

"Ezetimibe and simvastatin modulate gut microbiota and expression of genes related to cholesterol metabolism"

Catry, Emilie ; Pachikian, Barbara D. ; Salazar Garzo, Nuria ; Neyrinck, Audrey M. ; Cani, Patrice D. ; Delzenne, Nathalie M.

Abstract

AIMS: Hypolipidemic drugs are prescribed in the most of cases for the treatment of cardiovascular diseases. Several studies have showed that the gut microbiota is able to regulate the host cholesterol metabolism. This study aimed to investigate the potential impact of hypolipidemic drugs on the gut microbiota in mice, and to correlate it to the regulation of cholesterol metabolism. MAIN METHODS: Male C57Bl/6J mice were divided into four groups fed either a control diet alone (CT), or supplemented with simvastatin (0.1% w/w, Zocor®, MSD), or ezetimibe (0.021% w/w, Ezetrol®, MSD) or a combination of simvastatin and ezetimibe (0.1% and 0.021%, respectively) for one week. KEY FINDINGS: The combination of ezetimibe and simvastatin is required to observe a drop in cholesterolemia, linked to a huge activation of hepatic SREBP-2 and the consequent increased expression of genes involved in LDL cholesterol uptake and cholesterol synthesis. The gut microbiota analysis revealed no change in t...

Document type : Article de périodique (Journal article)

Référence bibliographique

Catry, Emilie ; Pachikian, Barbara D. ; Salazar Garzo, Nuria ; Neyrinck, Audrey M. ; Cani, Patrice D. ; et. al. *Ezetimibe and simvastatin modulate gut microbiota and expression of genes related to cholesterol metabolism*. In: *Life Sciences*, Vol. 132, no. 132, p. 77-84 (2015)

DOI : 10.1016/j.lfs.2015.04.004

Available at:

<http://hdl.handle.net/2078.1/162012>

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Ezetimibe and simvastatin modulate gut microbiota and expression of genes related to cholesterol metabolism



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ARTICLE INFO

Article history:

Received 5 December 2014

Received in revised form 9 March 2015

Accepted 15 April 2015

Available online 25 April 2015

Keywords:

Ezetimibe

Simvastatin

Microbiota

Cholesterol

SREBP-2

Lactobacillus

ABSTRACT

Aims: Hypolipidemic drugs are prescribed in the most of cases for the treatment of cardiovascular diseases. Several studies have showed that the gut microbiota is able to regulate the host cholesterol metabolism. This study aimed to investigate the potential impact of hypolipidemic drugs on the gut microbiota in mice, and to correlate it to the regulation of cholesterol metabolism.

Main methods: Male C57Bl/6J mice were divided into four groups fed either a control diet alone (CT), or supplemented with simvastatin (0.1% w/w, Zocor®, MSD), or ezetimibe (0.021% w/w, Ezetrol®, MSD) or a combination of simvastatin and ezetimibe (0.1% and 0.021%, respectively) for one week.

Key findings: The combination of ezetimibe and simvastatin is required to observe a drop in cholesterolemia, linked to a huge activation of hepatic SREBP-2 and the consequent increased expression of genes involved in LDL cholesterol uptake and cholesterol synthesis. The gut microbiota analysis revealed no change in total bacteria, and in major Gram positive and Gram negative bacteria, but a selective significant increase in *Lactobacillus* spp. in mice treated with the ezetimibe and a decrease by the combination. The changes in lactobacilli level observed in ezetimibe or combination treated-mice are negatively correlated to expression of genes related to cholesterol metabolism.

Significance: The present study showed that ezetimibe taken alone is able to modify the composition of gut microbiota in favor of *Lactobacillus* spp. These results suggest that members of the genus *Lactobacillus* play an important role in cholesterol metabolism, even in normocholesterolemic mouse model.

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1. Introduction

Cholesterol is an important component of mammalian cell membranes which takes part in the control of membrane fluidity, permeability and trafficking [1]. Impaired LDL-cholesterol (LDLc) level in the bloodstream leads to the development of cardiovascular diseases, such as atherosclerosis [2,3]. Today 17 million people die of cardiovascular disease each year and this number will double by 2030 [4]. Cholesterol metabolism regulation is mediated by several transcription factors, among which the sterol regulatory element binding protein 2 (SREBP-2) is the key regulator [5]. SREBP-2 upregulates the

expression of cholesterol synthesis genes including the rate limiting enzyme of cholesterol synthesis, the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAR). Moreover, SREBP-2 activation increases the expression of the low density lipoprotein receptor (LDLr), responsible for the tissue uptake of LDLc [6,7]. To date, many hypolipidemic drugs have been developed to reduce and to prevent hypercholesterolemia. Among these drugs, statins are competitive inhibitors of HMGCoAR, used since the late 1980s [8]. More recently ezetimibe, a non-competitive inhibitor of the Niemann-Pick C1-like 1 (NPC1L1) transporter mediating cholesterol absorption in the apical brush border membrane of jejunum enterocytes, has been introduced as hypocholesterolemic treatment [9]. In fact, the addition of ezetimibe to ongoing statin therapy provides an effective means for further reducing LDLc levels and bringing more high-risk patients to their LDLc goals [10].

Of interest, studies have shown that the gut microbiota is able to regulate cholesterol metabolism [11,12]. Indeed germ-free mice fed a high fat diet have an altered cholesterol metabolism characterized by an elevation of hepatic cholesterol storage, an overexpression of hepatic SREBP-2 and an increased dietary cholesterol absorption [11]. Moreover other studies have demonstrated that the modulation of gut microbiota by nutrients influences also the host cholesterol metabolism. We have

Abbreviations: BA, bile acid; DGGE, denaturing gradient gel electrophoresis; CE, cholesteryl esters; FC, free cholesterol; TC, total cholesterol; E/S, ezetimibe plus simvastatin; EZ, ezetimibe; HDLc, high density lipoprotein cholesterol; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLc, low density lipoprotein cholesterol; LDLr, low density lipoprotein receptor; NPC1L1, Niemann-Pick C1-like 1; SIM, simvastatin; SREBP-2, sterol regulatory element binding protein 2; TBP, TATA binding protein; TG, triacylglycerols.

