Interactions between the intestinal microbiota and bile acids in gallstones patients

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Summary

Cholecystectomy, surgical removal of the gallbladder, changes bile flow to the intestine and can therefore alter the bidirectional interactions between bile acids (BAs) and the intestinal microbiota. We quantified and correlated BAs and bacterial community composition in gallstone patients scheduled for cholecystectomy before and after the procedure, using gas–liquid chromatography and 16S rRNA amplicon sequencing, followed by quantitative real-time polymerase chain reaction of the phylum Bacteroidetes. Gallstone patients had higher overall concentrations of faecal BAs and a decreased microbial diversity, accompanied by a reduction in the beneficial genus *Roseburia* and an enrichment of the uncultivated genus *Oscillospira*, compared with controls. These two genera may thus serve as biomarkers for symptomatic gallstone formation. *Oscillospira* was correlated positively with secondary BAs and negatively with primary BAs, while the phylum Bacteroidetes showed an opposite trend. Cholecystectomy resulted in no substantial change in patients' faecal BAs. However, bacterial composition was significantly altered, with a significant increase in the phylum Bacteroidetes. Given that cholecystectomy has been associated with a higher risk of colorectal cancer and that members of the Bacteroidetes are increased in that disease, microbial consequences of cholecystectomy should be further explored.

Introduction

Bile acids (BAs) are saturated, hydroxylated sterols synthesized from cholesterol in hepatocytes and stored within the gallbladder. After a meal, release of cholecystokinin stimulates the gallbladder to contract, causing concentrated bile to flow into the duodenum. Bile acids act as detergents and have an important role in solubilizing dietary lipids and fat-soluble vitamins to facilitate their absorption in the small intestine (Hofmann, 1999). In addition to their role in the digestion of lipids, BAs generally inhibit bacterial growth and prevent bacterial infections in the small intestine (Lorenzo-Zuniga *et al.*, 2003). In the colon, BAs are deconjugated from glycine or taurine by bile salt hydrolases, which are common enzymes in the various genera of the intestinal microbiota and contribute to bile resistance (Jones *et al.*, 2008). Unconjugated BAs are more hydrophobic than conjugated ones, and can be absorbed passively and more easily across the colonic epithelium (Stremmel and Hofmann, 1990). Bile acids can be further modified by bacteria, generating secondary and tertiary forms, mostly through 7α-dehydroxylation. This enzymatic activity results in the conversion of the primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) to the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, and is known to be present only in specific intestinal bacteria belonging to the genus *Clostridium* (Begley *et al.*, 2005). These bacteria are non-dominant members of the microbiota, and their relative abundance can range from as low as 10^{-6} (Ridlon *et al.*, 2006) up to 10^{-3} per gram faeces (Berr *et al.*, 1996; Wells *et al.*, 2003). Overall, by modifying BAs, these bacteria contribute to the metabolism and the enterohepatic recycling of BAs, and also provide resistance to *Clostridium difficile* infections (Buffie *et al.*, 2015).

Gallstones affect 10–15% of the adult population in the west (Shaffer, 2005), and cholecystectomy (CS) is the most common surgical procedure to treat them. After CS, bile enters the duodenum directly, independent of the timing of meals. Bile acids secreted continuously into the 'empty' lumen might irritate the intestine and stimulate its motility. Indeed, post-CS patients often experience an increase in bowel movements, and general gut transit time is shortened by about 20% (Fort *et al.*, 1996; Sauter *et al.*, 2002). Moreover, continuous exposure to BAs can be harmful, leading to chronic diarrhoea and an increased

