A selenium-enriched diet helps to recover liver function after antibiotic administration in mice

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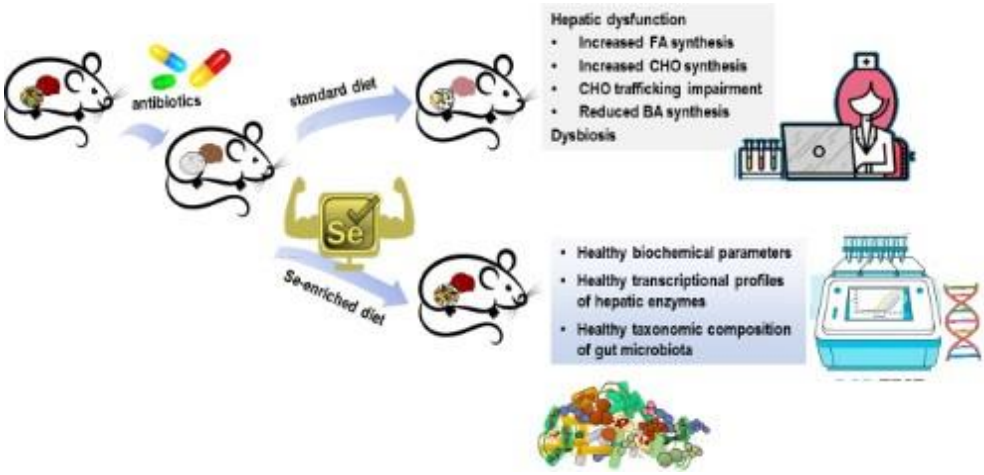
Abstract

Antibiotic (Abx) treatments or inadvertent exposure to Abx-contaminated food and water can adversely affect the health. Many studies show strong correlations between Abx and liver damage and point to gut dysbiosis as a contributing factor because the gut microbiota (GM) forms a complex network with the liver. Selenium (Se) is a beneficial micronutrient able to shape the composition of the GM. We analyzed here the ability of a low dose (120 μg/kg bodyweight/day) Se-enriched diet to ameliorate the effects of a 7-day previous intervention with an Abx-cocktail (200 mg/kg bodyweight/day of ampicillin, metronidazole, and neomycin; 100 mg/kg bodyweight/day of vancomycin; and 20 mg/kg bodyweight/day of amphotericin-B) over the global health and the homeostasis of cholesterol and bile acids in the mouse liver. We found that Se restored lipid metabolism preventing the increased synthesis and accumulation of cholesterol caused by Abx treatment. Integrating these results with previous metataxonomic and metabolomic data in the same mice, we conclude that part of the effect of Se against liver dysfunction (cholesterol and bile acids metabolism and transport) could be mediated by the GM. We provide data that contribute to a more complete view of the molecular mechanisms underlying the beneficial action of Se on health, pointing to a possible use of low doses of Se as a functional food additive (prebiotic) to prevent the negative effects of antibiotics.

Key words: antibiotics, hepatic dysfunction, selenium, prebiotic, gut microbiota restoration.

Graphical abstract



Highlights

- Acute antibiotic treatments (Abx) cause sustained liver damage over time.
- Abx perturb the hepatic lipid metabolism and transport.
- A low dose of selenium (Se) in the diet restores hepatic homeostasis in Abx-mice.
- The hepatoprotective effects of Se might be partially mediated by the gut microbiota.
- Low doses of Se can be used as a functional food additive (prebiotic).

1. Introduction

Antibiotics (Abx) are natural or synthetically produced chemicals widely used to inhibit bacterial growth (bacteriostatic) or to kill bacteria (bactericidal). For over 70 years, Abx have been a mainstay of medicine, used worldwide on an enormous scale as a cure for many infectious diseases affecting humans and animals, with the confidence that they were completely safe. However, multiple evidences show that Abx treatments and even inadvertent exposure to the rising concentrations of Abx in food and water, can have short- and long-term adverse effects on health (Ramirez *et al.*, 2020; Shah *et al.*, 2021). In the last decade, many prospective and retrospective studies have shown strong correlations between Abx and liver damage (Bjornsson, 2017; Fu et al., 2022; Li et al., 2022; Park et al., 2021). In fact, hepatotoxicity is usually listed among Abx contraindications and is one of the factors taken into consideration for Abx regulation and/or withdrawal from the market (Lee and Senior, 2005). Abx hepatotoxicity cases, which are relatively infrequent (1-10 per 10^5 prescriptions) (Polson, 2007), usually idiosyncratic and with varied clinical manifestations, are equally distributed between hepatocellular and cholestatic types, depending on the magnitude of the increase of transaminases relative to alkaline phosphatase (Iluz-Freundlich *et al.*, 2020). The hepatocellular type lesions have a higher risk of acute evolution and sometimes result in death/liver transplantation. In contrast, the cholestatic type lesion presents a high probability of chronic evolution and the clinical manifestations persist after Abx withdrawal (Robles *et al.*, 2010).

The gut microbiota (GM) forms a complex network with the liver, the so-called microbiota-gut-liver- axis, which also connect with the brain as the gut microbiota also forms a network with the nervous system (Ding *et al.*, 2020). The liver liberates compounds (lipids, primary bile acids, etc.) that reach the GM through the biliary tract, portal vein, and systemic mediators. As a metabolite producing factory, the GM transforms these biomolecules into

diverse metabolites such as secondary bile acids (BA) (Mohammed *et al.*, 2022), amino acids, and the short-chain fatty acids (SCFA) butyrate, acetate and propionate, many of them travelling through the portal vein and reaching back the liver, where they modify the hepatic functions (Khan *et al.*, 2019). Though a comprehensive understanding of the exchange between the microbiome and the liver still evades us, an accumulating body of research suggests that the disparate observations in liver disease-related studies could be unified and explained by the microbiome, and thus the GM has been regarded as a new environmental factor contributing to liver disease development (Ding *et al.*, 2020; Tripathi *et al.*, 2018; Wang *et al.*, 2021).

When exposed to Abx, microbial communities immediately change their composition depending on the susceptibility of the different taxa to Abx. Several studies have shown that GM possesses a capacity to recover from Abx treatment, although usually with a new composition as some species or strains disappear and others find a new niche to colonize (Palleja *et al.*, 2018; Ribeiro *et al.*, 2020; Shah *et al.*, 2021). The exchange of specific microbial populations in the GM dysregulates the production of microbial metabolites and can lead to several gastrointestinal, immunologic, and neurocognitive altered conditions (Blesl and Stadlbauer, 2021; Mohammed *et al.*, 2022; Ramirez *et al.*, 2020; Ribeiro *et al.*, 2020; Wang *et al.*, 2021).