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reported that supplementation with dietary inulin-type fructans, which promotes several bacterial gender levels in the gut, reduces lipidemia, lowers hepatic cholesterol content and inhibits hepatic SREBP-2 pathway, in $n = 3$ polyunsaturated fatty acid-depleted mice [13]. The current study investigated the potential impact of two hypolipidemic drugs, simvastatin and ezetimibe, administered separately or in combination, on hepatic and intestinal molecular targets of cholesterol metabolism as well as on gut microbiota modulation. The aim was to highlight the potential link between bacterial composition and drug-modulation of cholesterol metabolism.

2. Material and methods

2.1. Animals and diets

Nine-week-old male C57Bl/6J mice (Charles River, Brussels, Belgium) were housed in a colony of four mice per cage with a 12 h light/dark cycle at 22 °C. Mice were acclimatized for one week with free access to control (CT) diet (AO4, SAFE, Augy, France) and water.

Mice were separated into four groups. CT mice were fed a CT diet, EZ mice were fed a CT diet supplemented with ezetimibe (0.021% w/w; Ezetrol® 10 mg, Merck, New Jersey, USA), SIM mice were fed a CT diet supplemented with simvastatin (0.1% w/w; Zocor® 20 mg, Merck, New Jersey, USA) and E/S mice were fed a CT diet supplemented with ezetimibe and simvastatin (0.021% w/w and 0.1% w/w, respectively) for seven days *ad libitum*. The two hypolipidemic drugs were powdered and mixed with CT diet by trituration.

After the period of treatment, at the end of the dark cycle, mice have been anesthetized with isoflurane (Forene®, Abbott, Queenborough, Kent, England) before exsanguination and tissue sampling, and then they were killed by cervical dislocation.

The experiments were approved by and performed in accordance with the guidelines of the local ethics committee. Housing conditions were specified by the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement no. LA1230314).

2.2. Blood and tissue sampling

Portal and cava vein blood samples were collected in EDTA tubes and centrifuged (3 min at 13,000 g) for further analysis. Liver, cecal tissue and cecal content were removed, weighed and immediately frozen in liquid nitrogen. The samples were stored at -80 °C for further analysis.

2.3. Blood biochemical analysis

Plasma triacylglycerols (TG), total (TC) and high density lipoprotein cholesterol (HDLc) concentrations were measured in non-thawed cava vein blood using kits (DiaSys Diagnostic and Systems, Holzheim, Germany) coupling an enzymatic reaction and spectrophotometric detection of the final product. LDLc was estimated by the Friedewald formula [14]. Bile acids (BA) were measured using kit (DiaSys Diagnostic and Systems, Holzheim, Germany) in non-thawed portal vein blood.

2.4. Real-time quantitative PCR

Total RNA was isolated from the liver, jejunum and ileum by TriPure reagent (Roche, Basel, Switzerland). cDNA was prepared by reverse transcription of 1 µg total RNA using Reverse Transcription System kit (Promega, Leiden, The Netherlands). Real-time qPCRs were performed with a StepOnePlus™ instrument and software (Applied Biosystems, Foster City, CA, USA) using Mesa Fast qPCR™ (Eurogentec, Seraing, Belgium) for detection. Ribosomal protein L19 (RPL19) RNA was chosen as a housekeeping gene. Primers and gene details are summarized in Table 1. All samples were run in duplicate in a single 96-well reaction plate and data were analyzed

Table 1

Sequences for the primers used in real-time qPCR. SREBP-2, sterol regulatory element binding protein 2; HMGCoAR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDLr, low density lipoprotein receptor; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; CYP7A1, cytochrome 7A1; CYP7B1, cytochrome 7B1; RPL19, ribosomal protein L19; NPC1L1, Niemann-Pick C1-like 1.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
SREBP-2	GTGGCTCTCGTTTACTGAAGT	GTATAGAAGACGGCTTCACCAA
HMGCoAR	CCTGACACTGAAGTGAAGCG	TCCTTCCAGAACACAGCAGC
LDLr	CTGTGGGTCATAGGCTATCT	GCGGTCCAGGGTTCATCTTC
ABCG5	TGGCCCTGCTCAGCATCT	ATTTTAAAGGAATGGCATCTCTT
ABCG8	CCGTCTCAGATTCCAATGA	GGCTTCCGACCATGAATG
CYP7A1	GGGATTGCTGTGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
CYP7B1	TAGGCATGACGATCTGAAA	TCTCTGGTAAAGTGAGCTGAAA
RPL19	GAAGGTCAAAGGGAATGTGTCA	CCTTGTCTGCTTCAGCTTGT
NPC1L1	GGCTCCATCTGGAGTAGCTG	ATGCCACTACCATCCAGAC

according to the $2^{-\Delta\Delta CT}$ method [15]. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

2.5. Gut microbiota analysis

The metagenomic DNA from cecal content was extracted using a QIAamp-DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative PCR for total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides-Prevotella* spp., *Roseburia* spp. and *Lactobacillus murinus/animalis* was performed as described before in [16] using Mesa Fast qPCR™ (Eurogentec, Seraing, Belgium) for detection. The cycle threshold of each sample was compared with a standard curve (performed in duplicate) made by diluting genomic DNA (five-fold serial dilution) (BCCM/LMG, Ghent, Belgium and DSMZ, Braunschweig, Germany). Prior to isolating the DNA, the cell counts were determined in culture and expressed as "colony-forming unit" (CFU) [16]. Denaturing Gradient Gel Electrophoresis (DGGE) was performed to study the effect of the treatments in lactobacilli group. For that purpose a 16S-rRNA nested PCR approach was used. The first PCR round was performed with lactobacilli group-specific primers SGLAB0159F and SGLAB0667R as described previously [17] followed by a second amplification with primers 338F-GC with a GC clamp of 40 bp and 518R [18]. All amplification products were checked by electrophoresis on a 1.5% agarose gel. DGGE was performed with the use of a PhorU system (Ingeny) in $0.5 \times$ TAE buffer at 60 °C. PCR products were loaded onto 8% v/v polyacrylamide gels in $0.5 \times$ TAE. The electrophoretic conditions were the following: 16 h at 120 V in a 40 to 60% urea-formamide denaturant agent gradient. The gels were stained in $1 \times$ TAE buffer with SYBR Gold and visualized with UV radiation. Individual bands were cut out from the gel and re-amplified with the original primer pairs. PCR and the PCR products were subjected to sequencing using the services of Macrogen (Amsterdam, The Netherlands). The sequences were compared with those available in the GenBank database using NCBI BLAST (<http://www.ncbi.nlm.nih.gov/>).

