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Attempted Isolations of Cholesterol-to-Coprostanol Reducing Bacteria in the Human Gut

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Abstract

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of death globally. In early development of atherosclerosis, retention of cholesterol in artery walls is a key step. Cholesterol both endogenously produced and absorbed from the diet ends up in the gut, where bacteria may reduce it to coprostanol. Unlike cholesterol, coprostanol cannot be reabsorbed into circulation from the intestines, leading to long-standing hypotheses that high cholesterol-to-coprostanol conversion may lower blood cholesterol and thus the risk of ASCVD. Metagenomic evidence for both conversion being health-associated and ASCVD being a microbiota-modulated disease is mounting, emphasising the potential importance of microbial cholesterol metabolism in the gut. This project thus aimed to isolate cholesterol-converting bacteria from the human gut to further characterise them. Conversion was initially studied in a pure *Eubacterium coprostanoligenes* culture as a positive control. However, the type strain was quickly outcompeted by a contaminant whereupon culturing of faecal samples from two healthy donors was initiated. Over four sample series, several media compositions and cultivation approaches were investigated. Coprostanol was not observed in any culture. Pathway intermediates could not be analysed and as such it is possible, although improbable, that partial conversion took place. There was also no way of detecting the potential presence coprostanoligenic but non-converting bacteria. Altogether, these results reiterate on the previously established fastidiousness of coprostanoligenic bacteria. Further attempts would benefit from more research determining the conditions supporting cholesterol conversion, as a better understanding of those conditions is a first step in characterising the bacteria performing conversion. Eventually, this might enable research on supporting their growth using prebiotics or the feasibility of their application as next-generation probiotics for improved cardiovascular health.

Keywords: cholesterol, coprostanol, gut microbiota, atherosclerosis

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1. Introduction

1.1. Background

The sterol lipid cholesterol is fundamental to human life, being a key factor in cell membrane structure as well as a precursor to several hormones and other steroids [1]. However, the levels of cholesterol in blood need to be regulated as high levels of total cholesterol may trigger and aggravate several common cardiovascular diseases (CVD). CVD is the leading cause of death globally, accounting for 32% of all mortalities worldwide or almost 18 million deaths a year [2]. In 2019, 85% of these deaths were due to stroke or ischaemic heart attack [2]. Both are severe consequences of an initial disease called atherosclerosis, whereupon plaque consisting of cellular detritus and lipids, primarily cholesterol, accumulates in the artery walls. With time, this causes the arteries to narrow, eventually rupturing and inducing blood clots [3]. In turn, imbalanced cholesterol levels are a significant risk factor for developing atherosclerosis.

As such, there is a vested global interest in the management of total blood cholesterol, and therefore an interest in cholesterol metabolism. Indeed, endogenous cholesterol metabolism is quite well understood. An average human will every day synthesise about 700-900 mg cholesterol (mainly in the liver and small intestine) and absorb another 300-600 mg from food [4]. This will be used for cell renewal, as substrate for steroid hormone and bile acid formation, and the up to 1 g that is left over will enter the colon via bile excretion. There, intestinal bacteria may reduce it to its saturated analogue coprostanol, which unlike cholesterol is not efficiently reabsorbed into circulation and thus eliminated in the faeces.

The theory that an intestinal microbial metabolism promoting high cholesterol-to-coprostanol conversion may lead to lower circulating levels of cholesterol and thus reduce the risk of CVD has been around since at least 1983, when an inverse correlation between levels of circulating cholesterol and the faecal coprostanol/cholesterol ratio was first reported [5]. More recently, unpublished work by the Bäckhed group shows that, in a small-scale Swedish patient-control cohort study, the cholesterol-to-coprostanol conversion rate was significantly lower in stroke patients than in people with asymptomatic atherosclerosis. Across the board, evidence of atherosclerosis being a microbiota-influenced and -affected disease is mounting [6–12]. Combined with the knowledge that the gut microbiota substantially affects variation in blood lipids [13], this indicates that microbial cholesterol metabolism in the gut may impact human cardiovascular health. This, in turn, emphasises the importance of further investigation into cholesterol-to-coprostanol conversion and the bacteria that perform it, bringing us to the aims of this project.

1.2. Aims

This project had three main aims, each building upon the previous one:

- Characterisation of growth conditions using a known cholesterol-reducing bacterial isolate (i.e., *Eubacterium coprostanoligenes*)
- Implementation of culturing and detection methods for complex cholesterol-reducing cultures from faecal incubations

- Isolation and characterisation of known or yet unknown cholesterol-reducing species of human origin.

1.3. Demarcations

The project was limited to *in vitro* work, as well as the study of *E. coprostanoligenes* and faecal incubations from two human donors. Additionally, only cholesterol-to-coprostanol reduction was studied, with no intent towards translational research on potential health effects of this conversion nor related bile acid metabolism. There was also no intention to elucidate which cholesterol-reducing pathway(s) the investigated bacteria use, excepting the potential for pathway intermediates to be used as indicators of conversion during analysis. The limitation to *in vitro* work additionally means that any potential modulations to cholesterol conversion caused by the host or the microbiota as a whole were not studied.

2. Theory

During the last decades, a so-called 'microbiome revolution' [14] has taken place. Today, we know that the number of bacteria in and on an average human adult outnumber their own cells [15], with 95% of those bacteria harboured in the gastrointestinal tract. This is the gut microbiota, and ever-increasing evidence shows that it influences much of human physiology. For example, it plays a decisive role in immune system development and regulation [16], ferments otherwise indigestible dietary fibres [17], and synthesises neurotransmitters such as serotonin [18], amongst countless other functions together affecting human physiology and regulating our metabolism [19–21].

The associations between an altered gut microbiome, both in composition and function, and cardiometabolic diseases such as diabetes and atherosclerosis are well-established [7,8,21–26] and still mounting [27,28]. Most of this knowledge stems from culture-independent metagenomics studies. In such studies, technological advances allowing for investigations at increasing resolutions have provided and continue to provide information on low abundant and rare bacteria. However, there is an enormous gap between this genomic knowledge and the culturability of these same bacteria. It is estimated that up to 70% of gut microbes remain uncultured [29], meaning that very little is known about the physiology of many, potentially important, gut bacteria. As long as this remains the case, the full functional potential of the gut microbiota cannot be properly investigated nor exploited for medical use, making this project a part of a larger attempt at closing the gap between the metagenomics and characterisation of gut bacteria.

2.1. Endogenous cholesterol metabolism

As mentioned in chapter 1.1, cholesterol is essential to all mammalian life, being one of the key compounds for regulating cell membrane structure [30]. In humans, it plays an additional important role as a precursor to vitamin D, steroid hormones such as the 'stress hormone' cortisol and sex hormones like testosterone and oestrogens, and emulsifying bile acids [1]. We are capable of both endogenous cholesterol biosynthesis and absorption of exogenous cholesterol from food. In the latter case, cholesterol is absorbed from the intestine, packaged

into chylomicrons, and taken up by the liver [31]. Chylomicrons are also called ultra-low-density lipoproteins, with lipoproteins being a class of particles used to transport lipids around the bloodstream. They consist of a centre of cholesterol esters and triglycerides surrounded by a phospholipid membrane, using apolipoproteins as carriers and stabilisers, thus allowing hydrophobic lipids to travel around the water-based circulatory system [1]. As indicated, they are generally classified by their density.

From the liver, cholesterol is carried into circulation by very-low-density lipoproteins, which are processed into low-density lipoproteins (LDLs) that cells can take up [31]. A majority of plasma cholesterol is esterified. The excess is either stored in lipid droplets in various tissues and blood, or shuttled back to the liver in high-density lipoproteins (HDLs) [32]. From there, cholesterol is either sent onwards to organs capable of steroid hormone formation (mainly the gonads and adrenal glands) or excreted into the gallbladder as non-esterified, oxidised bile acids [31]. Bile acids are emulsifiers that participate in lipid digestion, secreted into the duodenum and then largely reabsorbed from the ileum and colon in a process called enterohepatic circulation [33]. Additionally, they take part in several signalling pathways and thus modulate human metabolic pathways [33], and can be metabolised by the gut microbiota, thus modulating their signalling capabilities and potentially further affecting host health [34].

In total, roughly 50% of the cholesterol – both from the diet and bile – in the digestive system is reabsorbed into circulation via the portal vein [35]. Furthermore, transintestinal cholesterol excretion (TICE) in essence allows enterocytes to directly excrete plasma-derived cholesterol into the intestinal lumen [36]. Both these pathways contribute to the regulation of cholesterol metabolism and help bring leftover cholesterol from diet, bile, and shed intestinal cells into the colon, where it can be metabolised by the gut microbiota. In addition to the cholesterol-to-coprostanol conversion this thesis focuses on, it has recently been established that gut microbes in the genus *Bacteroides* are capable of cholesterol sulfonation [37,38]. As of yet very little is known of the health implications or potential of this pathway.

2.1.1. Pathogenesis of atherosclerosis

In the case of cholesterol input (biosynthesis and dietary intake) exceeding output (conversion to bile acids and steroid hormones, cell renewal, and microbial conversion), excess cholesterol will collect in the blood, causing hypercholesteremia. The correlation between atherosclerotic cardiovascular disease (ASCVD) and hypercholesteremia was established more than 100 years ago [39] – and while there are innumerable risk factors, both behavioural and genetic, for developing atherosclerosis, elevated cholesterol levels appear to be the only one that on its own can drive the disease [40].

Today, atherogenesis is generally explained by the 'response-to-retention' theory as put forward by William and Tabas in 1995 [41]. According to this theory, hypercholesteremia, or more specifically elevated levels of LDL-bound cholesterol, is associated with altered permeability of the arterial wall [3]. In vasculature where the endothelial barrier is compromised, atherogenic lipoproteins are retained and oxidised in the endothelium [41]. This causes adherence of monocytes to the endothelium, which migrate into the lining and are activated into macrophages, in turn causing activation of signalling factors, leading to further influx and activation of macrophages [3]. When scavenging and 'eating' lipoproteins, these immune cells may turn into lipid-rich foam cells, aggregating into a so-called fatty streak. Due to the immune response, the inner lining of the artery will thicken and transform into a fibrous cap on top of

the fatty streak, forming a stable plaque. Up to this point, there generally are no clinical complications. With time, though, the lipid core of the plaque will necrotise, with these necrotic cells releasing signals that cause cell death in the cap as well [40]. Thus, the cap will thin and become more vulnerable to the mechanical stress of blood pumping. Eventually, the plaque will rupture and heavily promote thrombosis, obstructing blood flow and – depending on location – causing cardiovascular events such as strokes or myocardial infarction.