risk for colorectal carcinogenesis (Barrasa *et al.*, 2013). Thus, changes in BA excretion and profile are important for human health. Furthermore, recent epidemiological studies have suggested that CS is correlated not only with an increased risk of colorectal cancer (Goldacre *et al.*, 2012), but also cardiovascular disease (Chavez-Tapia *et al.*, 2012) and non-alcoholic fatty liver disease (Ruhl and Everhart, 2013). However, only the latter study showed an exclusive association with the procedure itself rather than with gallstone disease. The suggested connection between CS and pathology raises the importance of shedding more light on the bacterial and biliary changes that occur following this procedure. Analysis of patients before and after a procedure is advantageous, since each patient is her/his best control, and paired statistical tests can be used, improving statistical power. Here we compared the faecal profiles of BAs and bacterial communities in gallstone patients scheduled for CS and controls with normal gallbladders, in order to identify disease-specific modifications. We then followed the gallstone patients after their CS in order to investigate how microbiota and BAs change following the procedure,

enabling us to gain new insights into biliary disease, determine possible associations between BAs and specific bacteria, and infer causative processes.

Results

Gallstone patients have increased faecal BA concentrations compared with control subjects

Past studies that examined faecal BAs of post-CS patients showed conflicting results and were limited by comparing post-CS patients with controls rather than to pre-CS gallstone patients (Brydon *et al.*, 1982; Breuer *et al.*, 1986; Fromm *et al.*, 1987; Zuccato *et al.*, 1993). We thus quantified the five major faecal BAs, using gas–liquid chromatography, in gallstone patients before and after CS, as well as in controls (Appendix S1). In agreement with previous studies (Mamianetti *et al.*, 1999), gallstone patients had significantly higher overall faecal BA concentrations compared with controls (median: 8.17 ng/μg dry faeces versus 6.23 ng/μg, Mann–Whitney test $P = 0.015$, see Fig. 1 and Table S1). Notably, faecal
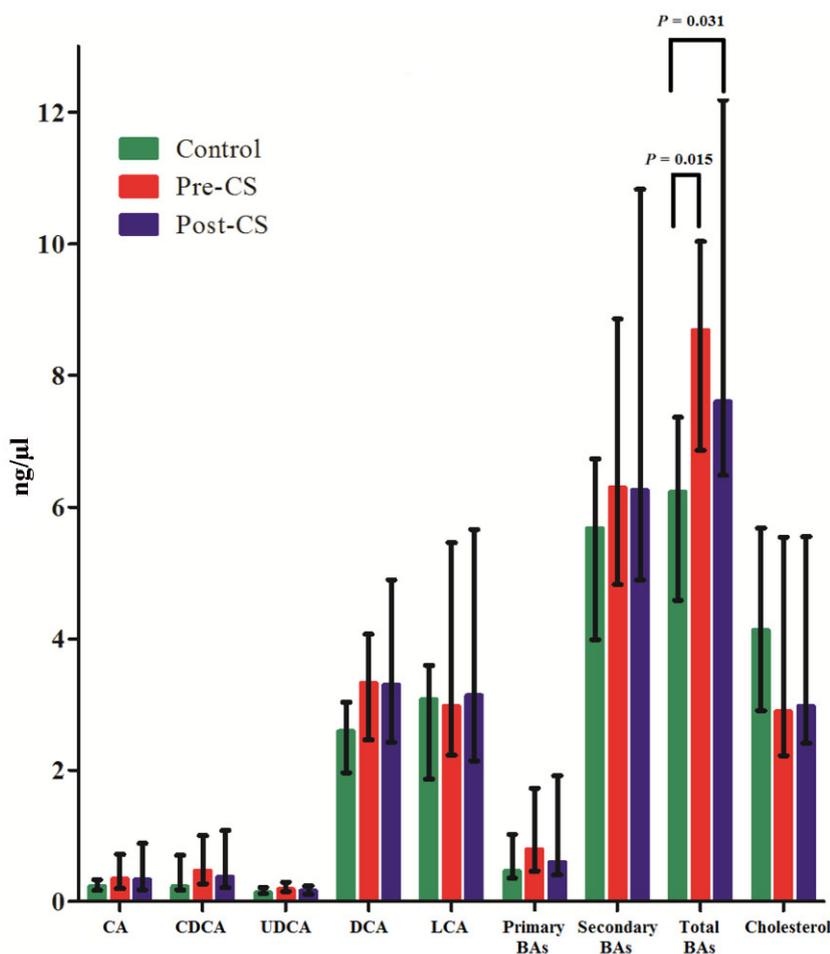
Fig. 1. Faecal bile acids and cholesterol concentrations (ng/μg of faecal matter) in gallstone patients and controls.