2.6. Western blotting

Cytoplasmic and nuclear proteins were extracted following the manufacturer's instruction (NE-PER®, Thermo Scientific, Waltham, USA) from 50 mg of hepatic tissue. Equal amount proteins were separated by 10% SDS/PAGE and transferred to the nitrocellulose membrane, blocked in tris-buffered saline Tween-20 (TTBS) containing 5% non-fat dry milk for 1 h at room temperature. The membranes were incubated overnight at 4 °C with the rabbit anti-SREBP-2 antibody (Abcam, Cambridge, UK) in TTBS containing 1% non-fat dry milk. Signals were revealed using ECL western blotting substrates (SuperSignal West Pico Substrate, Thermo Scientific, Waltham,

Table 2

Plasma and portal lipid concentration of CT, SIM, EZ and E/S mice. TG, CHOL, HDLc and LDLc concentration (mM) in cava vein and BA concentration (μ M) in portal vein of mice fed a control diet (CT; $n = 7$ -to-8), a control diet supplemented with simvastatin (SIM; $n = 6$ -to-7), a control diet supplemented with ezetimibe (EZ; $n = 8$) or a control diet supplemented with the combination (E/S; $n = 7$ -to-8) for one week. Data are the mean \pm SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis.

Parameters	CT	SIM	EZ	E/S
<i>Cava vein</i>				
Triglyceridemia (mM)	0.93 \pm 0.04 ^a	0.88 \pm 0.11 ^a	0.94 \pm 0.08 ^a	0.43 \pm 0.07 ^b
Cholesterolemia (mM)	1.52 \pm 0.07 ^a	1.35 \pm 0.03 ^a	1.29 \pm 0.09 ^a	0.92 \pm 0.07 ^b
HDLc (mM)	0.69 \pm 0.02 ^a	0.57 \pm 0.02 ^b	0.49 \pm 0.02 ^b	0.52 \pm 0.03 ^b
LDLc (mM)	0.39 \pm 0.07	0.36 \pm 0.03	0.38 \pm 0.07	0.20 \pm 0.05
<i>Portal vein</i>				
Bile acids (μ M)	93.14 \pm 25.02	68.08 \pm 18.3	82.12 \pm 16.5	90.4 \pm 8.94

USA). TATA binding protein (TBP) was used as loading control. The corresponding bands were scanned and quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>).

2.7. Statistical analysis

Results are presented as mean \pm SEM. Statistical significance between groups was assessed by one-way ANOVA. Dixon's Q-test was performed to statistically reject outliers (95% confidence level). When the sampling was not normal, a nonparametric one-way ANOVA using Wilcoxon test was performed. If variances between groups are unequal, we have carried out a Welch's ANOVA. The statistically significant ANOVA tests were followed by post hoc Tukey's multiple comparison tests using JMP®10 (SAS, North Carolina, USA). Data with different superscript letters were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis. Associations between variables were assessed by Pearson correlation test (GraphPad Prism version 4.00). $p \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Hypolipidemia is induced by combination of ezetimibe and simvastatin

Hepatic lipid content is not significantly affected by the different treatments, compared to control (CT) mice (data not shown). The