2.2. Atherosclerosis and the microbiome

There are significant associations between ASCVD and specific microbial taxa within the commensal microbiota [6,7], as well as previously established correlations to pathogenic bacteria [9,10]. Overall, symptomatic atherosclerosis is associated with alterations in both the function and composition of the gut metagenome. For example, a metagenome-wide association study comparing ASCVD patients and controls found that patients generally were enriched for facultative anaerobes, oral bacteria, and potentially inflammatory species, on the whole displaying less fermentative and potentially less anoxic guts [6]. Symptomatic atherosclerosis patients being enriched in Proteobacteria, a typically low-abundant phylum often indicating increased inflammation [42], has also been observed in the small cohort study by the Bäckhed group mentioned in chapter 1.1 as well as in a larger 200-participant study [43]. The latter study also found controls to be enriched for the families *Lachnospiraceae* and *Ruminococcaceae* [43], both of which have been associated with high faecal coprostanol levels [44]. Another study found the genera *Roseburia* and *Eubacterium* to be enriched amongst healthy controls [7]. Both genera are typically considered beneficial, with many species producing important compounds such as butyrate [45,46].

Furthermore, there are suggestions that the gut microbiome may regulate inflammatory pathways related to the development of atherosclerosis [7], as well as produce metabolites involved in atherogenesis and plaque development [10]. The most well-studied example of such a compound is trimethylamine N-oxide (TMAO). It is synthesised by the liver from the microbial metabolite trimethylamine, which in turn is produced from dietary precursors such as L-carnitine and choline, common in e.g. red meat [47]. TMAO promotes atherogenesis in both mouse models and humans, and plasma levels are predictive of CVD risk in humans [25]. On the other hand, the microbiota also produces compounds such as butyrate and other short chain fatty acids (SCFAs). Fermentation products from dietary fibres, SCFAs are anti-inflammatory and generally considered to have positive effects on cardiometabolic health due to their roles in maintenance of gut barrier integrity, metabolic homeostasis, and immune regulation [48,49].

Altogether, there are strong associations between the functional potential and structure of the gut microbiome and atherosclerotic disease, with ASCVD generally being associated with a loss of functional potential in the guts. With profound effects on host health still being uncovered, the potential of exploiting and possibly modulating the microbiome in treatment of ASCVD is only growing.

2.3. Coprostanoligenic bacteria

The existence of cholesterol-reducing, or coprostanoligenic, gut bacteria was theorised by the turn of the 20th century [4], with such a reduction proven in the 1950s using anaerobically

incubated faecal samples [50]. It took until 1973, however, for the first successful isolation of a coprostanoligenic strain. Isolated from rat cecum, this bacterium was identified as *Eubacterium* ATCC 21408 and had an absolute requirement for neutral sterols [51]. Also in 1973, more gut anaerobes from the genera *Clostridium*, *Bacteroides*, and *Bifidobacterium* were reported to perform cholesterol reduction [52], while later reports could not replicate this finding [53,54]. One of these replication studies, however, reported the isolation of a coprostanoligenic strain from human faeces [53]. More strains were later isolated from baboon faeces [54,55]. Almost all of these, as well as *Eubacterium* ATCC 21408, required brain-based plasmenylethanolamines as growth factors [54]. Subsequently, all these strains – including *Eubacterium* ATCC 21408 – have been lost [4].

In 1994, incubations from a hog manure lagoon resulted in the isolation of *Eubacterium coprostanoligenes* (or *Eubacterium* ATCC 51222) [57]. A small, Gram-positive coccobacillus, it does not have an absolute requirement for neutral sterols or plasmenylethanolamines but does require phosphatidylcholine [57]. It produces hydrogen, can survive in aerobic conditions for 48 h, and is still available today.

In 2007, another attempt at isolating a cholesterol-reducing bacterium of human origin succeeded, and resulted in the discovery of *Bacteroides* sp. strain D8, a Gram-negative rod [58]. Notably, this is also the first isolate not belonging to the genus *Eubacterium*. As there was no link between strain abundance and rate of cholesterol conversion, *Bacteroides* sp. strain D8 is unlikely to be primarily responsible for cholesterol conversion in humans. In pure cultures, cholesterol conversion began on day 3 after inoculation and required a week to complete [58]. This was also found to be case for *E. coprostanoligenes*, but the two species displayed different medium preferences. *E. coprostanoligenes* neither grew nor converted cholesterol in standard brain medium, the medium in which *Bacteroides* sp. strain D8 performed best, while the *Bacteroides* grew but did not perform conversion in basal cholesterol medium, in which *E. coprostanoligenes* converted [58].

A few years later, it was reported that five *Lactobacillus* strains reduced cholesterol to coprostanol when grown in MRS broth supplemented with 0.01% cholesterol [59]. However, there has seemingly been no follow-up on this research.

Over the years, several mechanisms for cholesterol-to-coprostanol conversion have been proposed, illustrated in Figure 1. In the 1950s, a direct reduction of the 5,6-double bond (path II) was initially reported [60], while later studies established that reduction proceeds essentially exclusively through a four-step indirect pathway (path I) with 5-cholesten-3-one, 4-cholesten-3-one and coprostanone as intermediates [61,62]. Today, all evidence indicates conversion through this pathway. Furthermore, these intermediates have all been shown to form in cultures of *Eubacterium* ATCC 21408 [51,56], *E. coprostanoligenes* [63,64], and *Bacteroides* sp. strain D8 [58].

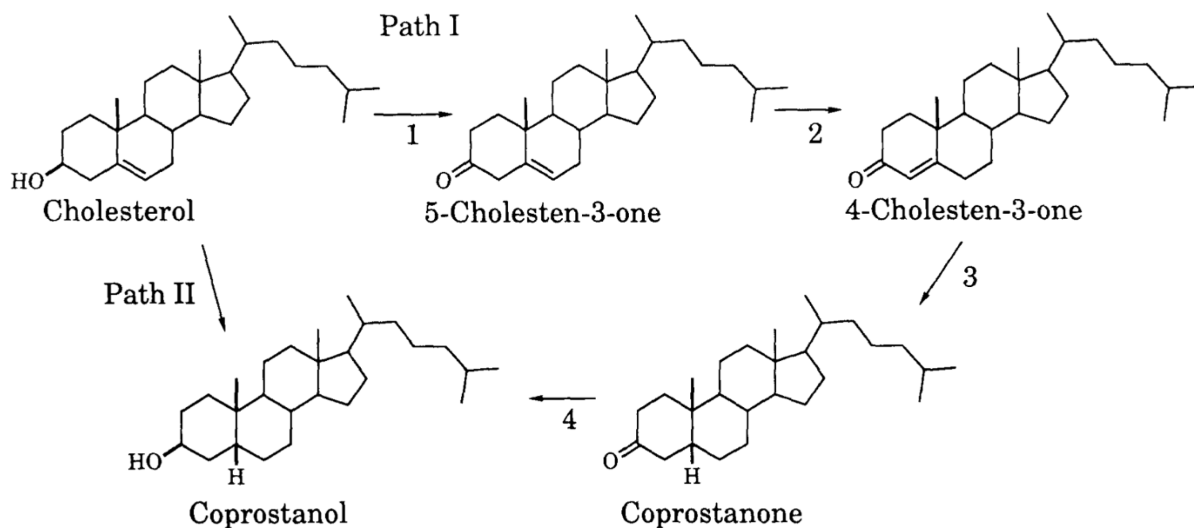

Figure 1. Proposed pathways for microbial cholesterol-to-coprostanol reduction. In path I (indirect), numbers mark the reaction steps: (1) oxidation, (2) isomerisation, (3) and (4) reduction. Borrowed from [63].

2.4. Cholesterol-to-coprostanol conversion at host level

Previous research [65–67] has shown that healthy people can be divided into 'conversion groups' depending on their rate of cholesterol reduction. This rate is calculated as the amount of coprostanol divided by the total amount of measured neutral sterols in the faeces. There is a mainly bimodal division of 'low' and 'high' converters, with different studies using between 30% and 50% conversion rate as arbitrary cut-offs between the two groups [65–68]. Using a 30% conversion cut-off, high converters were estimated to have more than 10^8 coprostanoligenic bacteria per gram of fresh faeces, while low converters were estimated to have less than 10^6 coprostanoligenic bacteria/g faeces [65]. An individual's conversion rate is thus closely correlated to the abundance of cholesterol-converting bacteria in the gut [65], and additionally independent of age and sex [65,66,69].

Without external disturbances, what evidence there is indicates that an individual's conversion status is stable over time [67,70]. However, it can be affected by antibiotic treatment, with stronger effects seen for antibiotics generally targeting Gram positive and/or anaerobic bacteria [69]. In children, microbial cholesterol metabolites are very low or undetectable under 12 months of age, with conversion rates resembling those of an adult established around 3-4 years of age [71–74].

The influence of diet on conversion rate is also contentious. In animal models, there have been reports of mice on an obesogenic diet showing significantly increased coprostanol levels when supplemented with inulin [75] and of rats on a lactose-rich diet exhibiting lower conversion rates than controls on a sucrose-based diet [76]. The fat composition of their diet has also been shown to affect the sterol excretion of rats, with increased linoleic acid found to significantly increase coprostanol formation [77]. In humans, meanwhile, a vegetarian diet has been repeatedly shown as having little to no impact on conversion rate [70,78,79]. Nonetheless, there is also evidence for the microbiota preferably using plant sterols rather than cholesterol when those make up a higher proportion of total sterols [80], seemingly indicating that a vegan diet could reduce conversion.

There are furthermore indications of correlations between cholesterol conversion and intestinal disease. Lower conversion rates have been found in patients with ulcerative colitis [81] and in people from colon cancer-prone families [82] as compared to controls. Additionally, Crohn's disease has been shown to be linked to a decrease in potentially cholesterol-converting *ismA+* species [64] (see next section). Taken together, these correlations suggest that cholesterol-reducing bacteria are sensitive to inflammation, and further show that much remains to be discovered regarding what ecological niche they occupy.