BAs of post-CS patients remained higher than in the controls (medians of 7.6 ng/μg and 6.23 ng/μg, respectively, $P = 0.031$, Fig. 1). These findings support the recent observation that the mRNA level of the rate-limiting enzyme in BA synthesis is increased over 400% in gallstone patients compared with controls (Herrera *et al.*, 2009). Thus, it is plausible that the modified faecal BA content reflects an underlying abnormal BA metabolism associated with gallstone formation, rather than being a symptom of their presence.

Since the influence of CS on faecal BAs is debated and since BAs composition may be related to other harmful processes such as carcinogenesis, it is important to know whether the procedure can alter the composition of faecal BAs. We therefore performed paired comparisons of each individual's pre-CS and post-CS faecal BA profiles ($n = 11$, Fig. S1), as well as an independent global comparison of all pre-CS ($n = 13$) and post-CS ($n = 17$) samples (Fig. 1). Except for an increase in DCA concentration in post-CS samples ($P = 0.043$), both tests yielded no statistically significant differences, suggesting that CS does not markedly affect faecal BA composition in gallstone patients. These results complement a study that examined long-term effects of CS in 12 female patients more than 5 years after the procedure and detected no significant changes to the BA pool composition (Kullak-Ublick *et al.*, 1995).

Bacterial modifications in the microbiota of gallstone patients

Recent studies in rodents have demonstrated that higher concentrations of BAs in the colon increase the abundance of some members of the Firmicutes, especially 7α-dehydroxylating species (Ridlon *et al.*, 2013). In contrast, the abundance of members of the Bacteroidetes phylum and other Firmicutes species can decrease, especially when colonic DCA levels are very high (Islam *et al.*, 2011). We were interested to test which bacterial taxa differ between gallstone patients and controls, and whether these differences correlate with BA levels. We thus obtained 454-pyrosequencing data of 16S rRNA gene amplicons from DNA extracted from faecal samples of gallstone patients ($n = 14$) and controls ($n = 16$), and inferred taxonomy using the QIIME pipeline (Caporaso *et al.*, 2010; for details, see Appendix S1). Analysis of Similarity (ANOSIM) of the unweighted UniFrac distances showed no overall separation between these two groups (R = 0.0267, $P > 0.231$), but there was a substantial reduction in bacterial diversity in the gallstone patients (median Shannon index of 2.14 versus 2.51 in the controls, $P = 0.049$, Mann–Whitney test). Comparable reductions in bacterial diversity have been observed in several metabolic disorders (Karlsson *et al.*, 2012; 2013; Qin

Table 1. Bacterial taxa identified by LEfSe as differentiating gallstone patients from controls.

Taxon	Median controls ($n = 16$)	Median patients ($n = 14$)	MaW *P*-value (two-tailed)
Roseburia	0.0467	0.0132	0.021
Bacteroides uniformis	0.0057	0.0009	0.033
Ruminococcaceae	0.3076	0.4765	0.009
Oscillospira	0.0252	0.1210	0.041

et al., 2012). We then compared patients and controls using the biomarker discovery method LEfSe [linear discriminant analysis effect size (Segata *et al.*, 2011)] with α = 0.05 (Table 1). In gallstone patients before CS, the genus *Roseburia* and the species *Bacteroides uniformis* were decreased compared with controls, while the family *Ruminococcaceae* and the genus *Oscillospira* were increased. Notably, *Oscillospira* abundance was previously shown to increase following a switch to an animal-based diet that led to elevated faecal BAs (David *et al.*, 2014). As we have also shown elevated faecal BAs in gallstone patients, it appears that *Oscillospira* benefits from increased concentrations of BAs in the colon and could be a potential biomarker for gallstones.