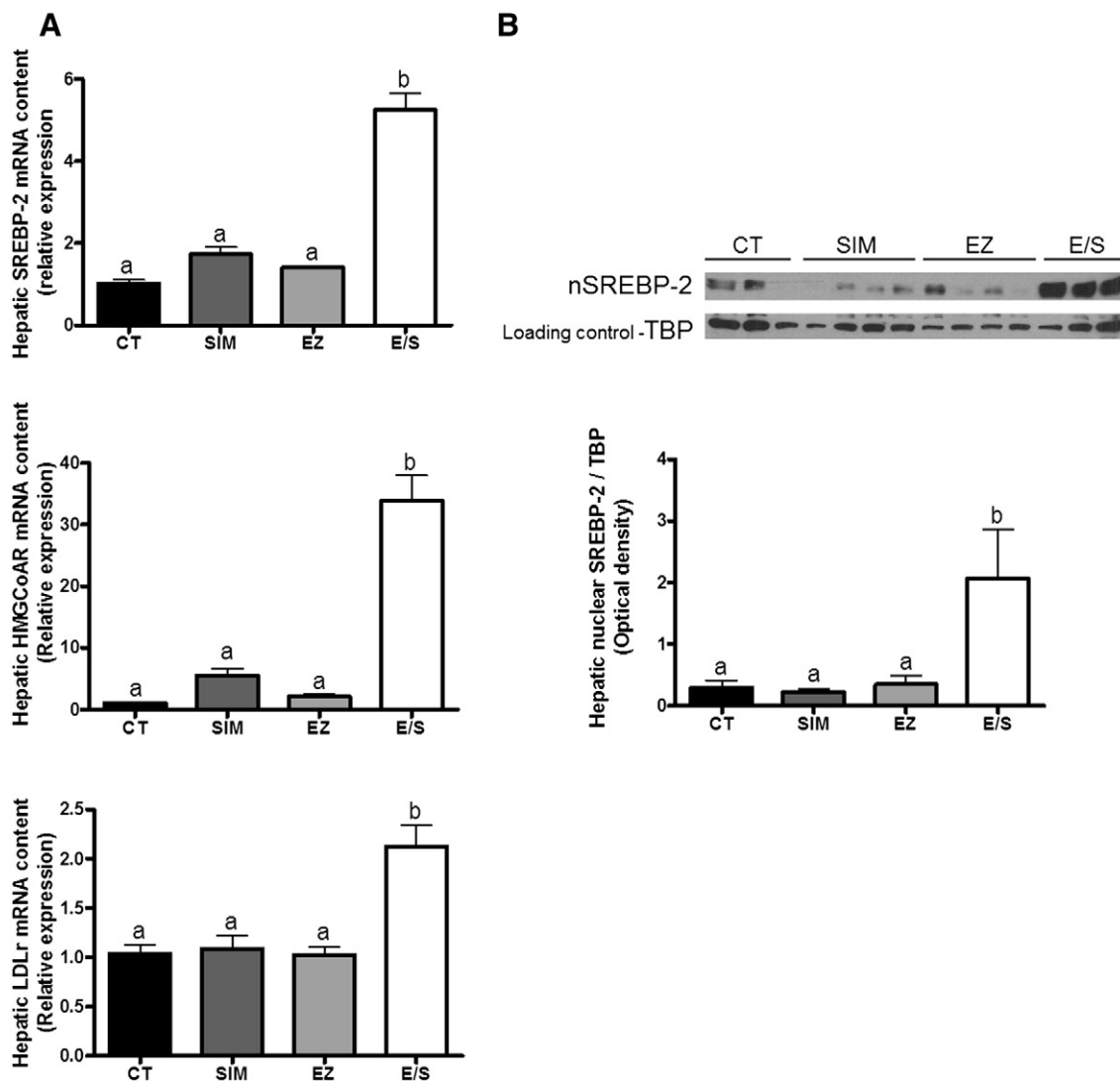


Fig. 1. mRNA content of SREBP-2 (A) and two of its target genes (HMGCoAR, LDLr) (A) and nuclear content of the active SREBP-2 (nSREBP-2) form (B) in the liver of mice fed a control diet (CT; $n = 8$), a control diet supplemented with simvastatin (SIM; $n = 8$), a control diet supplemented with ezetimibe (EZ; $n = 7$ -to-8) and a control diet supplemented with both simvastatin and ezetimibe (E/S; $n = 8$) for one week. The bands corresponding to the nuclear SREBP-2 in the immunoblot were scanned and quantified. Data are the mean \pm SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis.

combination of the two drugs in E/S mice was essential to decrease significantly the triglyceridemia (TG) and the cholesterolemia (CHOL) (Table 2). EZ or SIM alone had no effect on triglyceridemia and total cholesterolemia. All treatments led to a reduction in HDLc compared to controls. Only the combination treatment affected significantly the LDLc concentration compared to CT mice but none treatment affected the BA portal vein concentration (Table 2).

3.2. Combination of ezetimibe and simvastatin led to a synergistic and drastic effect on SREBP-2 activation in the liver

SREBP-2 mRNA expression was increased in the liver of E/S mice compared to the three other groups and this was accompanied by a significant increase in the mRNA expression of LDLr and HMGCoAR, two SREBP-2 target genes (Fig. 1A). Hepatic SREBP-2 gene expression was increased by both SIM and EZ treatment alone, although for the latter it was not significantly different from CT mice due to the drastic effect of the combination. SIM and EZ supplementation did not modify LDLr mRNA expression, whereas HMGCoAR mRNA was significantly increased by five-fold in the liver of SIM mice compared to CT mice (Fig. 1A). SREBP-2 is synthesized as an inactive precursor embedded in the endoplasmic reticulum. By using western blot, we quantified the nuclear active form of SREBP-2 released following two specific protein cleavages. The band analysis revealed a higher nuclear content of the active SREBP-2 protein in the liver of E/S mice compared to all groups (Fig. 1B), confirming that the combination of ezetimibe and simvastatin acts in synergy to activate the SREBP-2 pathway (Fig. 1B).

Other pathways that are involved in cholesterol homeostasis are the hepatic cholesterol biliary excretion and the hepatic BA synthesis. We measured by RT-qPCR the expression of several important genes involved in these pathways. The expression of the heterodimer ATP-binding cassette G5 and G8 (ABCG5/G8), involved in the hepatic cholesterol excretion in the biliary canaliculus, was not changed by EZ

supplementation but was increased by two-fold in the liver of SIM mice compared to CT mice. The combination enhanced the effect of SIM alone as we observed a five-fold increase in their expression in the liver of E/S mice compared to CT mice (Fig. 2A). Cytochrome P450 7A1 (CYP7A1) and CYP7B1 are rate-limiting enzymes in the hepatic classical and alternative BA synthesis pathways, respectively. Hepatic CYP7A1 mRNA expression was not modified by any treatment (Fig. 2B). CYP7B1 expression was not affected following SIM or EZ treatment. However, the combination of E/S significantly decreased CYP7B1 mRNA expression by two-fold (Fig. 2B).

3.3. SREBP-2 and related gene expression in the upper and lower intestine are differently modulated upon ezetimibe and simvastatin treatments

In the jejunum, both SIM and EZ alone increased the gene expression of SREBP-2, HMGCoAR and LDLr. This increase was even more important when the two drugs are combined in the jejunum (Fig. 3A). Unlike the jejunum and the liver, EZ had no effect on the mRNA expression in the ileum, whereas SIM, alone or in combination with EZ, raised the mRNA expression of HMGCoAR, LDLr and SREBP-2 (Fig. 3B).

The jejunum NPC1L1 mRNA expression is decreased by SIM, EZ and combination treatments, compared to CT mice (Fig. 3C).