2.4.1. The metagenomics of conversion

The first gene involved in microbial sterol metabolism was identified in 2020, when metagenomic analysis resulted in the identification of *ismA* ('intestinal sterol metabolism A'). This is a family of genes encoding for microbial cholesterol dehydrogenases found in a clade of mostly uncultured bacterial species [64]. Across six cohorts, the presence of *ismA*+ species in the human metagenome was associated with decreased levels of both faecal and circulating cholesterol, as well as increased levels of intestinal and faecal coprostanol and cholestenone. 20 metagenomic *ismA*+ species with an average relative abundance of 1.4% were identified across the datasets. Phylogenetic analysis indicated that most belong to *Clostridium* cluster IV [64], with *E. coprostanoligenes* being the only cultured strain amongst them. While this study found that a majority of coprostanol formation in human gut can be explained by *ismA*+ microbes, there were a subset of samples amongst the six cohorts without any *ismA+* species that still contained coprostanol [64]. This could be a consequence of the limit of detection in sequencing, but an important possibility is that cholesterol-to-coprostanol conversion also could be performed by microbes encoding non-*ismA* cholesterol dehydrogenases with less specificity for coprostanol or with highly circumstance-dependent activity. *Bacteroides* sp. strain D8 not appearing to encode *ismA* bolsters this hypothesis.

Furthermore, unpublished metagenomic analysis by the Bäckhed group shows that faecal conversion rate and abundance of *ismA* (specifically homologues of ECOP170, the version found in *E. coprostanoligenes* and the hydroxysteroid dehydrogenase with highest specificity towards coprostanol [64]) are positively correlated. Both measures additionally associate positively with faecal gene richness, which in turn is strongly associated with metabolic health [83]. Across three patient cohorts, people with high *ismA* counts have better metabolic health markers, and on average CVD patients have lower *ismA* counts than control groups (which include people with asymptomatic plaques). While *ismA* abundance correlates strongly with conversion rate, it is not a linear relation, further contributing to the hypothesis that other genes and bacteria may be capable of conversion.

Looking at the microbiome as a whole in this unpublished project, *ismA* abundance is strongly associated with methanogenesis and cellulosome modules breaking down complex fibres, as well as high abundance of the genera *Methanobrevibacter* and *Oscillospira*, and the species *Ruminococcus bromii*. *Methanobrevibacter* is methanogenic [84], while *Oscillospira* is associated with leanness and reduced in patients with inflammatory disease [85]. *R. bromii*, meanwhile, is key in opening up complex fibres for the remaining microbiota [86]. Low *ismA* count, on the other hand, correlate to sugar degradation and degradation of less complex fibres, as well as nitrate reduction and some other pathways associated with anaerobic respiration. People with low *ismA* counts were enriched for the genera *Fusobacterium* and *Enterocloster*, both negatively associated with host health [87,88], and the inflammatory *R. gnavus*, which has been found to be enriched in symptomatic atherosclerosis patients [89]. Altogether, lower

conversion rate and lower *ismA* counts both seem to be associated with disease, further incentivising research into cholesterol-converting bacteria.

3. Methods

3.1. Culturing of *Eubacterium coprostanoligenes*

3.1.1. Media formulations and preparation

Cultures of *E. coprostanoligenes* were grown in three different media: basal cholesterol medium (BCM), standard brain medium (SBM), and cholesterol brain medium (CBM), as well as on plates of cholesterol brain agar (CBA), modified basal cholesterol agar (MBCA), and LYBHI. These media formulations are found in Table 1-5 below, in the listed order. Fresh media and plates were left to equilibrate in the anaerobic chamber for at least 18 hours before use.

Table 1. Basal cholesterol medium recipe, adapted from [57].

Ingredient (manufacturer)	**Amount per litre**
Cholesterol (NF grade, MP Biomedicals)	2.0 g
Egg yolk lecithin (>60% L-α-phosphatidylcholine, Sigma-Aldrich)	0.5 g
Casitone (BD Difco)	10.0 g
Yeast extract (Oxoid)	10.0 g
$CaCl_2 \cdot 2 H_2O$ (Sigma-Aldrich)	1.0 g
Sodium thioglycolate (Sigma-Aldrich)	0.5 g
0.02% resazurin (Sigma-Aldrich) solution	5 ml

Cholesterol, lecithin, and a small amount of water were stirred together on a heat plate at 50˚C until the cholesterol dissolved before remaining ingredients were added and the pH adjusted to 7.5 with 1 M KOH.

Table 2. Standard brain medium recipe, as previously described [58].

Ingredient (manufacturer)	**Amount per litre**
Lyophilised calf brain	20.0 g
Casitone (BD Difco)	10.0 g
Yeast extract (Oxoid)	10.0 g
K_2HPO_4 (Sigma-Aldrich)	5.0 g
Sodium thioglycolate (Sigma-Aldrich)	0.5 g

Calf brain had previously been acquired from a slaughterhouse and lyophilised in-house, and was spoon-mashed to a powder before introduction to the medium. The medium was stirred on a heat plate at 50˚C until roughly homogenous. Before autoclavation, the pH was adjusted to 7.2-7.4 using 1 M KOH.

Table 3. Cholesterol brain medium recipe, adapted from [58].

Ingredient (manufacturer)	**Amount per litre**
Lyophilised calf brain	20.0 g

Cholesterol (NF grade, MP Biomedicals)	25.0 g
Egg yolk lecithin (>60% L-α-phosphatidylcholine, Sigma-Aldrich)	5 g
Casitone (BD Difco)	10.0 g
Yeast extract (Oxoid)	10.0 g
K_2HPO_4 (Sigma-Aldrich)	5.0 g
Sodium thioglycolate (Sigma-Aldrich)	0.5 g

For CBA plates, 14 g agar (BD Difco) per litre was added before sterilisation. Powdered calf brain, cholesterol, and lecithin were stirred together in half the water volume on a heat plate at 50˚C until homogenous. Then, remaining ingredients were added and the pH adjusted to 7.2-7.4 using 1 M KOH.

Table 4. Modified basal cholesterol agar recipe, adapted from [57].

Ingredient (manufacturer)	**Amount per litre**
Cholesterol (NF grade, MP Biomedicals)	20 g
Egg yolk lecithin (>60% L-α-phosphatidylcholine, Sigma-Aldrich)	6.5 g
Casitone (BD Difco)	10.0 g
Yeast extract (Oxoid)	10.0 g
$CaCl_2 \cdot 2\ H_2O$ (Sigma-Aldrich)	1.0 g
Sodium thioglycolate (Sigma-Aldrich)	0.5 g
Agar (BD Difco)	20 g
0.02% resazurin (Sigma-Aldrich) solution	5 ml
0.4% trypan blue (Life technologies) solution	3.3 ml

This MBCA recipe is a blend between the BCM and modified lecithin agar (MLA) recipes presented in [57]. It aims to mimic liquid BCM closer with its easier preparation while maintaining the higher cholesterol and lecithin content found in MLA. When making the media, cholesterol, lecithin, and a small amount of water were stirred together on a heat plate at 50˚C until the cholesterol dissolved. Then, remaining ingredients were added and the pH adjusted to 7.2 using 1 M KOH. MBCA without trypan blue was also used.

Table 5. LYBHI recipe, as per [90] (then described as LYHBHI).

Ingredient (manufacturer)	**Amount per litre**
Brain heart infusion (Oxoid)	37.0 g
Yeast extract (Oxoid)	5.0 g
D-(+)-Cellobiose (Sigma-Aldrich)	1.0 g
D-(+)-Maltose · 1 H_2O (Sigma-Aldrich)	1.0 g
Cysteine (Sigma-Aldrich)	0.5 g
0.2% porcine haemin (Sigma-Aldrich BioXtra) solution	2.5 ml
Agar (BD Difco)	20 g

LYBHI is a rich, non-selective medium commonly used in-house for the cultivation of various gut bacteria, and was used as such in this project as well.

3.1.2. Anaerobic culturing

Freeze-dried *Eubacterium coprostanoligenes* ATCC 51222 was obtained from LGC (United Kingdom). The sample was introduced into an anaerobic Coy chamber (Coy Industries, United States) with an atmosphere of 85% N_2, 10% CO_2 and 5% H_2 and revived in BCM according to ATCC instructions [91]. Plates of tryptic soy agar with sheep blood – composed of 40.0 g tryptic soy broth (BD Difco) and 15 g agar (BD Difco) per 950 ml water, with 50.0 ml defibrinated sheep blood (Oxoid) added aseptically after autoclavation [92] – were inoculated with primary *E. coprostanoligenes* broth and incubated both aerobically and anaerobically at 37˚C for three days to check for contamination.

Liquid cultures kept inside the Coy chamber were incubated at 37˚C, shaken every weekday to avoid lipid precipitation, and transferred weekly using 1% inoculum. Thin layer chromatography (TLC) was performed weekly to assess cholesterol metabolism; see chapter 3.3 for details. Gram staining was also performed regularly to assess culture purity, with samples taken from each incubation using a 10 µl inoculation loop and plated on glass slides. Fixing was performed by drying the slides on a ~40˚C heat plate. Slides were dipped in a crystal violet solution for 1 min and rinsed with ddH_2O, whereafter they were dipped in an iodine solution for 1 min and rinsed with 99.5% ethanol. Finally, each slide was dipped in a safranin bath for 30 s and rinsed with ddH_2O, before drying on a ~40˚C heat plate and inspection.

After three culture generations, incubation into SBM and CBM was performed using 1% inoculum. The cultures were managed in the same manner as the BCM tubes. In addition to the constantly maintained two 1% inoculum-cultures of each media, BCM subcultures with inoculation concentrations of 2.5, 5, and 10% were also evaluated for cholesterol conversion. Samples for gas chromatography-mass spectrometry (GC-MS) were generally taken from week-old cultures of every third generation.

To evaluate any potential pressure build-up and effects thereof, gas-tight Hungate tubes were used. Working inside the Coy chamber, two tubes each were filled with BCM, SBM and CBM and inoculated with 1% transfer from a week-old culture of the same media. The tubes were then closed using rubber septa and plastic screw caps. Tubes were incubated at 37˚C for a week and shaken daily, with sampling for TLC on day 4 and 7. Samples for GC-MS were taken at the end of the week.