Numerous studies show that diet adaptation, by adjusting its composition or by including supplements, is one of the most effective methods to modulate GM for health promotion (Ağagündüz et al., 2022; Arman et al., 2022; Yeşilyurt et al., 2022). Among this supplements, selenium (Se) is one essential micronutrient with a key role in human health as it participates in the regulation of cellular redox homeostasis, protection from oxidative stress, immune response, cancer chemoprevention, etc. (Bjørklund et al., 2022; Kieliszek and Bano, 2022; Radomska et al., 2021; Soares de Oliveira et al., 2021; Solovyev et al.,

2021; Zhu et al., 2022). At least a part of the beneficial effects of Se-diet enrichment is mediated by the Se capability of shaping the GM (Callejón-Leblic *et al.*, 2021a; Yang *et al.*, 2020). Given that Abx treatment determines the composition of the microbiota, and that the microbiota, in turn, determines the biochemistry of BA, we addressed in this work the hypothesis that dysbiosis caused by Abx exposure may promote disease by altering lipid metabolism and BA homeostasis in the liver, and that diet supplementation with an adequate dose of Se would ameliorate the intestinal dysbiosis generated by acute Abx exposure and would restore liver homeostasis.

2. Materials and methods

2.1. Animals and treatments

Eight weeks-old male *Mus musculus* BALB/c mice (22-24 g, Charles River Laboratories, France) were used. The experimental procedure was as detailed in the paper by (Callejón-Leblic *et al.*, 2021a). As shown in Fig. S1, mice were randomly divided into three groups ~~(n=12-15 mice per group)~~ and caged in a preconditioned laboratory under controlled conditions of temperature (25/30 °C), humidity (40-60 %) and photoperiod (12 h light/12 h dark cycles), with free access to water and food for a total of three weeks. Mice in the C (control, n=12) and Abx groups (n=15 animals per group) were fed standard rodent chow during the three weeks of the experiment. The Abx+Se mice received a Se-supplemented diet (sodium selenite, 0.65 mg/kg feed, which is a dose 3 times higher than that typically found in the murine diet) during the last two weeks (recovery period). Mice in the Abx and Abx+Se groups received a mixture of Abx in the drinking water during the first week (days 3rd to 10th) and then, regular tap water for two additional weeks (days 10th to 24th). Fig. S1 shows the experimental design. The Abx mixture contained ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L), vancomycin (0.5 g/L), and the antifungal amphotericin B (10 mg/L). Feed and water were changed every two days to maintain their quality and were weighed to estimate daily consumption. The animals were also weighed every two days, coinciding with the times of feed change, to calculate the actual ingested doses of Abx and Se.

At the end of the experimental period, mice were anesthetized (isofluorane, 0.15%, inhaled), exsanguinated by cardiac puncture, euthanized by cervical dislocation, and dissected. Livers were immediately washed with cold saline buffer, weighed, and frozen in liquid N_2. Heparinized blood was centrifuged (3000 *g*, 10 min), and stored at -80 °C.

The research was approved by the Ethics Committee of the University of Cordoba (Spain) and by the Regional Government of Andalusia (project code number 02-01-2019-001). All experiments were performed at the Animal Experimentation Service of the University of Cordoba (SAEX-UCO) under the supervision of qualified and experienced professionals and complied with the ARRIVE guidelines, the European Community guidelines EU Directive 2010/63/EU for animal experiments and the Spanish Government Royal Decree 1386/2018.

2.2. Biochemical Analysis in plasma

Plasma was used to determine glucose (glucose oxidase/peroxidase method), lactate, total cholesterol (CHO, cholesterol oxidase/peroxidase assay), triglycerides (TAG, glycerol phosphate oxidase/ peroxidase assay), total bile acids (BA, enzymatic cycling assay), alanine transaminase (ALT/GPT, International Federation of Clinical Chemistry method), alkaline phosphatase (ALP-DEA, diethanolamine assay), total proteins (Biuret assay), albumin (ALB, bromocresol green assay), creatinine (CRE, Jaffe method), and urea (UREA/BUN-UV, urease/glutamate dehydrogenase assay) by using BioSystems® commercial kits and a BioSystems® Atom A-15 Analyzer at the SAEX-UCO.

2.3. Quantification of cholesterol and total bile acids in liver

For the determination of total hepatic CHO, about 20 mg of tissue was homogenized in a mixture of chloroform:isopropanol NP-40 (7:11:0.1). After centrifugation (15000 *g*, 10 min), the organic phase was air-dried at 50 °C and then placed under vacuum for 30 min to remove any traces of organic solvents. The dried lipids were dissolved in the assay diluent provided by the Total Cholesterol Assay Kit (Cell Biolabs, Inc.), used to measure total CHO content in the liver according to the manufacturer's protocol. This kit detects both cholesteryl

esters and free cholesterol in a colorimetric assay based on an enzyme driven reaction (cholesterol esterase/cholesterol oxidase). For hepatic BA analysis, about 50 mg of liver tissue was homogenized in cold-ice PBS followed by centrifugation at 10000 *g* for 20 min. The supernatant was used to measure the BA content in the liver with the colorimetric Total Bile Assay Kit (Cell Biolabs, Inc.) following the manufacturer's protocol. All determinations were performed in triplicate with a Thermo Scientific™ Varioskan™ LUX microplate reader.

2.4. Hepatic RNA extraction and cDNA synthesis

Total RNA was isolated from 20-30 mg of N_2-homogenized liver tissue with the AllPrep® DNA/RNA/Protein Kit (QIAGEN), following the manufacturer protocol, including a DNase I (180 U, RNase-Free DNA Set, Qiagen) step to remove residual gDNA. RNA purity and concentration were analyzed spectrophotometrically (Beckman Coulter DU-800 UV Spectrophotometer, with Hellma®Traycell cuvet). The integrity of RNA samples was evaluated with an Agilent™ 2100 Bioanalyzer system. The absence of gDNA was assessed by performing PCR on RNA samples with specific *Gapdh* intraexonic primers (Table S1). cDNA was synthesized from 2 µg of total RNA with the iScript cDNA Synthesis (BioRad) as described in the manufacturer protocol.

