/sitosterol metabolites ratio (in 22 subjects) after active beverage intake was higher than in the placebo group (expressed as median, 0.5 *versus* 0.3). Indeed, the positive correlation between fecal PS metabolites and total PS was greater after placebo than after active beverage intake. These observations would suggest that the gut microbiota was unable to metabolize the PS from active beverage, due to the abundant presence of these sterols.

A limitation of our study was the lack of a microbiota analysis, which did not form part of the objectives of the clinical study. Such

an analysis would have provided valuable information about the impact of high PS intake upon the intestinal microbial community, allowing it to be correlated to the fecal sterol conversion percentage found in our study. Nevertheless, the present study is of interest, since only one publication has evaluated the effect of a PS-enriched food upon the fecal sterol profile – this fact represents a limitation for the discussion of the results.

6. Conclusions

In the present intervention study focusing on the intake of a PS-enriched skimmed milk based fruit beverage (2 g/day over a 6-week period), which had a hypocholesterolemic effect, a decrease in fecal cholesterol metabolites was observed. These metabolites could be associated to pro-carcinogenic action. The PS-enriched beverage did not modify the fecal excretion of cholesterol, although an increase in coprostanone was observed, probably due to saturation of the gut microbiota. Furthermore, an expected significant increase in PS excretion - especially ethyl-coprostanol - was recorded. Besides, intake of the PS-enriched beverage modified the microbial converter profile with regard to cholesterol and sitosterol (decreasing the number of high converters), and stigmasterol (increasing the number of high converters).

Therefore, further studies on the effect of PS-enriched foods could have in the microbial metabolism of dietary sterols and on the impact of PS-enriched foods upon the gut microbiota composition and diversity are needed.

Statements of authorship

MJL was the main researcher. MJL and AA contributed to the study design and the writing of the study protocol. MC-T was in charge of sample analysis and data collection. MJL, AA and MC-T carried out the data analysis and writing the manuscript. JDB provided statistical advice and carried out the binary logistic regression for paired data analysis. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.08.012.

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