Growth on plates was also evaluated. CBA plates were initially inoculated with 10 µl from three-day old BCM cultures. After four days incubation at 37˚C in the Coy chamber, discrete colonies were transferred back into liquid BCM and handled like the other liquid cultures. Later on, LYBHI and MBCA plates (both with and without trypan blue) were inoculated with 100 µl from a two-day old liquid BCM culture and dilution-streaked every third day for three subcultures, until discrete colonies were achieved. At this point, 16s rRNA gene sequencing was performed, detailed in chapter 3.4.1. All plate cultures were Gram stained in order to examine microscopic morphology.

3.2. Culturing of complex faecal cultures

3.2.1. Media formulations and preparation

The BCM recipe (see chapter 3.1.1) was at one point changed to closer resemble the original recipe [57]. Amongst the ingredients, the only change was replacing egg-based lecithin with type IV-S soy lecithin (Sigma-Aldrich) and due to its lower purity increasing the amount to 1 g/l. Regarding the preparation, cholesterol, lecithin, and 150 ml H_2O was mixed in a standing blender on high for 10 min under a stream of N_2. This mixture was then combined with the remaining ingredients and the pH adjusted. Henceforth, these two variations will be referred to as 'egg BCM' and 'soy BCM', noting that this distinction also refers to the difference of non-electrically mixed and electrically mixed media.

Additionally, 'fibre BCM' (fBCM) was used to promote hydrogen production and thus pressurisation. A combination of three defined fibres was used for a total concentration of 0.1%, with a high concentration of potassium salts used to buffer against the pH drop fermentation causes. The recipe is found in Table 6.

Table 6. Basal cholesterol medium with fibres (fBCM), adapted from [57].

Ingredient (manufacturer)	Amount per litre
Cholesterol (NF grade, MP Biomedicals)	0.5/1.0/2.0 g
Soy lecithin, type IV-S (>30% L-α-phosphatidylcholine, Sigma-Aldrich)	1.0 g
Casitone (BD Difco)	10.0 g
Yeast extract (Oxoid)	10.0 g
$CaCl_2 \cdot 2 H_2O$ (Sigma-Aldrich)	1.0 g
Pectin (from apple, Sigma-Aldrich)	0.33 g
(+)-Arabinogalactan (from larch wood, Sigma-Aldrich)	0.33 g
Xylan (from corn core, Tokyo Chemical Industry)	0.33 g
KH_2PO_4 (Sigma-Aldrich)	5.0 g
K_2HPO_4 (Sigma-Aldrich)	10.0 g
Sodium thioglycolate (Sigma-Aldrich)	0.5 g
0.02% resazurin (Sigma-Aldrich) solution	5 ml

fBCM was prepared in the same manner as soy BCM, excepting the pH being adjusted to 7.2 for optimal buffering ability.

Media containing compositionally complex fibre preparations was also used. As these preparations contain varying amounts of simple sugars, which in humans would be taken up in the small intestine and not reach the colon, the fibres were digested and dialysed before use in order to maintain physiology-mimicking conditions. 5 g each of milled rye, wheat, and oat bran (MRB, MWB, and MOB, respectively) (Lantmännen AB, Sweden) were mixed with H_2O to form a 'swallowable […] paste' [93]. 10X α-amylase stock (from hog pancreas, Sigma-Aldrich) in 15 mM $CaCl_2$ was added and the pH adjusted to 6.8 using 1 M NaOH. The mixtures were incubated at 37˚C for 1 h. Then, each fibre paste was put into a rinsed cellulose ester dialysis membrane (Repligen, United States), closed with plastic clamps, and dialysed against H_2O at 4˚C for 72 h. The water was exchanged and sampled daily. The results of the dialysis were confirmed using polysaccharide TLC (see section 3.3 for technical details and Figure S1 for the results), whereafter the fibres were lyophilised and the yield calculated.

These fibres were then added to SHIRM (simulated human intestinal redox model) medium, with the recipe in Table 7. SHIRM is a rich medium designed to mimic human digestion, thus allowing for cultivation of a wide range of gut bacteria, and was used as it normally contains defined fibres that could easily be replaced with compositionally complex ones without larger adjustments.

Table 7. SHIRM (simulated human intestinal redox model) medium, adapted from [94].

Ingredient (manufacturer)	Amount per litre
D-(+)-glucose (Sigma-Aldrich)	0.5 g
Yeast extract (Oxoid)	3.0 g
Proteose-peptone (Merck)	3.0 g
Mucin, type II (from porcine stomach, Sigma-Aldrich)	1.0 g
NaCl (Merck)	0.9 g
KH_2PO_4 (Sigma-Aldrich)	0.45 g
K_2HPO_4 (Sigma-Aldrich)	0.45 g
$CaCl_2 \cdot 2\ H_2O$ (Sigma-Aldrich)	0.12 g
$MgSO_4 \cdot 7\ H_2O$ (Sigma-Aldrich)	0.009 g
0.2% porcine haemin (Sigma-Aldrich BioXtra) solution	5 ml
Vitamin mix I (Table S1)	1 ml
Vitamin mix II (Table S2)	1 ml
Digested and dialysed MRB/ MWB/ MOB, normalised to 0.25% fibre content	3.6/ 2.9/ 11.4 g

Two bottles of media were prepared per fibre. To one of the two bottles, 2 g/l cholesterol (MP Biomedicals) and 1 g/l type IV lecithin (Sigma-Aldrich), pre-mixed with H_2O in a blender for 10 min, were added. Working in a LAF bench after autoclavation, the pH was adjusted to 1 using 6 M HCl. 10 mg/l pepsin (from porcine gastric mucosa, Sigma-Aldrich) was added and the media aerobically incubated at 37˚C for 30 min. Meanwhile, pancreatic juice was prepared by combining one part each of 12 g/l autoclaved bovine bile (Sigma-Aldrich) and 12.5 g/l sterile-filtered $NaHCO_3$ (Sigma-Aldrich) before adding 0.9 g/l pancreatin (from porcine pancreas, Sigma-Aldrich). The media and pancreatic juice were then combined in a 7:3 ratio, with sterile-filtered 0.5 g/l L-cysteine (Sigma-Aldrich) added before introducing the media into the Coy chamber for immediate use.

3.2.2. Cultures in basal cholesterol media

On several occasions, fresh faecal samples from two healthy volunteers were acquired and immediately introduced into the Coy chamber. To check for conversion, TLC was performed on the original samples by dissolving ~10 ml faeces in 400 µl water and then proceeding with extraction as described in chapter 3.3.

On three occasions, approximately 1 g sample was dissolved in 25 ml medium each. Then, ten-fold dilution series were prepared down to dilutions of 10^{-9}. Two factors differed between the three occasions: tube type and media composition. Series I was kept in, and the serial dilution performed in, 15 ml polypropylene tubes (TPP) using egg BCM. In series II, glass tubes with non-tight screw caps and soy BCM were used. Series III was performed in Hungate tubes. Additionally, fBCM with varying cholesterol content was used: the initial dilution series and

the first transfer were performed in 0.05% cholesterol, the next two transfers were done with 0.1% cholesterol, and after that 0.2% cholesterol fBCM was used. The initial dilution series used for series III was also plated on CBA and incubated at 37˚C in the Coy chamber. After 4 days, discrete colonies were incubated in Hungate tubes with soy BCM and treated as the remaining Hungate tubes.

Incubation took place at 37˚C. All cultures were shaken every weekday. Transfer into fresh BCM was performed every two to four days, with 1% inoculum used for series I and 5% for series II and III, with 10% inoculum additionally evaluated in series III. Series I and II were kept inside the Coy chamber. The Hungate tubes used for series III were prepared inside the Coy chamber, with transfers done using syringe and needles in a LAF bench. TLC was performed regularly to monitor cholesterol metabolism in all three series, while GC-MS was performed on selected samples in each series.

3.2.3. Cultures with complex fibres

A fresh faecal sample from a healthy donor was introduced into the Coy chamber immediately upon acquisition, with 1 g sample enriched in 25 ml LYBHI for 3 h. Meanwhile, triplicate Hungate tubes of six different SHIRM medium variations were prepared, containing: milled rye bran and cholesterol (rye/chol), milled rye bran and no cholesterol (rye), and ditto for milled wheat bran (wheat/chol and wheat) and oat (oat/chol and oat). These were inoculated with 2.5% of the faecal enrichment in LYBHI and incubated at 37˚C. Tubes were shaken daily. TLC was performed both on the faecal sample (dissolved in water before extraction) and on the enriched LYBHI to check for conversion. Samples for GC-MS and 16S rRNA gene sequencing were taken daily. On day 3, the cultures containing cholesterol were transferred into Hungate tubes with soy BCM using 2.5% inoculum. TLC was performed on day 6.

The subcultures in soy BCM were also incubated at 37˚C and shaken regularly. Transfers were performed every 3-5 days, initially using 2.5% inoculum and increasing to 5% in generation 4. TLC was performed weekly, while GC-MS was performed on samples from generations 1, 4 and 7. Samples for 16S rRNA gene sequencing was taken from generation 1 on day 3 after inoculation.

3.3. Chromatography methods

TLC was used for qualitative assessment of cholesterol reduction. Samples were extracted using Folch's method [95], adding 125 µl of a 2:1 mixture of chloroform and methanol to 400 µl bacterial culture. The samples were vortexed well and spun down, whereafter 2 µl was taken from the bottom phase and applied on an HPTLC silica gel 60 plate (Merck). A 98:2 solution of chloroform and methanol was used as running buffer. As standard, a mixture of 0.1% w/v of cholesterol (NF grade, MP biomedicals) and coprostanol (Sigma-Aldrich) with 0.05% w/v egg-yolk lecithin (>60% L-α-phosphatidylcholine, Sigma-Aldrich) in H_2O was used, extracted like the samples.

After running, plates were sprayed first with a saturated solution of $Fe(II)SO_4$ in glacial acetic acid, using Zak and Epstein's [96] reagent for sterol staining, and then with 10 M H_2SO_4. The plates were dried on a ~120˚C heat plate. Cholesterol presented as purple bands, while

coprostanol ran slightly higher and a lighter colour. The limit of detection was established to be slightly below 100 µM for extracted samples, corresponding to ~25 µM in the bacterial cultures.