2.5. Real-time qRT-PCR in liver

Primers used in qRT-PCR are listed in Table S1. All primers uniquely and specifically amplified their target gene, as determined by agarose gel electrophoresis and Sanger sequencing of amplicons. PCR reactions were set up from 50 ng of cDNA in triplicate by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a final volume of 20 µL, using adequate 96-well plates. The PCR amplification protocol consisted of one activation

step (95 ºC, 4 min) and 40 two-step cycles with a denaturalization (95 ºC, 15 s) step and a hybridization/extension step (60 ºC, 45 s). A melting curve was performed after each run to check contamination in the well. Fluorescence was recorded at the end of the extension step (CFX96 Touch Real-Time PCR Detection System, Bio-Rad) and plotted *vs.* cycle number to set the threshold (T) in the exponential phase of the reaction above the baseline, and to infer the cycle threshold (C_T) of each PCR product. A calibration curve was set up to convert the C_T values in transcript number per pg of total RNA as previously described (Prieto-Alamo *et al.*, 2003). This PCR protocol was also applied for determining primer amplification efficiencies (Table S1).

2.6. Statistical analysis of biochemical and transcriptional data

The experimental groups Abx and Abx+Se were compared with the control group (C) using a Dunnett Multiple Comparisons Test, including the Bartlett statistic test for analyzing the homogeneity of variances and the Kolmogorov and Smirnov test for assessing normality. A *t*-Student test was used to compare data of Abx and Abx+Se mice. The GraphPad InStat v.3.05 for Windows (GraphPad Software) was used. Differences were considered to be statistically significant at $p<0.05$.

3. Results & Discussion

3.1. Effects of Abx and Se on body weight, food consumption, and survival.

To study the putative contribution of Se to health recovery after antibiotic treatment, mice were given a mixture of antibiotics in the drinking water for one week before they were fed either a normal murine diet (Abx group) or a Se-enriched diet (Abx+Se group) for two additional weeks. The antibiotic (Abx) mixture composition was chosen to completely deplete the gut microbiota (GM) as described in the literature (D'Amato et al., 2020; Reikvam et al., 2011; Tao et al., 2020; Zarrinpar et al., 2018) and contained ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L) and vancomycin (0.5 g/L). Ampicillin, a third-generation aminopenicillin, and neomycin, an aminoglycoside, are used against gram-positive and gram-negative anaerobic bacteria (NIH, 2012). Metronidazole is a nitroimidazole antibiotic effective against susceptible anaerobic bacteria and protozoa and a few facultative anaerobes (*i.e.*, *Helicobacter pylori*) as part of a combined therapy regimen (NIH, 2012). Vancomycin is a glycopeptide antibiotic with primary activity against gram-positive bacteria by inhibition of bacterial cell wall synthesis (NIH, 2012). The cocktail also contained the antifungal amphotericin B (10 mg/L), hence targeting the different types of intestinal microorganisms. Selenium supplementation added to the diet of the Abx+Se animals (0.65 mg sodium selenite/kg chow) resulted in a concentration three times higher than that of a normal murine diet (containing about 0.20 mg/kg of Se, (Raines and Sunde, 2011), a non-toxic but biologically active dose (Sunde and Raines, 2011;(Callejón-Leblic et al., 2021a). Considering the daily water and food intake records of the mice, a daily intake of 0.04 mg/kg body weight can be deduced for mice on standard diet, and 0.12 mg/kg body weight for mice receiving the Se-supplemented diet. The bioavailability of ingested Se was established on our previous work with mice subjected to identical experimental conditions. In those works we measured a two-fold increase in the amount of selenium bound to albumin

in blood and in the amount of selenium incorporated into selenoproteins such as GPX. These results demonstrated that selenium, administered in the form of selenite, was bioaccessible and able to be utilized in various biological processes (Callejón-Leblic et al., 2021b).

There were no deaths and no differences in feed consumption were observed among the three experimental groups (Fig. 1C). However, mice in the Abx and Abx+Se groups showed an evident body-weight reduction during the Abx ingestion period (Fig. 1A), associated with a marked reduction in water consumption (Fig. 1B). Similar data has been reported for mice under a comparable treatment (Tao *et al.*, 2020). After removal of Abx on day 7th, water and food intake of the Abx mice was not significantly different from that of the control (C) group, but the body weight of Abx mice was slightly lower than that of the C animals at the time of sacrifice (Fig. 1A). No statistically significant differences were observed in the weight or appearance of mice livers at this point.

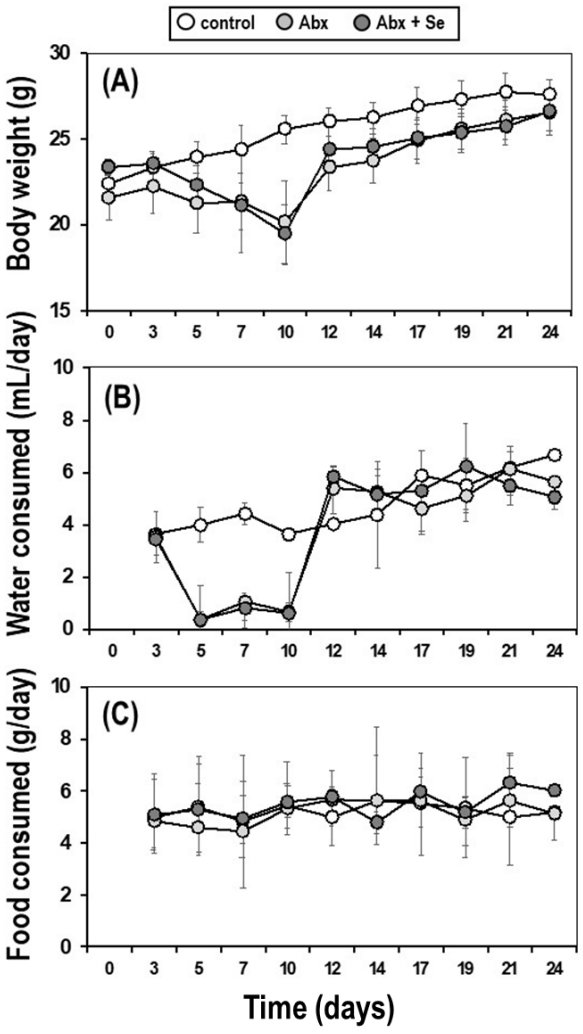


Figure 1. Effect of treatments over body weight (A), water (B) and food consumption (C) of mice. The mice body weights and the amount of water and chow consumed were recorded every two days and the mean ± SD ~~of all mice in each group (n=12-15)~~ is plotted.