3.4. The combination of ezetimibe and simvastatin reveals a selective modification in *Lactobacillus* spp. count in the cecum

To investigate possible changes in gut microbiota composition induced by treatments, we carried out an analysis of the microbiota by qPCR. The abundance of the total bacteria measured in the cecal content was not affected by any treatment (Fig. 4A). Similarly, the abundance of *Bacteroides-Prevotella* spp. and *Roseburia* spp. did not differ between groups (Fig. 4B). Two Gram-positive bacteria, namely *Bifidobacterium* spp. and *Lactobacillus* spp. were analyzed by qPCR of 16S-rRNA. *Bifidobacteria* level is not modified by the treatments.

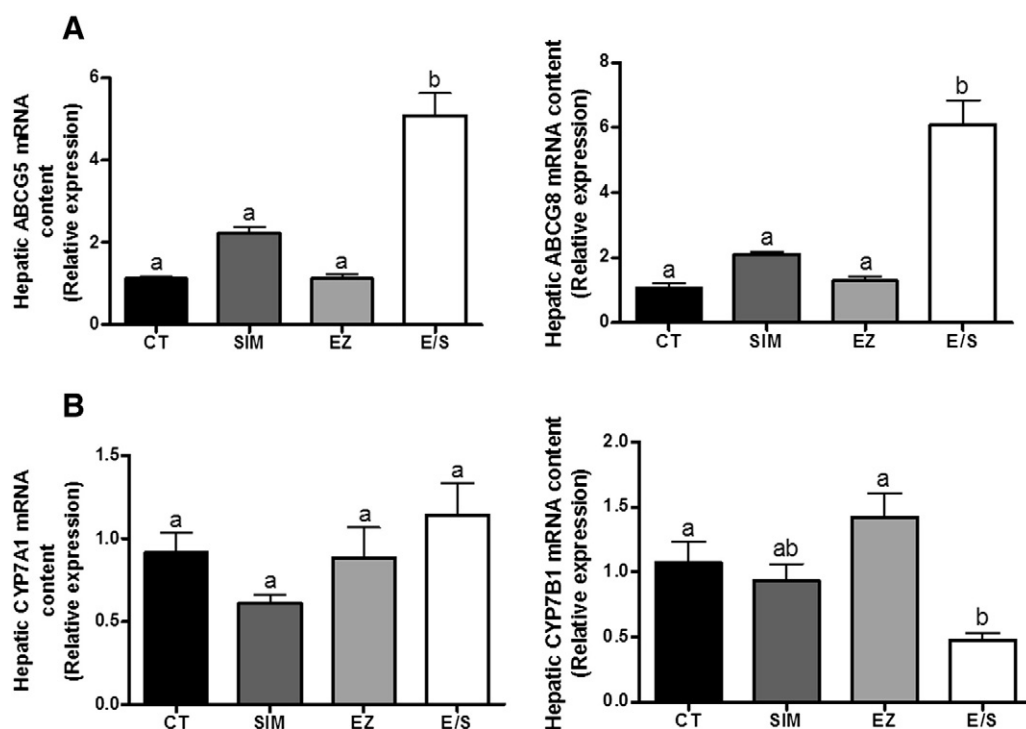


Fig. 2. mRNA content of several genes involved in hepatic cholesterol secretion into the bile (ABCG5 and ABCG8, A), in hepatic BA synthesis (CYP7A1 and CYP7B1, B) in the liver of mice fed a control diet (CT; $n = 7$ -to- 8), a control diet supplemented with simvastatin (SIM; $n = 7$ -to- 8), a control diet supplemented with ezetimibe (EZ; $n = 8$) and a control diet supplemented with both simvastatin and ezetimibe (E/S; $n = 7$ -to- 8) for one week. Data are the mean \pm SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis.