Meanwhile, GC-MS was used for quantitative and more thorough analysis. 45 µl of internal standard (~4 mM Cholesterol-d7, Avanti Polar Lipids) was added to glass vials and evaporated before addition of 50 µl sample. Extraction was then performed using the BUME (butanol and methanol) method as described previously [97]. 100 µl of the BUME extracts were evaporated under nitrogen flow before recovery into 100 µl each of dichloromethane and N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane, based on a derivatisation method previously presented by Szucs et al [98]. After 30 min in a 70˚C oven, the samples were put into the GC-MS system (Agilent Technologies, United States), consisting of a 7090A gas chromatograph coupled to a 5975C mass spectrometer equipped with a DB-5MS UI (30m x 250 µm x 0,25µm) column. Selected ion monitoring was used, with mass-to-charge ratios found in Table S3, while running parameters can be found in Table S4.

Polysaccharide TLC was performed in a 2:1:1 mobile phase of 1-butanol, glacial acetic acid, and H_2O. Samples were directly applied onto an HPTLC silica gel 60 plate (Merck), with 5 mM glucose (Sigma-Aldrich) as standard. Dried plates were sprayed with a 95:5 mixture of 1% orcinol in methanol and concentrated H_2SO_4, dried again, and developed on a ~100˚C heat plate.

3.4. 16S rRNA gene sequencing

3.4.1. Full-length gene amplification

Amplification and sequencing of the full 16S rRNA gene was performed in order to investigate the purity of *E. coprostanoligenes* cultures, with cultures of interest grown on plates. Firstly, colony polymerase chain reaction (PCR) was performed for amplification. The surface of well-defined colonies were picked separately with sterile 10 µl filter tips. Each sample was then immersed into 49 µl PCR reaction mix, composed of 25 µl 2X HotStarTaq master mix (Qiagen), 17 µl H_2O, 5 µl 10X Coral load (Qiagen), and 1 µl each of 10 µM 27F and 1492R primers (see Table S5 for sequences). Purified bacterial DNA was used as a positive control, and 1 µl H_2O as negative control.

The PCR programme started with 5 min initial denaturation at 95˚C. 26 cycles consisting of 30 s 94˚C denaturation, 45 s 52˚C annealing, and 90 s 72˚C extension followed. The programme finished with 7 min elongation at 72˚C before cooling to 4˚C. Gel electrophoresis (120 V) was then performed to check for the desired 1.5 kb-DNA fragment, using TAE buffer and a 1.2% agarose gel.

DNA purification was then performed based on the NucleoSpin Gel and PCR Purification Kit (Macherey-Nagel). The volume of each PCR product was brought to 100 µl with H_2O. After transfer to microcentrifugation tubes, 200 µl NT1 buffer was added. The samples were mixed, quickly spun down, loaded onto columns, and spun down for 1 min at 11000 g, the speed used for all subsequent centrifugations. Flow-through was then discarded, 700 µl of NT3 buffer added, and the samples spun down for 1 min again. Next, the columns were placed into new collection tubes and spun down for 2 min. Open columns were incubated at room temperature (RT) for 5 min in order to dry. 30 µl elution buffer NE was then added to the columns, which were incubated for another 2 minutes before being put into new microcentrifuge tubes and

centrifuged for 1 min. Samples were quantified by Quant-iT PicoGreen (Invitrogen) according to protocol [99], and sent to Eurofins Sweden for sequencing.

3.4.2. Sequencing of the V4 variable region

Sequencing of the V4 variable region of the 16S rRNA gene was performed in order to identify composition of and potential circumstantial changes to complex bacterial incubations. Firstly, 500 µl bacterial sample was centrifuged at 13000 g for 5 min. The pellet was dissolved in 500 µl buffer ASL (Qiagen) and the solution transferred to Lysing matrix E-containing tubes (MP Biomedicals). Samples were then vortexed for 2 min, put in a 90˚C heatblock for 10 min at 1400 rpm, incubated on ice for 2 min, and put in a bead beater at 5.5 m/s for 60 s. After 5 min incubation on ice, the bead beating was repeated. The samples were centrifuged (4˚C, 12700 rpm) for 5 min, with the supernatants collected into new tubes. 200 µl buffer ASL was then added to the pellet-containing tubes, which were vortexed for 15 s. Subsequently, the heating, incubation on ice, one round of bead-beating, and centrifugation were repeated as described, with the supernatant added to the supernatant-containing tubes. Tubes were inverted after addition of 260 µl ammonium acetate and incubated on ice for 5 min. Samples were then centrifuged (4˚C, 12700 rpm) for 10 min, the supernatants transferred to new tubes, and 600 µl ice-cold isopropanol added to each tube. After 30 min incubation on ice, samples were centrifuged under the previously described conditions for 30 min. The supernatants were discarded and the pellets washed with 500 µl chilled 70% ethanol before 5 min centrifugation (4˚C, 12700 rpm). Next, the supernatants were discarded once more. The pellets were left to dry in room temperature (~45 min) before dissolving them in 200 µl buffer TE (Qiagen) and leaving them at 4˚C overnight.

Purification was performed using the QIAamp DNA Mini Kit (Qiagen). 2 µl RNAse was added to the samples, which were briefly vortexed and spun down. After 15 min incubation at 37˚C, 15 µl proteinase K and 200 µl buffer AL were added and the tubes inverted before 10 min incubation at 70˚C. Then, 200 µl 99.5% ethanol was added, the tubes inverted, and the samples loaded onto QIAamp columns. Columns were centrifuged for 1 min (RT, 16000 rcf) and the collection tubes exchanged. 500 µl buffer AW1 was added, the samples spun for 1 min (RT, 16000 rcf), and the flow-through discarded. This process was repeated using buffer AW2. Samples were then centrifuged for 1 min (RT, 16000 rcf) in fresh collection tubes. After moving the columns to Eppendorf tubes, 50 µl buffer EB was added, and the samples incubated for 5 min at RT before centrifuging them for 1 min (RT, 16000 rcf). Finally, this incubation and centrifugation was repeated with a further 30 µl buffer EB. DNA concentrations were checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Next, amplification was performed with PCR in 25 µl reactions using dual barcoded primers (515F and 806R) degenerated in several positions to also allow for detection of Archaea [100]. A master mix of – per sample – 17.60 µl H_2O, 2.50 µl AccuPrime buffer II 10X (Invitrogen), 1.25 µl DMSO (5% of volume), 0.50 µl BSA (0.4 mg/ml, Thermo Scientific), 0.50 µl barcoded forward primer (0.2 µM), and 0.15 µl Taq enzyme (Invitrogen) was prepared and aliquoted into a 96-well plate. To each well, 0.50 µl of an individual barcoded reverse primer (0.2 µM) was added, as was a sample of 2 µl gDNA (corresponding to 20-50 ng) or a negative control of 2 µl H_2O. The PCR programme started with 2 min denaturation at 94˚C, followed by 25 cycles of 15 s 94˚C denaturation, 30 s 52˚C annealing, and 30 s 68˚C extension, finishing with 2 min elongation at 68˚C before cooling to 4˚C. To check for the desired 300 bp DNA fragment, gel electrophoresis (120 V) was performed using TAE buffer and a 1.2% agarose gel.

Post-PCR purification was performed using the same protocol as detailed in chapter 3.4.1. Quantification was done using the Qubit dsDNA HS assay kit (Invitrogen), performed to protocol [101] with 2 µl sample. For samples with insufficient concentrations, the PCR protocol and clean-up was repeated with new combinations of barcoded primers and 28 cycles.

Samples were aliquoted into 1.5 ml tubes to attain two pools, together containing 40 ng of each sample. For samples containing less than 40 ng DNA in total, 15 µl was put into the pool to yield as much DNA as possible without diluting the remaining pool too much. These pools were then purified further using the AMPure XP kit (Beckman Coulter). After resting at RT for 30 min, the kit was vortexed well and 100 µl of each sample pool mixed with 160 µl AMPure XP bead suspension, followed by 5 s vortexing and 10 min incubation at RT. The samples were then put into a magnetic particle collector (Dynal MPC-S). After 5 min incubation at RT the liquid was discarded, taking care not to disturb the beads, and the samples removed from the particle collector before addition of 200 µl 70% ethanol, vortexing, and 1 min incubation in the particle collection. The liquid removal and ethanol washing were repeated once. Finally, samples were dried at 37˚C for 5 min in the particle collector and the pellet resuspended in 30 µl buffer NE. DNA concentration was then measured with Qubit, before sequencing on a MiSeq system with the reagent V2 kit and 250 bp paired-end sequencing (Illumina).

4. Results

4.1. Culturing of *E. coprostanoligenes*

In order to confirm establishment of cholesterol conversion in the pure *E. coprostanoligenes* cultures, TLC was performed. As shown in the TLC plate in Figure 2(a), the second generation of *E. coprostanoligenes* cultures appeared to convert cholesterol (samples 2.1 and 2.2). Note that the standards run slightly higher than the samples due to being dissolved in DMSO rather than water, and that the samples were not extracted. Conversion in these two cultures was later confirmed by GC-MS – on day 3 after inoculation, sample 2.1 contained 5004 µM cholesterol and 289 µM coprostanol, while sample 2.2 contained 4564 µM cholesterol and 353 µM coprostanol, corresponding to conversion rates of 5.5% and 7.2%.