3.2. Changes in plasma biochemical parameters

Table S2 shows the values of some biochemical parameters measured in the plasma of mice included in the different experimental groups. Some of these data (those with significant differences) have been also plotted in Fig. 2 for better visualization.

Abx treatment did not significantly modify the levels of glucose, lactate, triglycerides (TAG), total cholesterol (CHO), total proteins, albumin or creatinine levels in plasma (Table S2), but increased the activity of ALT (alanine transaminase, x1.9-fold) and ALP (alkaline phosphatase, x1.4-fold) (Table S2, Fig. 2). The elevation of ALT and ALP in plasma is often associated with liver damage or dysfunction. Typically, elevated ALT reflects hepatocellular injury while elevated ALP indicates impaired bile flow, though the distinction between hepatocellular disease and cholestasis is not absolute (Iluz-Freundlich *et al.*, 2020). ALT catalyzes the reversible transference of the α-amino group from alanine to the α-keto group of ketoglutaric acid to produce pyruvate and glutamate. Hence, ALT variations in plasma of Abx mice may reflect pharmacological action of antibiotics on liver functionality (Kobayashi *et al.*, 2020), but also an altered amino acid catabolism, which would be corroborated by the increased amounts (x1.6-fold) of plasmatic urea (Pi *et al.*, 2019) measured in these animals (Table S2 and Fig. 2).

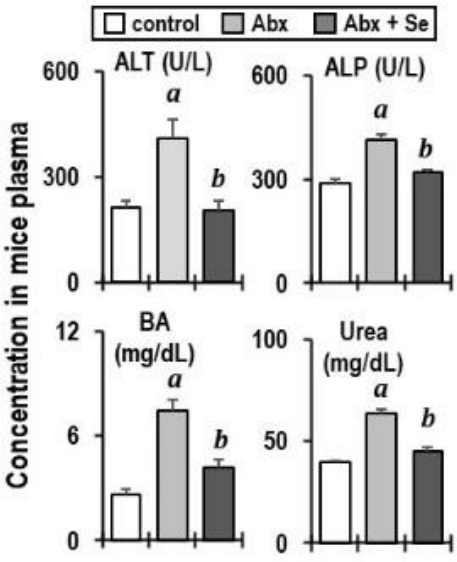


Figure 2. Changes in biochemical parameters measured in mice plasma caused by Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (*t*-Student) is expressed as ***b***..

ALP is a plasma cell membrane-bound glycoprotein that catalyzes the hydrolysis of organic phosphate esters at basic pH and whose precise physiological function remains unknown (Sharma *et al.*, 2014). Although it is present in many organs, the majority of alkaline phosphatase in serum (more than 80 %) is released from liver and bone, and in small amounts from intestine (Lowe *et al.*, 2020). ALP is considered a marker of bile duct obstruction as it is newly synthesized by bile duct epithelial cells in response to biliary blockage (Lowe *et al.*, 2020). Bile acids (BA) are amphipathic molecules synthesized from cholesterol in the liver. These primary BA are secreted as part of the bile by specific transporters located in the basolateral membrane of the hepatocyte. In the intestine, primary BA are modified by gut bacteria to generate secondary BA, which come back to the liver where they act as signal molecules. When enterohepatic bile flow is impaired, BA concentrations increase in serum and/or liver (Gijbels *et al.*, 2021; Pollock and Minuk, 2017). We found increased levels of plasmatic total BA (~3-fold) and a parallel elevated activity of ALP in the Abx group (Table S2 and Fig. 2). These results suggest Abx causing alteration of BA enterohepatic circulation and damages to hepatic tissues, including bile ducts, which would lead to the release of hepatic enzymes (*i.e.*, ALT and ALP) to blood (Pollock and Minuk, 2017).

What would cause BA metabolism and transport alteration in the Abx mice? Gut microbiota composition has profound effects on BA metabolism by promoting deconjugation, dehydrogenation, and dehydroxylation of primary BA, thus increasing the chemical diversity of BA pool (Sayin *et al.*, 2013). Gut microbiota also regulates BA synthesis in the liver by modulating the activity of intestinal FXR, the nuclear receptor farnesoid X receptor, which controls the hepatic BA synthesis through the induction of *Fgf19* (Fibroblast Growth Factor 19, termed FGF15 in rodents) expression in the enterocytes (Gonzalez *et al.*, 2016; Sayin *et al.*, 2013). The Abx administered to Abx-group mice would

reduce the amount and diversity of gut microbes, thereby altering the expression of microbial genes and derived metabolites able to regulate FXR signaling (Gonzalez *et al.*, 2016; Yoon and Yoon, 2018).

Our data in Table S2 and Fig. 2 clearly show that allowing mice to recover from Abx treatment in the presence of a Se-enriched diet prevented, at least partially, the metabolic alterations caused by Abx treatment. Selenium is an essential trace element with important roles in human health (Ferreira *et al.*, 2021; Zoidis *et al.*, 2018), acting as a cofactor of several enzymes in the liver, but its participation in bile secretion has yet to be defined (Manzotti *et al.*, 2019). However, increased dietary Se intake has been linked to decreased prevalence of gestational intrahepatic cholestasis (Manzotti *et al.*, 2019) and the administration of selenoneine, a novel organic Se compound, has been reported to attenuate hepatic steatosis in mice (Miyata *et al.*, 2020). In addition, and though remarkably different doses were used, the study of Hu *et al.* (Hu *et al.*, 2018) demonstrates that Se-diet enrichment causes decreased BA biosynthesis, which agrees with our results (Table S2 and Fig. 2). Selenium can also modulate BA metabolism by modifying the composition of the GM, and consequently, the observed Se effect in Abx+Se mice might be caused, at least in part, by GM ability to regulate secondary BA metabolism generation and to decrease hepatic BA synthesis in the liver by alleviating FXR inhibition in the ileum (Sayin *et al.*, 2013). These issues are addressed in the following sections.