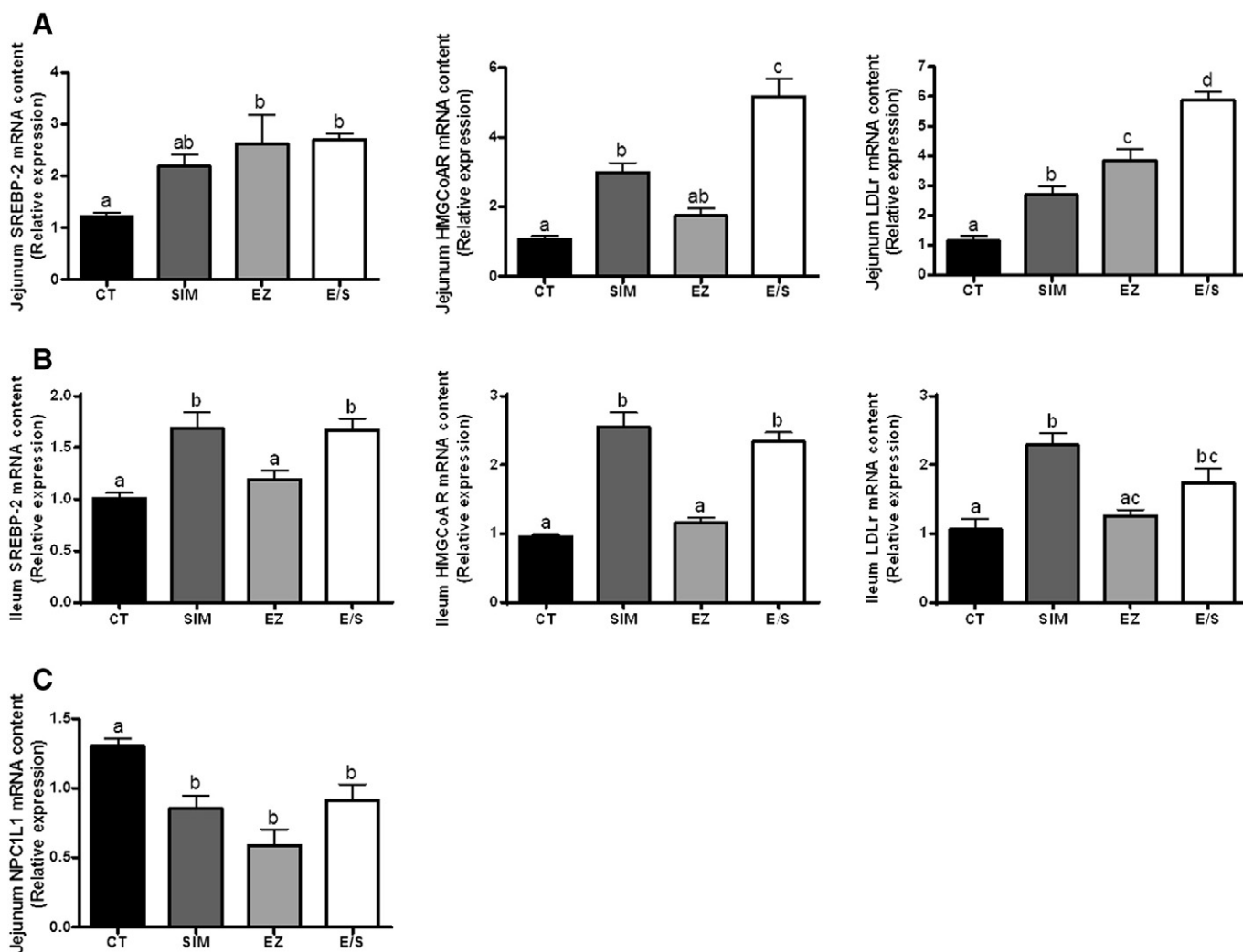


Fig. 3. mRNA content of SREBP-2 and two of its target genes (HMGCoAR, LDLr) in the jejunum (A) and in the ileum (B) and jejunum NPC1L1 mRNA content (C) of mice fed a control diet (CT; $n = 6$ -to-8), a control diet supplemented with simvastatin (SIM; $n = 8$), a control diet supplemented with ezetimibe (EZ; $n = 7$ -to-8) and a control diet supplemented with both simvastatin and ezetimibe (E/S; $n = 8$) for one week. Data are the mean \pm SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA.

Interestingly a significant drop in *Lactobacillus* spp. was observed in mice treated with ezetimibe.

According to this observation, a DGGE analysis was performed to assess cecal composition of *Lactobacillus* spp (Fig. 5A). The analysis revealed enrichment in the band that corresponds to *L. murinus/animalis* in EZ mice compared to the other groups. Unfortunately it was not confirmed by qPCR of 16S-rRNA analysis. However, the DGGE analysis suggests that the drop in *Lactobacillus* spp. observed in E/S mice is most probably due to a significant decrease of *L. murinus/animalis* count in the cecum, confirmed by qPCR of 16S-rRNA (Fig. 5B).

3.5. *Lactobacillus* spp. is negatively correlated with hepatic cholesterol metabolism related expression genes

Correlation analyses were performed between the cecal level of *Lactobacillus* spp., bacterial group modified by E/S treatment and several lipid parameters. The hepatic gene expression of SREBP-2, HMGCoAR and LDLr appeared negatively correlated with the cecal content of *Lactobacillus* spp. Conversely, the hepatic CYP7B1 mRNA, involved in alternative BA synthesis, was positively correlated to the *Lactobacillus* spp. In the intestine, only jejunum NPC1L1 mRNA expression was negatively correlated with the *Lactobacillus* spp. cecal level (Table 3). The intestinal

gene expression of SREBP-2, HMGCoAR and LDLr was not correlated to *Lactobacillus* spp. in the jejunum or ileum.

4. Discussion

The association of simvastatin and ezetimibe is often considered in a substantial proportion of cases when statin therapy seems to be not sufficient to prevent or decrease cardiovascular disease risks [8,19]. Indeed most clinical studies have reported a higher decrease in total cholesterolemia, LDLc and triglyceridemia in hypercholesterolemic patients treated with E/S combination, compared to the changes observed by EZ or SIM treatment alone [20–23]. Our study is performed on wild-type mice fed a standard diet, without cardiovascular risks. The decreased triglyceridemia and total cholesterolemia were promoted only with the combination E/S, whereas in other atherosclerotic models, statin [24,25] and ezetimibe [26,27] treatment, as well as E/S combination are well known to reduce total cholesterolemia and LDLc [28]. Ezetimibe, simvastatin and combination reduced significantly the HDLc concentration. It could be due to an increased HDLc uptake by the liver, since it is considered as an important process in reverse cholesterol transport [24]. ATP-binding cassette A1, involved in hepatic cholesterol efflux to lipid-poor lipoproteins A1 and to form the HDL nascent [2], is not altered by any treatment suggesting that there is no