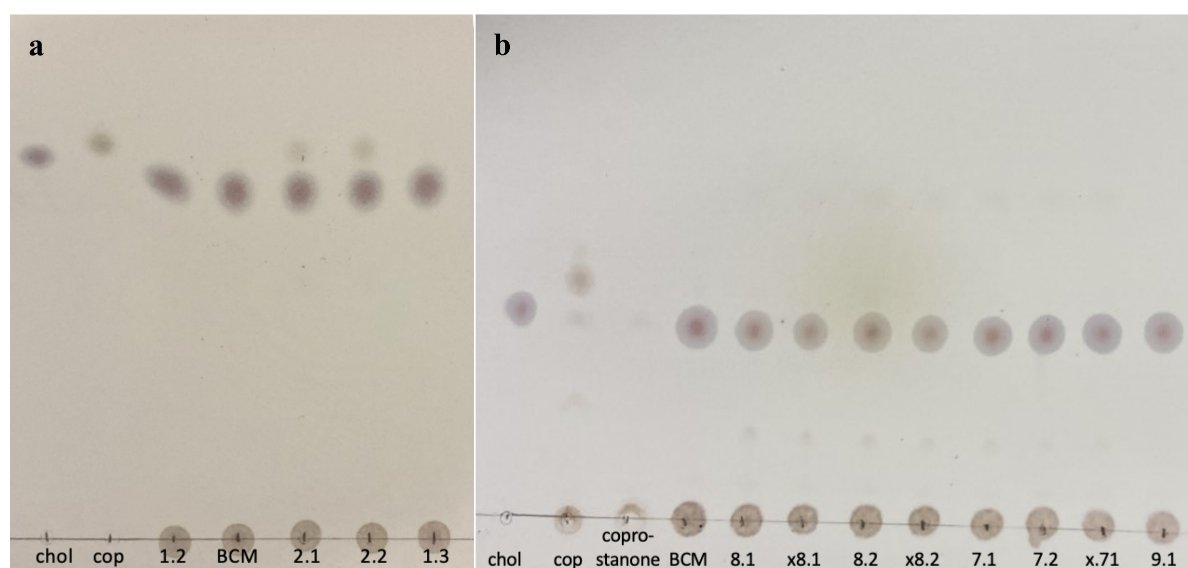

Figure 2. Thin layer chromatography of *E. coprostanoligenes* cultures. Standards are denoted 'chol' (cholesterol) and 'cop' (coprostanol); BCM indicates a media blank. The first number of the sample name indicates

generation, the other which duplicate it is. All samples are unextracted. **(a)** Generations 1 and 2. Note that the standards run higher due to being dissolved in DMSO. **(b)** Generations 7-9, with an x before the sample ID indicating inoculation concentration above 0.1%. The lane marked 'coprostanone' contains coprostanone.

Over the next eleven generations, no further *E. coprostanoligenes* culture displayed a putative coprostanol band in TLC analysis, as exemplified in Figure 2(b). On this plate, there is also a lane with coprostanone (Sigma-Aldrich), an intermediate of the indirect conversion pathway. This standard was added in an attempt to closer monitor conversion, but as repeated tries never resulted in a visible band it was quickly discarded. Increased turbidity and regular Gram staining confirmed the bacteria to still be alive. Additionally, the Gram staining was in line with the described morphology of *E. coprostanoligenes* as a small, Gram-positive coccobacillus growing singly and in pairs [57], exemplified in Figure 3. Due to these mixed results, 16S rRNA gene sequencing was performed to confirm the purity of the cultures. This established the cultures as *Cutibacterium acnes*, an aerotolerant anaerobe and a lipophilic species considered an opportunistic pathogen and part of the normal skin flora of adults [102]. Consequently, the cultures were discarded.

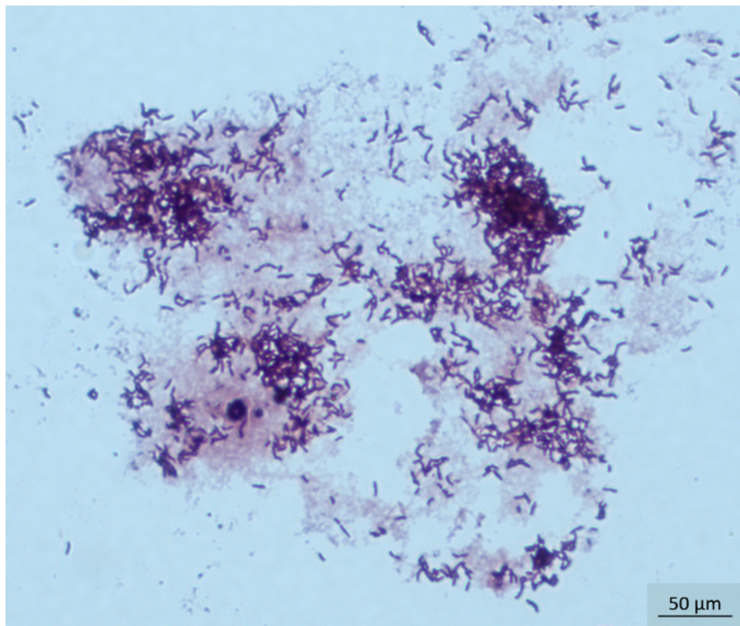

Figure 3. Gram-stained microscopy of putative *E. coprostanoligenes* culture.

4.2. Faecal cultures without fibres

In parallel to culturing of the *E. coprostanoligenes* type strain, we tried to establish cholesterol conversion in complex faecal cultures as a prelude to attempted isolation of coprostanoligenic strains. Series I (see chapter 3.2.2; faecal cultures in Falcon tubes) indicated a putative coprostanol band on TLC plates, with generation 2 shown in Figure 4. While very faint and thus difficult to compare its colour to that of the standard, this band ran at the same height as the coprostanol standard and was present in samples from both donors (A and B). Consistently present in the cultures diluted down to 10^{-5}, the band generally appeared after three to four days of incubation and became fainter over the course of generations. GC-MS analysis showed no coprostanol in the same samples shown in Figure 4, nor samples from generations 4, 7, or 10, implying that the putative coprostanol band in fact was a confounder.

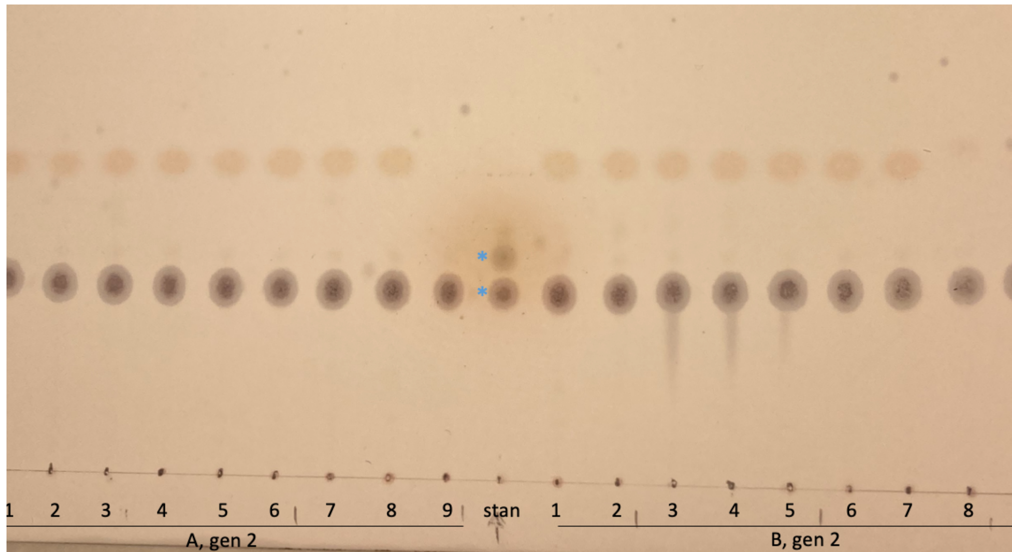

Figure 4. Thin layer chromatography of faecal samples in Falcon tubes (2nd gen, 5 days old). A and B denote the donors, while numbers 1-9 mark the dilution factor of the culture (10^{-1}-10^{-9}). The lane with cholesterol/ coprostanol standard is denoted 'stan' and the compounds marked in blue, cholesterol being the lower one.

Series II, performed in glass tubes, behaved very similarly to series I. The lower the dilution factor, the opaquer the cultures grew and the more the lipid precipitate dissolved. Kept for three generations and with cultures up to 10 days old, these incubations showed no putative coprostanol band in TLC, as exemplified in Figure 5.

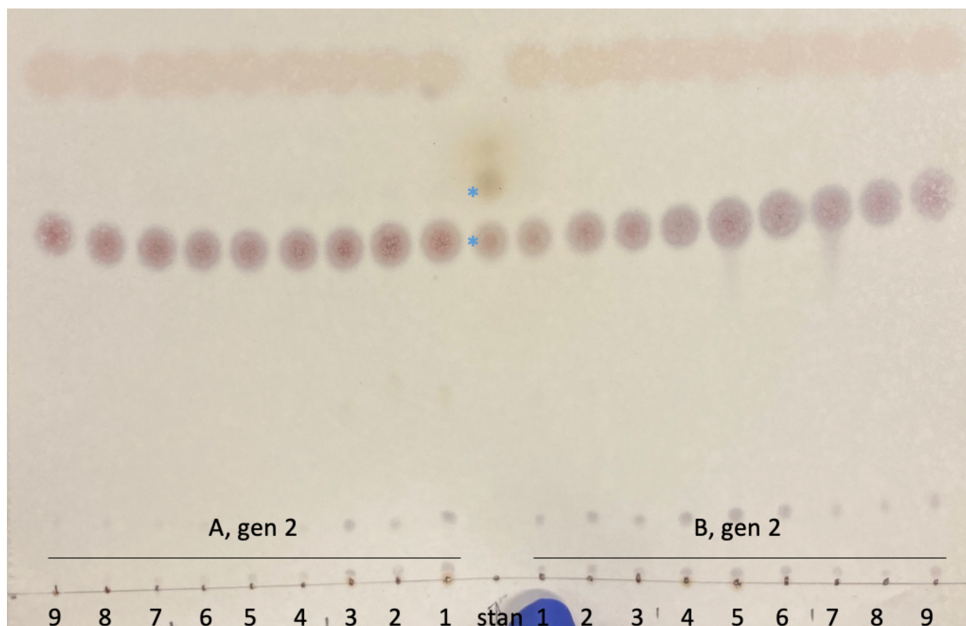

Figure 5. Thin layer chromatography of faecal samples in glass tubes (2nd gen, 4 days old). A and B denote the donors, while numbers 1-9 mark the dilution factor of the culture (10^{-1}-10^{-9}). The lane with cholesterol/ coprostanol standard is denoted 'stan' and the compounds marked in blue, cholesterol being the lower one.