3.3. A Se-enriched diet restores lipidic metabolism in the liver of Abx treated mice

Many metabolic pathways, including those that regulate BA, fatty acids (FA), cholesterol (CHO), and glucose homeostasis, are housed in the liver. To evaluate whether Abx treatment elicits alterations in liver metabolism that could explain the changes observed

in some biochemical parameters in plasma, we analyzed the transcriptional profiles of genes related to lipid metabolism and trafficking in the liver.

Data in Fig. 3 show that Abx treatment significantly increases the transcript counts of genes involved in lipogenesis. Sterol regulatory element-binding proteins (SREBP) controls lipid homeostasis by regulating genes encoding enzymes that are crucial for the synthesis of CHO and unsaturated FA. Two genes, *Srebf1* and *Srebf2* encode SREBP-1 and SREBP-2, respectively, which are processed by two proteases to release three transcriptionally active fragments, namely SREBP-1a, SREBP-1c, and SREBP-2, with different roles in lipid metabolism (Xue *et al.*, 2020). The scheme in Fig. 3 depicts this regulatory cascade.

SREBP-1c mainly promotes the synthesis of FA and triacylglycerides (TGA) as it acts as a well-established transcriptional activator of *FasN*, the gene encoding the cytoplasmatic fatty acid synthetase (FASN). FASN is a multifunctional protein containing six enzymatic domains that produces palmitate, a 16-carbon chain saturated FA, from acetyl-coenzyme A and using NADPH as electron donor. Mice in the Abx group showed increased abundance of *Srebf1* transcripts (x1.8-fold, Fig. 3A) and consequently of its target genes *FasN* (x3.6-fold, Fig. 3C) and *Lpin1* (x2.3-fold, Fig. 3C).

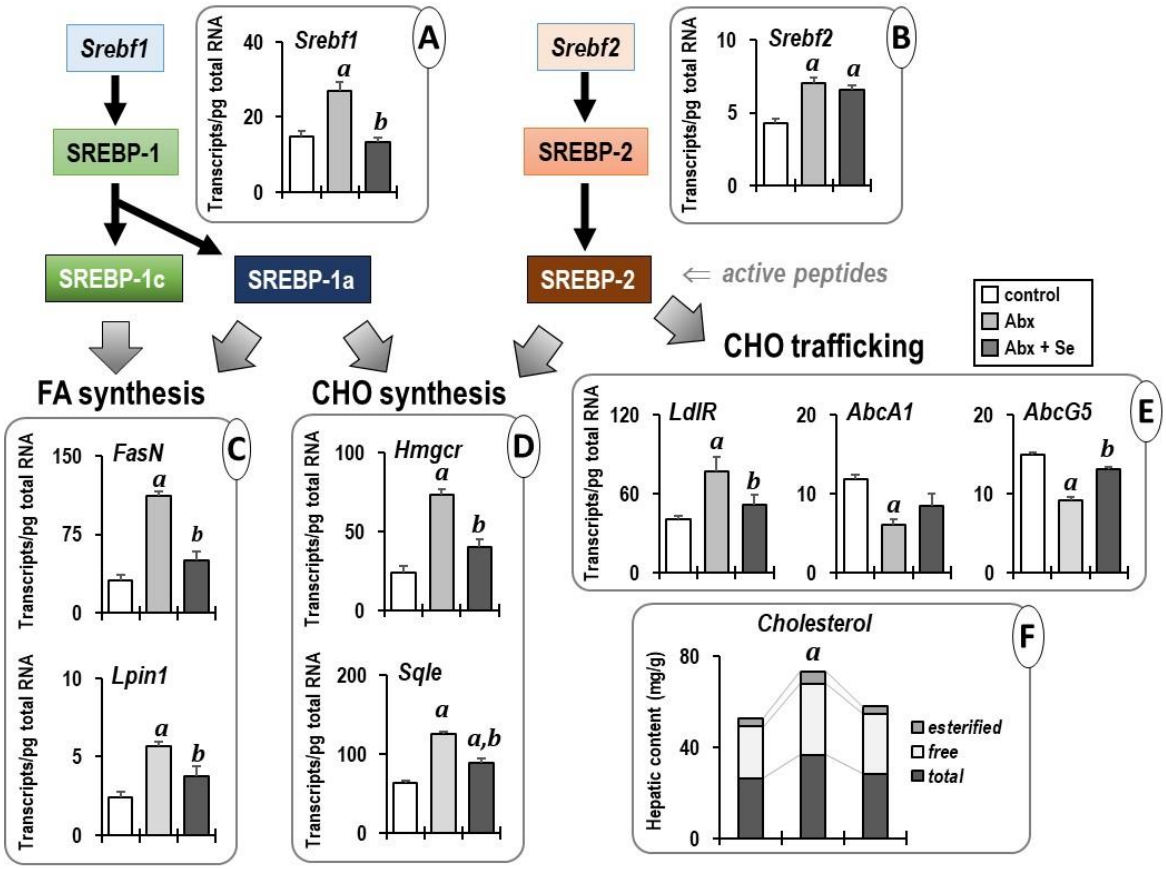


Figure 3. Changes in the hepatic transcriptional profiles of genes linked to lipid metabolism and CHO trafficking after Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (*t*-Student) is expressed as ***b***.

Lipins are magnesium-dependent phosphatidate-phosphatase enzymes that catalyze the limiting step in the synthesis of diacylglycerol, and therefore regulate the synthesis of several lipid species, including phospholipids. Diacylglycerol is also a regulator of signaling cascades including protein kinases C and D, involved in insulin resistance and autophagy (Lutkewitte and Finck, 2020). Our data hence indicate that Abx treatment stimulates the synthesis of FA and acyl-glycerides in the liver of treated mice. This result is in agreement with data in Table S2 and Fig. 2 and with previous reports proposing that Abx therapies

increase hepatic lipid accumulation (Zarrinpar *et al.*, 2018), which modifies the biology and function of intracellular organelles and initiates phosphorylation cascades (Marra and Svegliati-Baroni, 2018) that caused liver damage and the liberation of hepatic enzymes (*i.e.*, ALT).