Of note, *Roseburia* was previously shown to be reduced in human atherosclerosis and type 2 diabetes (Karlsson *et al.*, 2012; 2013; Qin *et al.*, 2012), reinforcing the association of its decline in metabolic disorders. Furthermore, an increase in *Roseburia* was associated with protection against diet-induced obesity in a rodent model, and dependent on farnesoid X receptor (FXR). Importantly, BAs are ligands of FXR and are known to exert their beneficial effects on gut epithelial integrity (Inagaki *et al.*, 2006) and metabolism (Ryan *et al.*, 2014) through this receptor. Taken together, these data imply that some FXR-mediated metabolic benefits that are dependent on the presence of *Roseburia* may be lacking in gallstone patients, despite high levels of BAs.

The phylum Bacteroidetes is increased following CS

Since CS changes the pattern of bile secretion to the intestine, replacing meal-dependent release of a relatively large BA dose with a more continuous drip of low concentration of BAs, this surgical procedure could affect the intestinal microbiota composition of CS patients. Generally, the faecal microbiota composition of patients who underwent CS was similar to its pre-CS state (Fig. 2 and Fig. S2). Furthermore, there was no substantial change in bacterial diversity following CS (median Shannon index of 2.19 in the post-CS samples versus 2.14 in the pre-CS, $P = 0.368$, Mann–Whitney test). To compare pre- and post-CS states as independent
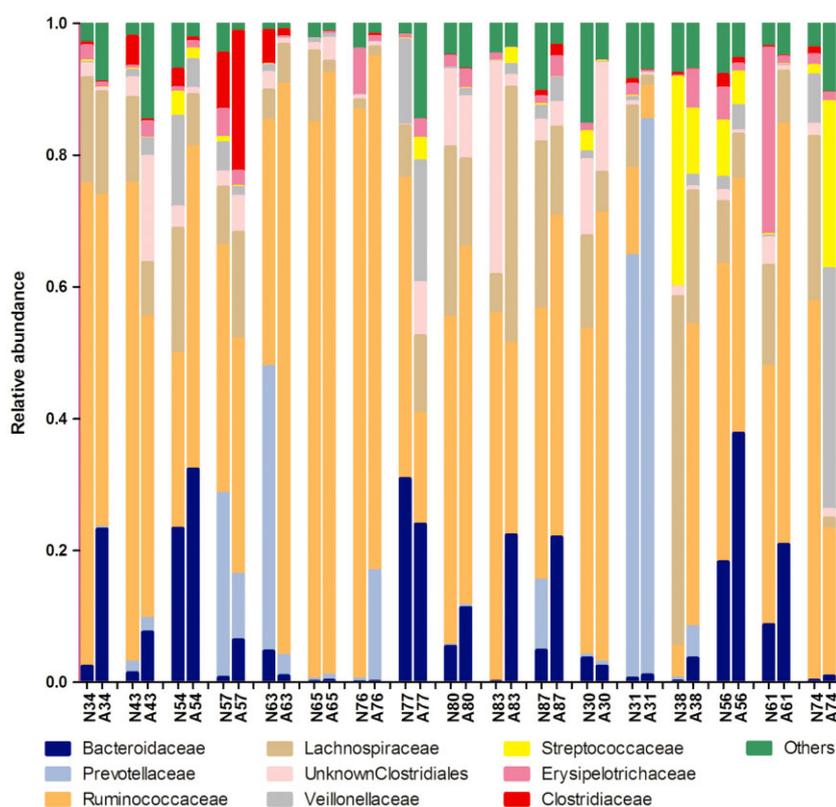
Fig. 2. Paired comparison of faecal microbiota composition of gallstone patients, at the family level, before (left, marked with the letter 'N') and after (marked with the letter 'A') cholecystectomy. Samples 30, 31, 38, 56, 61 and 74 were obtained from subjects who had undergone ES prior to CS.