4.3. Faecal cultures with fibres

As both conversion rate and *ismA* count are associated with methanogenesis, we hypothesised that the addition of fibres to faecal cultures may promote cholesterol conversion, as fibre degradation is the main hydrogen source in the gut. Series III, the cultures kept in Hungate

tubes, showed essentially the same pattern of turbidity as series I and II, with the exception of the 10^{-8} and 10^{-9} diluted cultures from neither donor growing opaque. Cultures diluted up to 10^{-6} showed gas-build up within two days of inoculation, possibly indicating hydrogen as a fermentation product. TLC results indicated a putative coprostanol band, exemplified in Figure 6, in all samples from both donors up to dilution 10^{-5}. However, GC-MS results showed no coprostanol in these samples, meaning that even the stronger putative band, seen in cultures with higher inoculum concentrations, was a confounder.

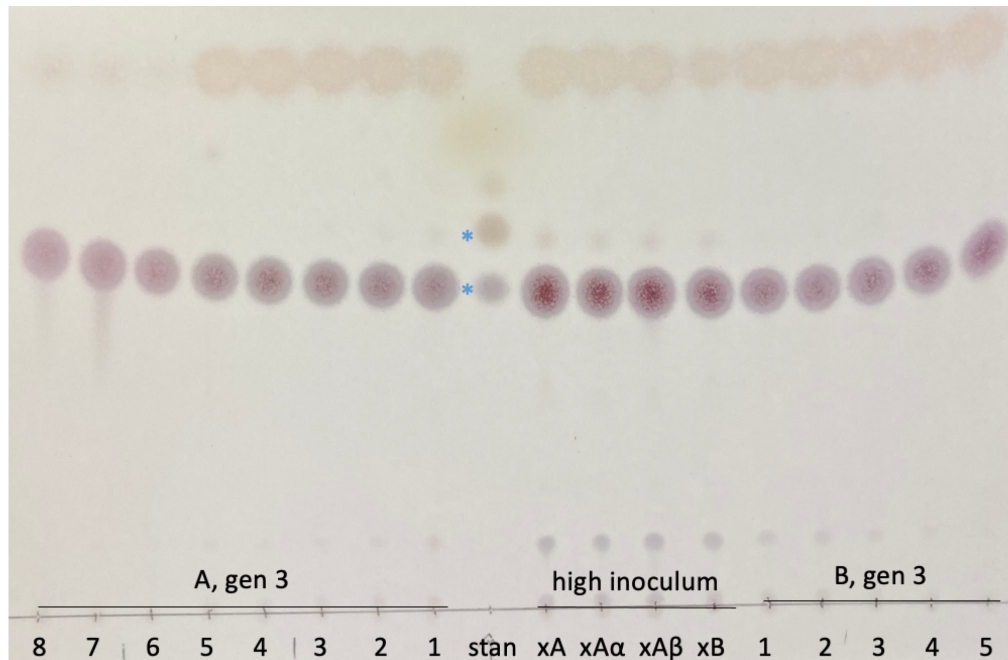

Figure 6. Thin layer chromatography of faecal samples in Hungate tubes (3rd gen, 4 days old). A and B denote the donors, while numbers 1-9 mark the dilution factor of the culture (10^{-1}-10^{-9}). An x before the sample ID indicates an inoculation concentration of 10%, where α and β separate the duplicates. The lane with cholesterol/ coprostanol standard is denoted 'stan' and the compounds marked in blue, cholesterol being the lower one.

Regarding the CBA plates inoculated from series III, three different colony morphologies were clearly observed: shiny, slightly domed, small circles; matte, flatter circles; and matte, slightly larger colonies with ragged edges. All were cream-coloured. No gas build-up nor putative coprostanol bands were observed in any of the soy BCM cultures in Hungate tubes inoculated from CBA colonies.

Next, compositionally complex fibres were added instead of defined ones. Initially cultivated in SHIRM medium, there were clear differences between cultures incubated with the different fibre preparations. The cultures with milled oat bran displayed gas-build up within a day, while the rye-based cultures followed on day 2, and the wheat-based cultures on day 3. Furthermore, the cultures with cholesterol added showed stronger pressurisation than those without, regardless of the fibre preparation. Seen in Figure 7, the SHIRM cultures with cholesterol all displayed a putative coprostanol band in TLC on day 1 after inoculation, as well as several other putative sterol bands not seen in earlier cultures. As expected, the controls without cholesterol did not display a putative coprostanol band nor a significant cholesterol band.

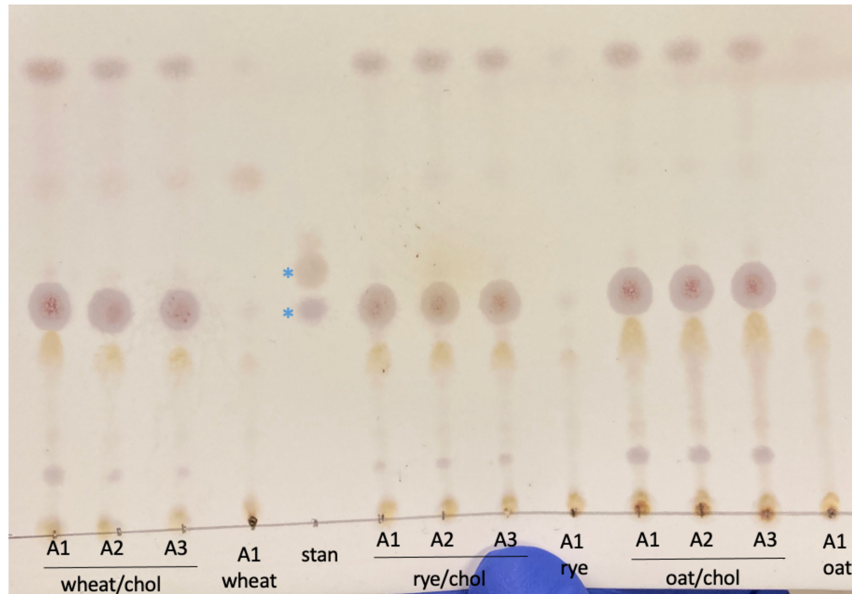

Figure 7. Thin layer chromatography of faecal samples with compositionally complex fibres (in SHIRM). A denotes the donor, while the number following it denotes the triplicate. The grain and 'chol' (i.e., cholesterol) mark which medium variation the culture was grown in. The lane with cholesterol/coprostanol standard is denoted 'stan' and the compounds marked in blue, cholesterol being the lower one.

The cholesterol-containing cultures which were transferred into soy BCM continued to display different behaviours based on which grain the corresponding SHIRM cultures contained. While all cultures became pressurised, the oat-based ones continued to show the highest gas build-up even in subsequent generations, and became pressurised at earlier timepoints. As exemplified in Figure 8, the apparent differences in sterol metabolism also carried over, with the oat-based cultures showing stronger non-cholesterol bands, in regard to both the putative coprostanol band and the unknown bands running lower than cholesterol. These differences faded over generations. All cultures, no matter what original grain, grew about equally opaque at comparable rates, and all lipid precipitates became loosened.

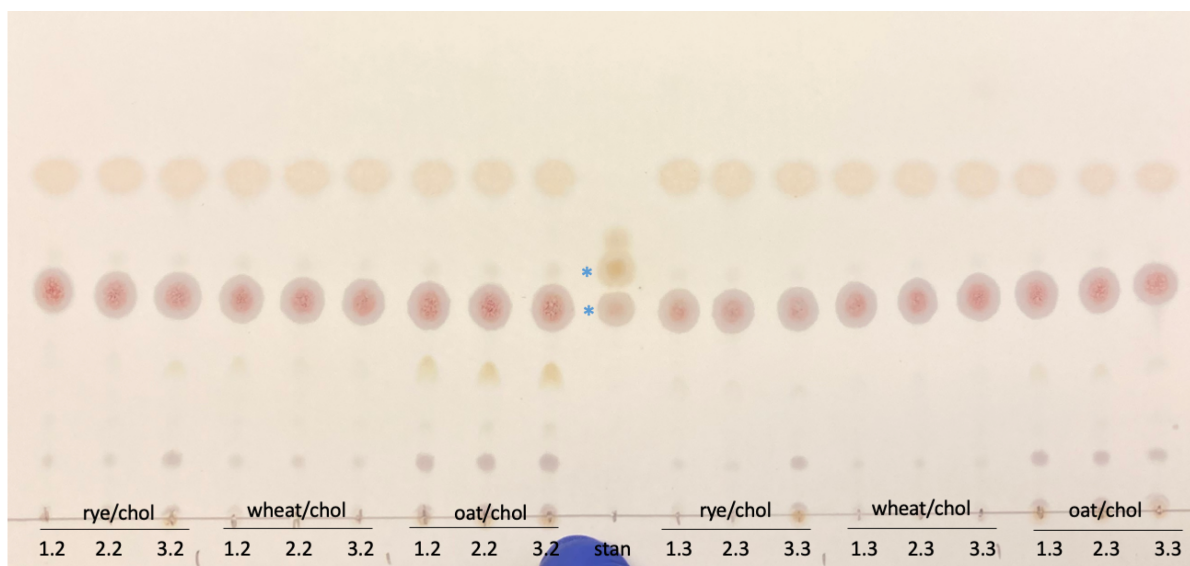

Figure 8. Thin layer chromatography of faecal samples with complex fibres (2nd and 3rd gen in soy BCM). All cultures originate from donor A. The first number of the sample ID marks the triplicate and the second the generation, while the grain and 'chol' (i.e., cholesterol) denote from which SHIRM variation the cultures stem. The lane with cholesterol/coprostanol standard is denoted 'stan' and the compounds marked in blue, cholesterol being the lower one.

Later GC-MS analysis showed that neither the SHIRM cultures nor soy BCM cultures of generations 1, 4, or 7 contained any coprostanol. That is, the putative coprostanol band seen in these cultures was in fact still a confounder and no conversion had been established.

4.3.1. 16S rRNA gene sequencing

Sequencing of the V4 variable region of the 16S rRNA gene was performed on the sample series with complex fibres in order to observe the effects of cholesterol and different fibres on the development and dynamics of community composition at taxa level. However, due to problems with the equipment, data is still pending.