Diet supplementation with selenite restored the expression levels of *Srebf1*, *FasN*, and *Lpin1* genes (Fig. 3A & C). The role of Se in lipid biosynthesis is not easy to infer from the literature, due to the narrow margin separating the toxicity of Se from its beneficial effects and the broad concept of dietary enrichment with Se, which sometimes involves the use of doses much higher than the standards. In this sense, Hu et al (Hu *et al.*, 2018) reported that supplemental Se alters (increase) hepatic FA, energy metabolism, and body mass in mice, but using concentrations 8-fold higher than the standard dose. Similarly, Zhang *et al.* (Zhang *et al.*, 2020a) found that dietary supranutritional Se (10-fold higher than the standard dose) increased the transcript abundance of *FasN*. However, Donalson (Donaldson, 1977) demonstrated, more than forty years ago, that Se is a competitive inhibitor of FASN. In addition, many recent studies using non-toxic Se doses also demonstrated that this oligoelement exerts a beneficial effect on lipids homeostasis [*i.e.*, (Hasani *et al.*, 2018; Nido *et al.*, 2016; Peng *et al.*, 2021; Shi *et al.*, 2020)]. In agreement with those works, our data demonstrated that a mild Se-diet enrichment restored *FasN* transcript levels by modulating the expression of the *FasN* upstream regulator *Srebf1*. The work by Speckmann *et al.* (Speckmann *et al.*, 2017) established that Se reduces local methylation of a specific CpG site within the *Srebf1* gene and decreases its expression, which corroborates our findings. We, hence, demonstrated that under a Se-enriched diet recovery, FA biosynthesis rate does not result affected by Abx treatment at the transcriptional level, in agreement with other works using non-toxic Se doses [i.e., (Hasani *et al.*, 2018; Nido *et al.*, 2016; Peng *et al.*, 2021; Shi *et al.*, 2020)].

3.4. Se partially restores cholesterol metabolism and trafficking in the liver of Abx-treated mice

The liver is considered a masterpiece in the regulation of plasma cholesterol levels since *de novo* synthesis of cholesterol occurs in this organ. Cholesterol is synthesized from acetyl-CoA through a cascade of reactions performed by enzymes that are mainly regulated by SREBP-2, a proteolytically activated peptide derived from the *Srebf2* gene product. SREBP-2 acts as a basic helix-loop-helix leucine zipper transcription factor, activating the transcription of hydroxymethyl glutaryl-CoA reductase (HMGCR), squalene epoxidase (SQLE), and other key enzymes in the CHO synthesis pathway (Coates *et al.*, 2021). Data in Fig. 3 indicate that Abx treatment caused the upregulation (x1.6-fold, Fig. 3B) of *Srebf2* gene. In parallel, we found increased transcript levels of SREBP-2 target genes *Hmgcr* (x 3.1-fold, Fig. 3D) and *Sqle* (x 2.0-fold, Fig. 3D). The liver is also a main actor in CHO traffic, as it imports excess CHO from peripheral tissues to redistribute it to other tissues or to be eliminated, unmodified or transformed into BA. Thus, we also evaluate the transcript levels of some genes encoding the proteins involved in the CHO trafficking. Cholesterol enters hepatocytes mainly from cholesterol-rich low-density lipoprotein (LDL) particles with the mediation of the low-density lipoprotein receptor (LDLR). Bonded, LDL and LDLR are internalized and CHO is finally stored unbounded in endosomes and lysosomes (Vos and van de Sluis, 2021). LDLR is transcriptionally regulated by SREBP-2, which also influences, though negatively, the ATP binding cassette transporter A1 (ABCA1) abundance. ABCA1 is a transmembrane transporter protein highly abundant in the liver where it controls the efflux of CHO to the circulation (Pasello *et al.*, 2020). Accordingly, we found increased amounts of *LdlR* transcripts and reduced mRNA levels of *AbcA1* in the liver of Abx treated mice, which could result in accumulation of CHO in the liver. We also found reduced expression of *AbcG5* (ATP Binding Cassette Subfamily G Member 5). The protein ABCG5 forms heterodimers with ABCG8 and mediates the export of hepatic free CHO to bile

(Malhotra *et al.*, 2020). Data in Fig. 3E show that this clearance pathway is also impaired in Abx treated mice. To assess that the observed dysregulation in the transcript levels of genes involved in the synthesis, the uptake, and the clearance of hepatic CHO, were effectively causing hepatic CHO accumulation, we measure the CHO level in the hepatic samples. Data in Fig. 3F show that Abx treatment enhanced the amounts of total CHO in hepatic cells, as well as the proportion of free cholesterol. These data sustain the hypothesis of Abx causing both CHO synthesis and CHO retention in the mice liver, which would lead to progressive liver disease (cholestasis, hepatomegaly, fibrosis, cirrhosis) (Vos and van de Sluis, 2021). As shown for FA, our data in Fig. 3 and 4 show that enrichment of the mice diet with a low dose of Se at least partially avoids the Abx effects on the CHO homeostasis, in agreement with previous results (Dhingra and Bansal, 2006).

3.5. A Se-enriched diet favors bile acid-homeostasis recovery in the liver of Abx-treated mice

The major catabolic pathway of CHO in mammals transforms it into BA in the liver, the only organ that has all the enzymes required for their synthesis. The increased levels of CHO in the hepatocytes of Abx treated mice suggested augmented levels of BA synthesis and export that might be the cause of the elevated level of BA observed in the plasma of these mice (Fig. 2). The hepatic enzymes involved in CHO biosynthesis function through two different routes. The canonical or neutral pathway is initiated by the 7α-hydroxylation of cholesterol catalyzed by the enzyme CYP7A1. The enzyme CYP8B1 further catalyzes the transformation of the steroid nucleus and the oxidative cleavage of the side chain. In the non-canonical or acidic pathway, cholesterol enters the hepatic mitochondria where it is hydroxylated via the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1) to generate 27-hydroxycholesterol, further metabolized by oxysterol 7α-hydroxylase (CYP7B1) (Stofan and Guo, 2020). Data in Fig. 4 show reduced expression of these four *Cyp* genes at the

transcript level (panels A, B, C & D) in the liver of mice after Abx treatment, which was paralleled by a decrease in the total concentration of BA in the liver of these mice (panel E).