groups, we randomly picked pre- and post-CS samples from different subjects (20 patients overall, pre-CS $n = 10$, post-CS $n = 10$). Unweighted UniFrac-based ANOSIM showed a modest degree of separation between the groups (R = 0.152, $P = 0.033$). We then looked for taxa that differed significantly post-CS in all our gallstone patient samples, by performing LEfSe with a threshold of $\alpha = 0.10$. This more lenient threshold increases sensitivity, but requires additional validations, through paired-sample analysis (see below). Notably, Operational Taxonomy Units (OTUs) belonging to the phylum Bacteroidetes increased post-CS, including the family *Bacteroidaceae* and the genus *Parabacteroides* (Table 2).

To further validate these observations, we analysed the abundance of the LEfSe-detected taxa described above in 17 sample pairs taken from the same individuals before and after CS. At the phylum level, there was a twofold increase in Bacteroidetes post-CS (Table 2 and Fig. 3A). Within this phylum, the class Bacteroidia and the order Bacteroidales also increased significantly post-CS, as did the family *Bacteroidaceae* and the genus *Bacteroides* (Table 2). To confirm this increase using an additional method, Quantitative Real Time Polymerase Chain Reaction (QPCR) measurement of the relative fraction of the Bacteroidetes phylum was performed (Table 3, Appendix S1). The latter method confirmed that there was a 100% increase in the median

Table 2. Discriminative taxa that are elevated following CS.[a]

	Paired comparison				Independent samples comparison		
	Decreased	Increased	Unchanged	Sig (P)[a,b]	Median pre-CS ($n = 14$)	Median post-CS ($n = 17$)	Sig (P)[c]
Bacteroidetes	4	13	0	**0.017**	0.085	0.178	0.095
Bacteroidia	4	13	0	**0.017**	0.085	0.178	0.095
Bacteroidales	4	13	0	**0.017**	0.085	0.178	0.095
Bacteroidaceae	3	14	0	**0.003**	0.02	0.076	**0.047**
Parabacteroides	6	11	0	**0.009**	0.001	0.002	**0.053**

a. Six patients who underwent endoscopic sphincterotomy prior to cholecystectomy were also included.
b. Wilcoxon Monte Carlo-based *P*-values at a confidence interval of 95%, two-tailed; in bold – $P \leq 0.05$.
c. Mann–Whitney-based *P*-values at a confidence interval of 95%, two-tailed; in bold – $P \leq 0.05$.
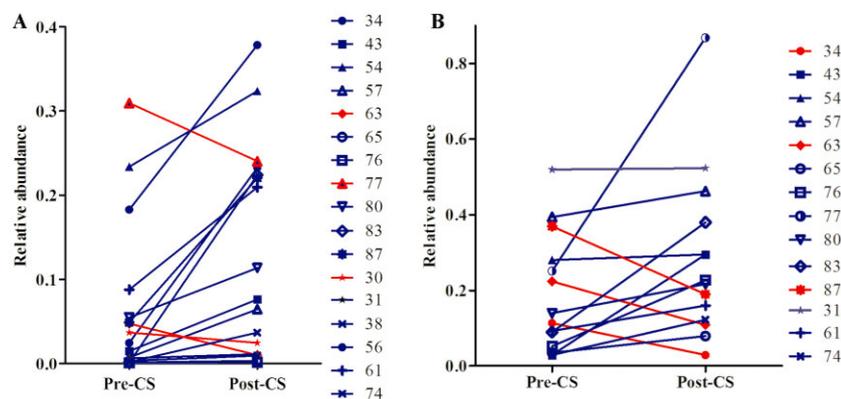
Fig. 3. Changes in the relative concentration of members of the Bacteroidetes in the faecal microbiota of gallstone patients following CS: (A) The relative fraction of the family *Bacteroidaceae* based on 16S amplicon sequencing. (B) QPCR-based quantification of the phylum Bacteroidetes. Red lines – decrease post-CS; blue lines – increase post-CS. Samples 30, 31, 38, 56, 61 and 74 were obtained from subjects who had undergone ES prior to CS.