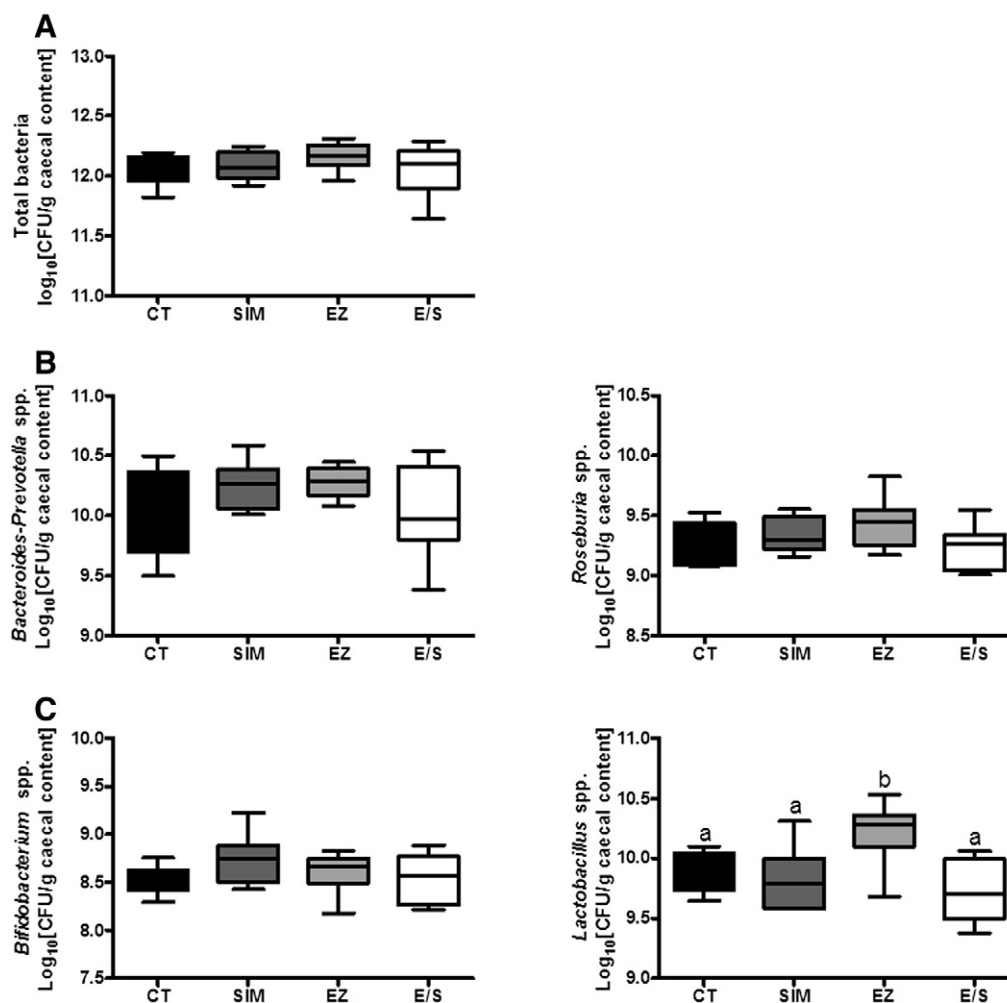


Fig. 4. qPCR analysis of 16S-rRNA of total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides-Prevotella* spp. and *Roseburia* spp. in the caecal content of mice fed a control diet (CT; $n = 7$ -to- 8), a control diet supplemented with simvastatin (SIM; $n = 8$), a control diet supplemented with ezetimibe (EZ; $n = 7$ -to- 8) and a control diet supplemented with both simvastatin and ezetimibe (E/S; $n = 7$ -to- 8) for one week. Data are the mean \pm SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis.

decrease in the HDL synthesis. Moreover, the combination of ezetimibe and simvastatin hugely increased the hepatic expression of SREBP-2, HMGCoAR and LDLr suggesting an enhanced activity of SREBP-2. We confirm this activation, assessed by the increase in the nuclear fractions of the protein by western blot. The intestine is the second place for cholesterol synthesis after the liver, so we analyzed the mRNA expression of these genes in the upper and lower intestine. In the jejunum combination of ezetimibe and simvastatin led to a more important upregulation of mRNA expression of SREBP-2, HMGCoAR and LDLr than in treatments not combined. Otherwise only simvastatin and combination were able to upregulate the pathway supporting the fact that the target tissue of ezetimibe action is the jejunum, and not the ileum. An *in vivo* study, performed on female pig, has been focused on the impact of the combination simvastatin and ezetimibe on cholesterol metabolism at the intestinal level [25]. They reported an increase in mRNA expression of SREBP-2 and its target genes, in EZ pigs and E/S pigs compared to CT [25]. In the opposite of our work, they did not distinguish the different intestinal segments.

In coherence with our observation, the overexpression of SREBP-2 in mice can be associated with an increase expression of genes involved in cholesterol excretion, as the heterodimer ABCG5/G8 [26]. One of most important ABCG5/G8 regulators is the liver X receptor. Interestingly, CYP7B1, involved in the *alternative* BA synthesis, is significantly decreased in E/S mice, suggesting the maintenance of a

high level of oxysterols, cholesterol derivatives produced by a shunt in the cholesterol synthesis. These cholesterol derivatives are able to activate the liver X receptor.

The major purpose of our work was to try to evaluate potential changes in the gut microbiota that could be due to the hypolipidemic treatment. Numerous studies have proposed that gut microbiota participates in the regulation of cholesterol metabolism [11,13,27]. Changing gut microbiota composition, mainly in favor of *Bifidobacterium* spp., led to an inhibition of the cholesterol synthesis pathway [13]. Members of the genus *Lactobacillus* play also an important role in the cholesterol metabolism. For example, *in vivo* studies with *Lactobacillus rhamnosus* GG or *Lactobacillus sakei* NR28 administration are able to decrease lipogenic genes expression in the mouse liver, such as Fatty Acid synthase or Stearoyl-coA Desaturase 1 [28]. Another study confirms these observations of the modulation of lipid metabolism by selected *Lactobacilli*. In fact, the combination of *Lactobacillus plantarum* KY1032 and *Lactobacillus curvatus* HYV7601 or *Lactobacillus curvatus* HYV7601 alone can modulate cholesterol metabolism by decreasing the expression of hepatic gene expression as HMGCoAR [29]. More, most of the *Bifidobacterium* spp. and *Lactobacillus* spp. strains have been shown to be able to reduce the cholesterol availability for intestinal absorption by assimilation [30], by binding it to the bacterial cellular surface or by incorporation into the bacterial membranes [31]. In addition, lactic acid bacteria are also able to increase the deconjugation of bile salt by