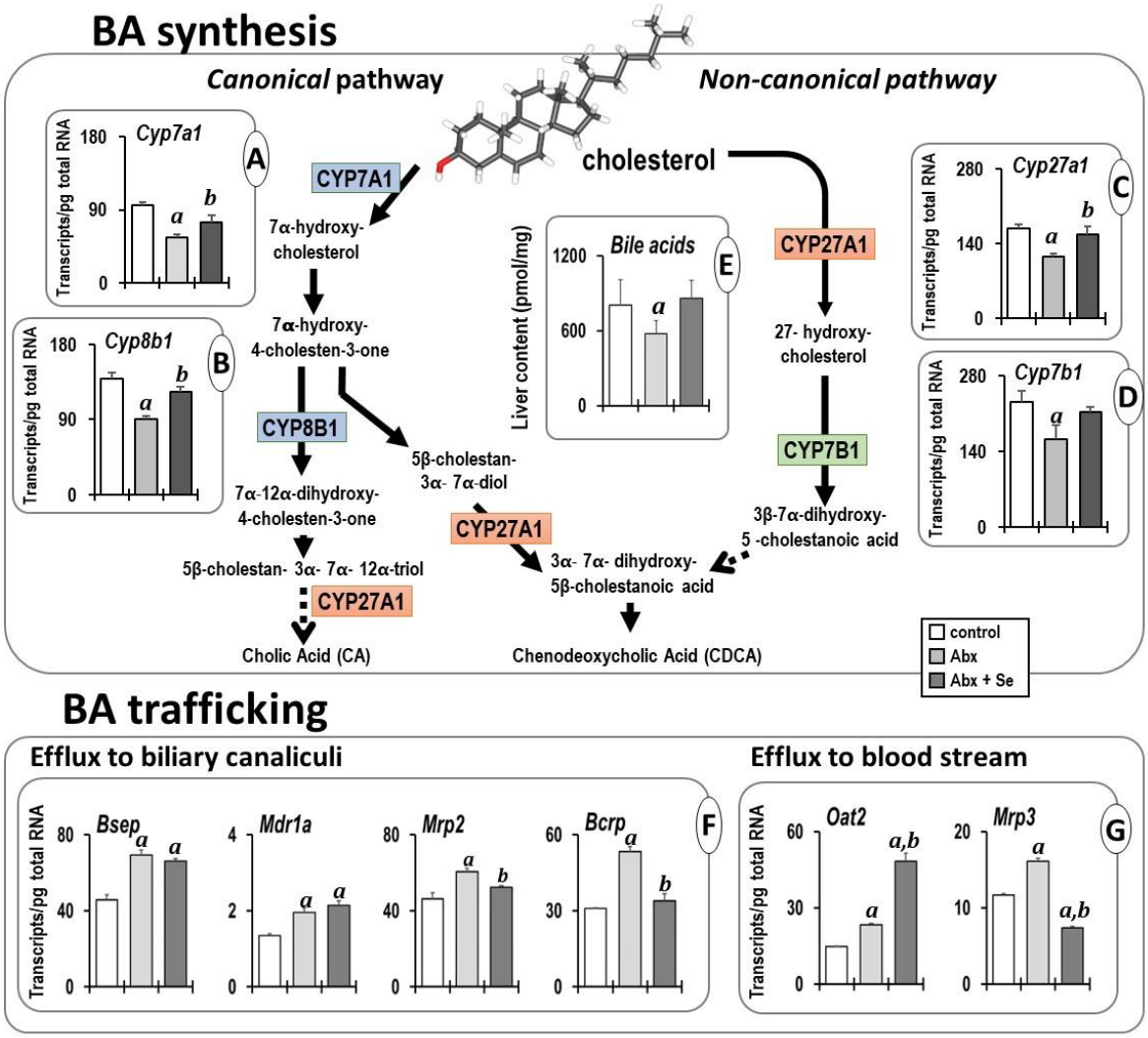


Figure 4. Changes in the hepatic transcriptional profiles of genes linked to BA biosynthesis and export after Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (t-Student) is expressed as ***b***.

Primary BA synthetized in the liver are conjugated to either glycine or taurine to be secreted into bile and then released, via the common bile ducts, into the intestinal tract, where they are required for the absorption of fats, steroids, and lipid-soluble vitamins to be

metabolized in the liver. Most BA are reabsorbed in the distal ileum by an active transport system to portal blood circulation and then return back to the liver, but others serve as substrate for intestinal microbial metabolism and undergo biotransformation to secondary BA before returning to the liver. Both primary and secondary BA constitute the circulating bile acid pool, with important functions as signaling molecules that modulate hepatic lipid, glucose, and energy metabolism. The total amount of BA in the liver is hence the result of BA synthesis, BA export to bile, and the blood and BA import. To assess the effects of Abx treatment over BA transport, we measure the expression levels of genes coding for several of these BA transporters (Fig. 4F & G). Our results suggest that, in addition to a low level of synthesis (Fig. 4A, B, C & D), there is an increased level of BA secretion (Fig. 4F & G) out of the liver of Abx mice, which would explain the lower BA content in the liver (Fig. 4E) and the higher amount in the plasma (Fig. 2). A supranutritional dose of Se in the diet of mice after Abx treatment contributed to recovering the control levels of total BA and transcripts of most assayed genes.

3.6. Associations between the liver function and the role of Se in restoring antibiotic induced dysbiosis of GM and the intestinal metabolome

In previous studies with these groups of mice we demonstrated that the Abx treatment used here deeply affected the taxonomic composition of mice GM and the intestinal metabolome and both effects remitted in the presence of a Se-supplement in the diet (Callejón-Leblic et al., 2021a; Callejón-Leblic et al., 2021c). That previous metabolome analysis showed increased levels of FA and derivatives, as well as significantly higher levels of steroids (*i.e.*, CHO) and BA (*i.e.*, 7-hydroxy-5-cholanic acid) in the intestinal content of Abx mice, which were alleviated in the Abx-Se group (Callejón-Leblic et al., 2021c). The results obtained in the present work have been interpreted in conjunction with these previous data to provide a comprehensive view of the molecular mechanisms underlying the

beneficial action of Se on health. Hence, Se-induced restoration of the relative abundance of *Ruminococcaceae*, some Bacteroidetes and *Lactobacillus* spp. could explain the recovery of BA homeostasis depicted in Fig. 2-4. The *Ruminococcaceae* are a family of *Clostridiales* belonging to the phylum Firmicutes that include several BA-7α dehydroxylating bacteria capable of regulating BA synthesis in mouse liver by removing tauro-β-muricholic acid, an FXR antagonist (Staley *et al.*, 2017). Depletion of these bacteria by Abx treatment (Callejón-Leblic et al., 2021c) would trigger the deregulation of enzymes that synthesize BA from CHO precursors and would explain the data in Figs. 2-4.