relative concentration of 16S rRNA genes belonging to this phylum, post-CS compared with pre-CS (medians of 0.11 and 0.22, respectively, $P = 0.024$, Mann–Whitney test). The trends observed in 16S amplicon sequence analysis and QPCR were also evident when examining the same individual before and after CS (Fig. 3).

It should be noted that microbial composition could also be affected by dietary habits. The patients in our study were advised to avoid a high-fat diet prior to surgery, but may have returned to their usual eating habits after the operation. A recent diet microbiota study showed an increase in members of the Bacteroidetes phylum after a switch to an animal-based diet (David *et al.*, 2014). We thus reran the statistical analyses on a subset of patients ($n = 16$) who had hypercholesterolaemia, and therefore were advised to avoid fried and high-fat foods both before and after the procedure. Importantly, these patients displayed the same trends as the entire cohort in terms of the increase in Bacteroidetes. The median value of Bacteroidetes among the hypercholesterolaemia patients alone increased from 0.07 before CS to 0.16 following the procedure. While this increase did not meet the standard threshold for statistical significance (Wilcoxon signed rank test for matched pairs $P = 0.29$), probably due to the reduction in group size and to a high inter-patient variability, the Bacteroidaceae family showed an even stronger, statistically significant, trend (median before CS: 0.02, median after CS: 0.05, $P = 0.045$). In conclusion,

despite the lack of change in faecal BA composition, CS appears to result in an increased abundance of the Bacteroidetes phylum, with specific increases in the family *Bacteroidaceae* and the genus *Bacteroides*. Whether this increase has any negative consequences for health remains to be determined. Members of the Bacteroidetes were found to be increased in colorectal cancer patients compared with controls (Sobhani *et al.*, 2011). Given that CS is associated with an increased risk of various sequelae (Chavez-Tapia *et al.*, 2012; Goldacre *et al.*, 2012; Ruhl and Everhart, 2013), it will be important to examine whether overabundance of Bacteroidetes is characteristic of these conditions.

Correlations between bacterial taxa and faecal BAs

We reasoned that some of the bacterial taxa that changed in abundance in Gallstone (GS) patients and after CS could correlate with BAs. Although microbial communities were modified following CS, the metabolic relations between BAs and bacteria are likely to remain similar. We therefore tested for correlations between BA concentration and bacterial relative abundance, focusing on taxa highlighted by the analyses described in the previous sections. For this analysis, all samples from both patients and controls that had both gas-liquid chromatography (GLC) and microbiota data [either pyrosequencing ($n = 45$), or QPCR ($n = 47$)] were used (Correlation analysis for these and additional bacterial taxa is provided in Table S2 and Fig. S3) Strikingly, the Bacteroidetes phylum was inversely correlated with secondary BA concentration ($P = -0.507$, $P < 0.001$; QPCR $P = -0.342$, $P = 0.022$), particularly with LCA concentration ($P = -0.554$, $P < 0.001$; QPCR $P = -0.404$, $P = 0.006$) and LCA fraction of all measured BAs ($P = -0.355$, $P = 0.017$; QPCR $P = -0.369$, $P = 0.013$). The total faecal BA and DCA concentrations were also negatively correlated (Spearman's $P = -0.307$, $P = 0.04$; $P = -0.345$, $P = 0.02$) with the Bacteroidetes phylum, but these correlations were not supported by the QPCR analysis (Spearman's

Table 3. Demographic data of the study groups.