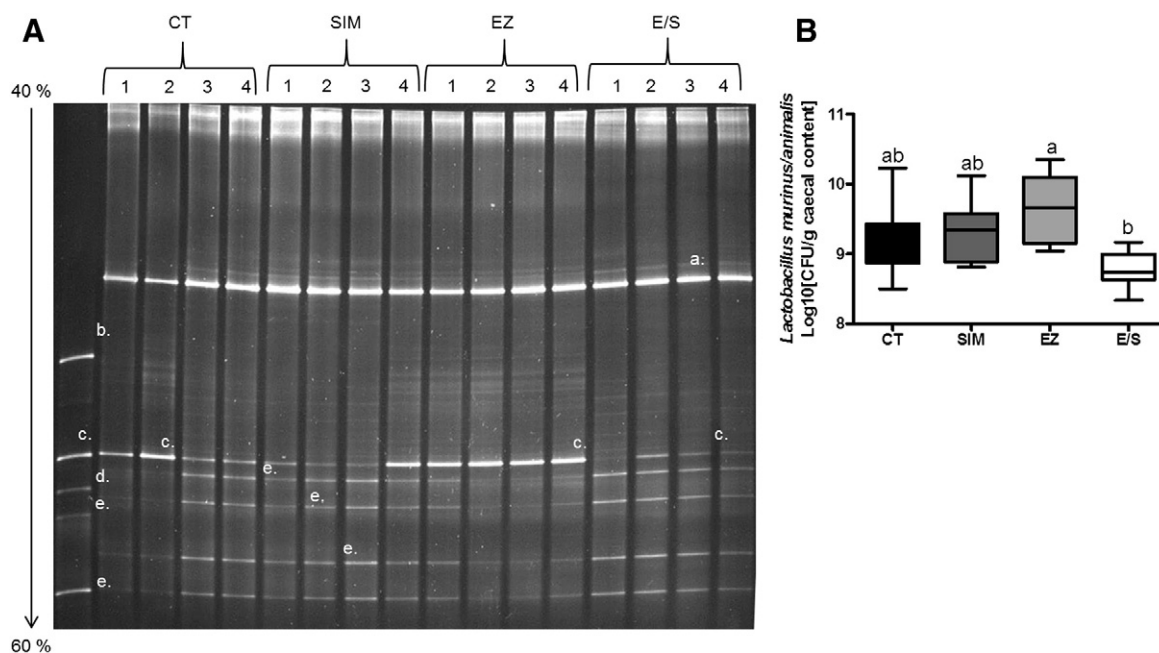


Fig. 5. Denaturing gradient (40 to 60%) gel electrophoresis (DGGE) performed with primers targeting the 16S-rRNA region of specific lactobacilli (A). The bands correspond to bacteria species as follows: *Lactobacillus gasseri/johnsonii* (a), *Lactobacillus acidophilus* (b), *L. murinus/animalis* (c), *Lactobacillus ruminis* (d) and *Lactobacillus reuteri* (e). qPCR analysis (B) of *L. murinus/animalis* and of *L. reuteri* in the cecum of mice fed a control diet (CT; n = 8), a control diet supplemented with simvastatin (SIM; n = 7-to-8), a control diet supplemented with ezetimibe (EZ; n = 8) and a control diet supplemented with both simvastatin and ezetimibe (E/S; n = 8) for one week. Data are the mean ± SEM. Data with one or more different superscript letters (a, b or c) were significantly different ($p \leq 0.05$) according to the post hoc ANOVA statistical analysis.

the action of the bile salt hydrolase enzyme [30,32]. These deconjugated bile acids are less reabsorbed by the enterohepatic circulation, resulting in higher biliary excretion in feces.

In vitro, the incubation of Caco-2 cells in the presence of *Lactobacillus acidophilus* ATCC 4356, has demonstrated that soluble factors produced by bacteria suppress NPC1L1 expression [33]. *In vivo*, we observed also a negative correlation between *Lactobacillus* spp. cecal level and the NPC1L1 expression. Another *in vivo* study in which rats were fed a cholesterol enriched diet and supplemented with the same strain *Lactobacillus* (at 10^9 CFU/day) confirmed that this microorganism is able to decrease the cholesterol absorption by inhibition of NPC1L1 transcription [34]. These data are in agreement with our preliminary results. The modification of gut microbiota in disfavor of *Lactobacillus* spp. observed in combination treated-mice, suggests that it could be more of a consequence than a cause of the change in host gene expression.

Table 3

Correlation between *Lactobacillus* spp. and lipid metabolism parameters. Pearson correlation test between *Lactobacillus* spp. cecal count and the expression of several genes involved in the regulation of cholesterol metabolism in the liver and in the jejunum of mice fed a control diet (CT; n = 6-to-8), a control diet supplemented with simvastatin (SIM; n = 8), a control diet supplemented with ezetimibe (EZ; n = 7-to-8) and a control diet supplemented with both simvastatin and ezetimibe (E/S; n = 8) for one week. $p \leq 0.05$ is significantly different according to the Pearson correlation test.

Parameters	<i>Lactobacillus</i> spp.	
	Pearson (r)	p-Value
Liver SREBP-2	-0.3696	0.0374
Liver HMGCoAR	-0.4408	0.0116
Liver LDLr	-0.4101	0.0198
Liver ABCG5	-0.2920	0.1109
Liver ABCG8	-0.2346	0.2040
Liver CYP7B1	0.4254	0.0152
Jejunum NPC1L1	-0.4457	0.0154

There is an increasing interest in the role of gut microbiota in the metabolism and pharmacokinetics/dynamics of some xenobiotics [35]. Finally, we cannot exclude that the modification of gut microbiota composition could lead to a change of ezetimibe metabolism, thereby modifying, the pharmacodynamics and/or pharmacokinetics of the drug. This type of study would be an interesting perspective of experimental research in the future.

5. Conclusion

The hypolipidemic drug, ezetimibe, taken alone does significantly affect the composition of gut microbiota in favor of *Lactobacillus* spp. but the association of ezetimibe and simvastatin is able to induce a drop in a specific species, the *Lactobacillus animalis/murinus*. One perspective would be to adapt the protocol in “pathological” model, to pay more attention on bacterial metabolic functions, rather than focusing on the phylogenetic analysis of the gut microbiota, in a hypercholesterolemic context.

Conflict of interest

None.

Acknowledgments

Supported by the FRS-FNRS (Fonds De La Recherche Scientifique) (convention FRSM 3.4574.07 and FRFC 2.4555.08) and by the Région Wallonne (Walnut 20). PDC is a research associate from the FRS-FNRS. PDC is the recipient of FNRS and FRSM (Fonds de la recherche scientifique médicale, Belgium) and recipient of ERC Starting Grant 2013 (European Research Council, Starting grant 336452-ENIGMO). NS is the recipient of a postdoctoral fellowship from the Spanish Ministry of Education, Culture and Sports.

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