In the same direction point the alterations in the Bacteroidetes phylum observed in the Abx and Abx+Se groups. Abx treatment caused a high increase in the abundance of *Bacteroides* spp. (*Bacteroidaceae*), *Parabacteroides* spp. (*Tannerellaceae*) and *Muribaculum* (*Muribaculaceae*) in absence of a Se-enriched diet (Callejón-Leblic et al., 2021a; Callejón-Leblic et al., 2021c).The Bacteroidetes are polysaccharides degraders and producers of short-chain fatty acids (SCFA), mainly acetate and propionate. Propionate participates in hepatic gluconeogenesis and reduces the expression of enzymes involved in the *de novo* synthesis of fatty acids and cholesterol. By contrast, acetate stimulates the hepatic synthesis of lipids, contributing to dyslipidemia, as well as the secretion of insulin and ghrelin by the pancreas and the gastric mucosa, respectively [reviewed in (Magne *et al.*, 2020)]. Bacteroidetes present taurocholic acid-specific bile salt hydrolases (BSH) (Ridlon *et al.*, 2006) and the reported enhanced growth of these microbes would be consistent with high levels of secondary BA able to activate nuclear FXR and membrane Takeda G protein-coupled receptor 5 (TGR5; *i.e.*, G protein-coupled bile acid receptor 1). Activation of FXR by BA inhibits transcription of the *Cyp7a1* and *Cyp8b1* genes in hepatocytes (Chiang and Ferrell, 2020). It could be assumed that Se restores the BA synthesis pathway (Fig. 3), and hence contribute to regulate glucose, lipid, and energy metabolism (Wu *et al.*, 2019) by

controlling the Bacteroidetes population (Callejón-Leblic et al., 2021a; Callejón-Leblic et al., 2021c) and the SCFA production. These results complement those obtained from the gut metabolomic analysis, where we did not find alterations in the levels of SCFA probably because of that study focused on a wide metabolite coverage by means of untargeted metabolomics (Callejón-Leblic et al., 2021c).

The Abx treatment significantly decreased the abundance of *Lactobacillus spp.* in the Abx mice, but it recovered to an approximate normal level 14 days later Abx withdraw when a Se-enriched diet was used (Callejón-Leblic et al., 2021a; Callejón-Leblic et al., 2021c). This beneficial effects of Se might be due to their capability to accumulate and bio-transform selenite (toxic) into Se-amino acids (*i.e.,* Se-methionine, Se-cysteine, and Se-methylcysteine), Se-nanoparticles, and/or volatile Se compounds (Martínez *et al.*, 2020). With an excess of Se, *Lactobacillus* and the host do not compete by dietary Se for the synthesis of selenoproteins and selenometabolites, and *Lactobacillus* specific Se-related proteins (*i.e.*, formate dehydrogenase or xanthine dehydrogenase) are sufficient to sustain its anaerobic growth by the fermentation of sugars or aromatic compounds (Sumner *et al.*, 2019). In parallel, selenoamino acids and other metabolites of Se could be used by the host to synthesize its selenoproteins, which are essential for the proper functioning of its immune response or its reproductive, endocrine or muscular systems due to their role in the redox regulation of signaling and the antioxidant system. (Zhang *et al.*, 2020b). Increasing evidence also demonstrates the ability of several *Lactobacillus* species to improve the anthropometric measurements and biomarkers related to lipid and glucose metabolism (Bagon *et al.*, 2021; Li *et al.*, 2020; Wang *et al.*, 2019). Elevated BSH enzyme activity in many *Lactobacillus spp.* has been reported to be responsible for the beneficial effects against hypercholesterolemia associated with increased abundance of *Lactobacillus spp.* in mice, as BSH modulates the expression of several genes such as FXR or LDLR, thereby reducing

cholesterol absorption and increasing cholesterol catabolism (Wang *et al.*, 2019). Thus, it can be assumed that the presence of Se in the diet regulates the abundance of certain *Lactobacillus* phylotypes involved in the proper liver functioning in mice.

3.7. Selenium supplements to Defer or Deter Abx-Related Complications

In the field of environmental toxicology, dietary selenium supplementation may help us to avoid side effects resulting from inadvertent exposure to antibiotics coming from food and water. From a clinical point of view, our results suggest that selenium supplements, at adequate doses, administered to people undergoing treatment with Abx may greatly reduce the adverse side effects that could alter the patient's life after treatment completion. Nonetheless, further studies directly in humans are required to determine the appropriate doses and to evaluate the consequences of Se supplementation in the long term to build clinical evidence for an effective understanding of the impact of Se in disease prevention.

4. Conclusions

From our data, we conclude that the alterations produced in liver metabolism as a consequence of Abx exposure affect FA, acylglycerides and CHO synthesis, as well as CHO trafficking and its catabolic degradation to BA. However, liver dysfunction is alleviated when recovery of mice after Abx withdrawal occurs under a Se-enriched diet. Interpreting our results in conjunction with our previous metataxonomic and metabolomic data in these mice, we further determined that the known role of Se against liver damage and hypercholesterolemia might be mediated, among others and at least in part, by the gut microbiota. We provide data that contribute to achieve a more comprehensive view of the molecular mechanisms underlying the beneficial action of Se on health. These data point to a potential use of low Se-doses as a functional food additive to alleviate the negative effects of antibiotics.

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6. Figure captions

Figure 1. Effect of treatments over body weight (A), water (B) and food consumption (C) of mice. The mice body weights and the amount of water and chow consumed were recorded every two days and the mean ± SD ~~of all mice in each group (n=12-15)~~ is plotted.

Figure 2. Changes in biochemical parameters measured in mice plasma caused by Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (*t*-Student) is expressed as ***b***.

Figure 3. Changes in the hepatic transcriptional profiles of genes linked to lipid metabolism and CHO trafficking after Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (*t*-Student) is expressed as ***b***.

Figure 4. Changes in the hepatic transcriptional profiles of genes linked to BA biosynthesis and export after Abx treatment under a standard or a Se-enriched diet. Bars represented the mean ± SEM of three independent determinations ~~(n=12-15 mice per experimental group)~~. Statistical significance (p<0.05) of the differences between each treatment group and the control (Dunnett's test) is expressed as ***a***; differences for Abx vs Abx+Se (*t*-Student) is expressed as ***b***.

7. Credit authorship contribution statement

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. PVHA and MJPA have equally contributed to this work and should be regarded as joint first authors

8. Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

9. Acknowledgements

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