		Patients	
	Controls	Pre-CS	Post-CS
Total	16	14	17
Gender (F/M)	10/6	9/5	11/6
Age (mean ± SD)	62.7 ± 11.1	61 (± 9.6)	62.9 (± 10.9)
Post-CS (months)			7.5 (± 3.6)
Obese (body mass index ≥ 30)	3	5	5

$P = -0.083$, $P = 0.589$; $P = -0.182$, $P = 0.230$). These findings are in agreement with a rodent study that has shown that members of this phylum are more sensitive to secondary BA exposure than other intestinal species (Islam *et al.*, 2011).

In contrast to Bacteroidetes, the genus *Oscillospira* was significantly positively correlated with the *fraction* of secondary BAs ($P = 0.355$, $P = 0.017$), but only weakly with the *total concentration* of secondary BAs ($P = 0.26$, $P = 0.084$). Specifically, this association can be attributed to a trend where *Oscillospira* was positively correlated with the relative fraction of LCA in the faeces (R = 0.357, $P = 0.016$) and negatively correlated with the relative fractions of CA (R = -0.392, $P = 0.008$), and the tertiary BA Ursodeoxycholic acid (UDCA) ($P = -0.412$, $P = 0.005$). Additionally, *Oscillospira* correlated negatively with faecal cholesterol concentration ($P = -0.419$, $P = 0.004$). The genus *Roseburia*, which showed negative association with *Ruminococcaceae* and *Oscillospira* ($P = -0.387$, $P = 0.007$ and $P = -0.344$, $P = 0.018$, respectively), was significantly positively correlated with faecal cholesterol ($P = 0.393$, $P < 0.008$), but not with BA concentrations.

Our finding that *Oscillospira* was significantly correlated with the *fraction* of secondary BAs and especially with LCA fraction (out of the total faecal BAs), but had a much weaker association with the *concentration* of these BAs, suggests that this genus is either directly or indirectly involved in the conversion of primary BAs to secondary BAs. Past studies showed that gallstone patients had significantly higher levels of 7α-dehydroxylating bacteria than individuals without gallstones (Wells *et al.*, 2000), and thus it is tempting to suggest that a BA 7α-dehydroxylating activity of *Oscillospira* may predispose individuals to cholesterol gallstones. Unfortunately this genus has not been cultivated yet, and so verification of its biochemistry and effects on BAs remains. These associations suggest that bacterial taxa such as *Roseburia* and Bacteroidetes are affected by the discharge of BA and cholesterol into the large intestine, while other taxa, such as *Oscillospira*, potentially play a more active role, and could be involved, directly or indirectly, in the generation of secondary BAs.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. BAs faecal concentrations of the five measured BAs and total BAs before and after cholecystectomy. N – pre-CS; A – post-CS. Samples 30, 31, 38, 56 and 61 were obtained from subjects who had undergone ES prior to CS.

Fig. S2. Paired comparison of faecal microbiota composition of gallstone patients, at the phylum level, before (left, marked with the letter 'N') and after (marked with the letter 'A') cholecystectomy. Samples 30, 31, 38, 56, 61 and 74 were obtained from subjects who had undergone ES prior to CS.

Fig. S3. Correlation plots of all significant correlations reported in *Results*. A–C: Bacteroidetes relative abundance (RA) versus secondary BAs concentration, LCA concentration and LCA fraction (of all measured BAs). Bacteroidetes RA was calculated using either pyrosequencing data (green circles) or QPCR data (blue triangles). D–H: *Oscillospira* relative abundance versus fraction of secondary BAs, LCA, CA, UDCA and cholesterol. I–K: *Roseburia* RA versus *Ruminococcaceae* RA, *Oscillospira* RA and cholesterol concentration.

Table S1. Faecal bile acids and cholesterol (ng/μg of faecal matter and relative fractions) in gallstone patients and controls.

Table S2. Correlations.

Appendix S1. Detailed description of experimental procedures.