5. Discussion

Overall, the results of this project reiterate on the previously established fastidiousness of coprostanoligenic bacteria. The loss of the *E. coprostanoligenes* type strain as well as the failure to establish cholesterol conversion in complex faecal cultures both affirm these culturability challenges. While bacterial isolation always is a stochastic process with a risk of failure, there are also several complicating factors that are worth closer consideration.

Firstly, the exact composition of the gas mix (85% N_2, 10% CO_2, and 5% H_2) in our anaerobic chamber has not been used in any previous successful isolation. Mostly, pure N_2 or gas mixtures with 10% H_2 have been used, as well as oxygen-depleted air. We have assumed the associations between *ismA* and methanogenesis as well as cellulosome modules to indicate a role of hydrogen in conversion, as fibre degradation produces H_2 and methanogenesis requires it as a substrate. If correct, this may mean that the partial hydrogen pressure is an important environmental factor for conversion. Cholesterol-to-coprostanol reduction does in fact correspond to an addition of hydrogen to the molecule, lending further credence to this hypothesis. Moreover, work on H_2S-producing bacteria was conducted in the Coy chamber concurrently. H_2S is a toxic gas that can, for example, modify protein structure [103], which might affect the atmosphere of the Coy chamber and subsequently dissolve in media stored within it, with potential but unknown consequences on the growth of our cultures.

This gas composition may have influenced loss of the *E. coprostanoligenes* strain, as may some other unknown factor(s) diverging our conditions from previous work. Presumably, however, the main factor was that *E. coprostanoligenes* is a fastidious and slow-growing bacterium thus easily overtaken by an opportunistic contaminant. Additionally, glycerol stocks of the strain were nonviable. This could in itself be a sign of fastidiousness in the form of extreme oxygen sensitivity, which would not be unexpected for a bacterium with a health-associated metabolism as increases in aerotolerant gut bacteria are typically linked to dysbiosis [104]. On the other hand, it would counter the previous characterisation of *E. coprostanoligenes* as slightly aerotolerant. As such, the failure of the stock is likely a consequence of the same fastidiousness and potentially suboptimal culturing conditions that led to the loss of the cultures. Notably, further stocks of *E. coprostanoligenes* were also unavailable from the culture collection ATCC, implying more overarching difficulties in propagating the bacteria.

Regarding the faecal cultures, the conversion rates of our sample donors are unconfirmed. While the TLC results consistently showed a putative coprostanol band in donor A, with a weaker one generally seen in donor B (exemplified in Figure S2), this was deemed insufficient confirmation due to the false positive repeatedly observed in faecal cultures. As such, GC-MS

was performed, with coprostanol observed in the sample from donor A. However, these were very suboptimal, water-dissolved samples, making the quantification untrustworthy. Due to the associations between conversion rate and both *ismA* count and abundance of coprostanoligenic bacteria, it is a reasonable assumption that it would be easier to capture such bacteria in samples from high converters. Regardless, the literature indicates a majority of healthy people to be high converters, with non-converters being a very small minority, wherefore neither of our donors converting at all seems unlikely. However, GC-MS confirmation is still needed: if both donors are low converters, the work as presented ought to be repeated with samples from a confirmed high converter. This would present its own challenges, as we due to the apparent fastidiousness of coprostanoligenic bacteria and the nonviability of the *E. coprostanoligenes* stock doubt the viability of frozen samples. Using fresh samples, this issue could be dealt with in two ways: one, incubate the sample and analyse its sterol content simultaneously, discarding the cultures if the conversion rate is unsatisfactory, or two, have confidence in the little evidence indicating that conversion rate is stable in an individual over time and confirm conversion with GC-MS before incubating a fresh sample.

In addition to these abovementioned general points at issue, series I of the faecal cultures may have failed due to lower total pressure or partial hydrogen pressure in the Coy chamber than necessary for conversion or due to some interaction with the plastic tubes, as indicated by the Falcon tubes turning pink after about 10 days of incubation. To address the latter possibility, series II was cultivated in glass tubes. To address the first possibility – and as series II proved futile – series III was cultivated in closed Hungate tubes and with the addition of fibre. This resulted in gas build-up in the less diluted cultures, presumably due to hydrogen formation from fibre degradation, but noting that headspace analysis was not conducted.

After those three attempts, compositionally complex fibres were added to the medium, the hypothesis remaining that hydrogen metabolism may be implicated in conversion. Additionally, the metagenomic analyses showing increased abundance of *ismA* in faecal communities with high proportions of *R. bromii* and high functional potential for cellulosome fibre degradation potentially link conversion to complex fibre metabolism. It should first be noted that the digestion and dialysis of the complex fibre preparation appear to have worked as intended. The TLC results (Figure S1) clearly show a decrease in simple sugars in the dialysed samples, though with some amounts still left, as well as traces of sugars in the water which the fibre preparations were dialysed against. The protocol of first incubating faecal samples in SHIRM additionally led to the establishment of gas production in soy BCM, which had not been seen before. Since the pressure build-up was stable even after 13 subcultures, it is unlikely to have been caused by fibre remnants, but rather by amino acid fermentation and/ or Stickland reactions [105]. Both are common when carbohydrates are depleted in the gut, as mimicked by BCM, and produce both H_2 and CO_2. Even without coprostanol formation in these cultures, establishing gas production in BCM cultures might be a step in the right direction for future isolation attempts based on our assumption that hydrogen metabolism is important for cholesterol conversion.

The TLC results indicate some form of sterol metabolism in essentially all faecal cultures, as they produced bands not seen in standards nor media blanks (exemplified in Figures 4-8). However, the potential products and the bacteria responsible for them are unknown. As the TLC visualisation according to our understanding relies on the formation of sterol acetates [106], the number of possible compounds is additionally limited to those that can be esterified. Importantly, the intermediates of the indirect conversion pathway do not have such a hydroxy group. As supported by the repeated failure of establishing a coprostanone standard, these could

not be detected with TLC (note that while the isomer used was not bioactive, the method should not be stereoselective). The SHIRM media (Figure 7) contain bile and possibly phytosterols – both of which should be detectable – with potential remnants of those in subsequent BCM cultures (Figure 8). However, cholesterol should be the only steroid substrate in all other relevant faecal cultures (a few cultures were transferred from brain-containing plates, but the risk of media carry-over was deemed minimal), and as the few cultures that were analysed with GC-MS at several timepoints did not decrease in cholesterol over time, there is an additional question of what substrate was used to form these TLC-visualised compounds. One method of investigation would be the use of radiolabelled cholesterol: compounds produced from cholesterol would then be radioactive, while confounders from some other substrate would not be. While this would not allow identification of the exact compounds observed in the TLC, it would however allow for much closer monitoring of cholesterol metabolism than was available during this project.

Intermediates were also indetectable with GC-MS as the derivatisation method used is not suitable for ketones. With neither method capable of detecting intermediates, we have no way of excluding or confirming the possibility of our cultures performing partway conversion, i.e., converting cholesterol to some intermediate(s) of the indirect pathway without completing the conversion to coprostanol. According to the literature, these intermediates make up an insignificant minority of neutral sterols in human faeces, the large majority being cholesterol and coprostanol. Still, little is known of the environmental conditions necessary for or promoting conversion, meaning we cannot exclude the possibility of intermediates accumulating under *in vitro* circumstances. Since no decrease in cholesterol concentration was observed in analysed cultures, it does however seem unlikely. Nonetheless, untargeted analysis or establishment of a chromatography method that can detect ketones would be the next step for further investigation of potential intermediate accumulation or other steroid products, possibly combined with the abovementioned radiolabelling.

Moreover, when *ismA* was expressed in *E. coli*, it was capable of oxidising both cholesterol to 5-cholesten-3-one (step 1 in Path I, Figure 1) and coprostanol to coprostanone (step 4 in reverse) [64]. We do not yet know what factors contribute to determining the direction of the reaction, nor what other enzymes might be involved in conversion. As such, there is a theoretical possibility of us having captured coprostanoligenic bacteria in such conditions that they metabolised any coprostanol formed. Cholesterol conversion is a secondary metabolism in *E. coprostanoligenes* and assumedly in other *ismA*+ strains (most earlier isolates required neutral sterols for growth, while no evaluation seemingly was done for *Bacteroides* sp. strain D8). Importantly, this means that we cannot say whether any of our experiments have managed to capture or culture any coprostanoligenic bacteria, only that we have not observed conversion. Assuming that at least donor A is a converter and that we at some point captured potentially coprostanoligenic bacteria, this would indicate that complete cholesterol-to-coprostanol conversion takes place only during very specific conditions difficult to mimic *in vitro*. In turn, this would provide a partial explanation to not only our isolation failure, but also the intense difficulties seen in isolating, culturing, and preserving coprostanoligenic strains across at least 70 years.

As of 2020, the discovery of *ismA* could allow for identification of potentially coprostanoligenic bacteria not detectable by sterol analysis, keeping in mind that all *ismA* homologues may not convert cholesterol. However, this approach would lead to other issues: it would not capture *ismA*- microbes capable of conversion, as hypothesised to exist, and as comprehensive metagenomic investigation does not function on the timeline required for bacterial culturing,

development of *ismA*-specific primers for qPCR or probes for protein blotting would be necessary, which in turn would require thorough investigation of homologues to ensure as complete coverage as possible. Additionally, identifying *ismA+* strains in this manner would provide no further information on how to induce cholesterol conversion *in vitro*, merely ensure a higher probability of coprostanoligenic bacteria being present in the sample.

Metagenomic information may also be integral for future isolation attempts. We hypothesised that the associations established between *ismA*, methanogenesis, and complex fibre degradation would via the addition of fibres promoting hydrogen production allow for establishing cholesterol conversion where the previously successful media had failed, but this could – so far – not be confirmed. Nonetheless, these associations and future metagenomic information could be important to more closely identify the environmental niche occupied by coprostanoligenic bacteria and the conditions needed for cholesterol conversion. If we are correct in assuming our donors are converters, we ostensibly cannot yet establish those selective conditions, making it even more difficult to capture and cultivate these low-abundant bacteria.

Similarly, the repeated loss of previous isolates may indicate that suboptimal culturing conditions have been used overall – conditions in which the bacteria can survive for some generations, but not conditions in which they can thrive for longer. The growth and cholesterol conversion of both *E. coprostanoligenes* and other coprostanoligenic bacteria might depend on small details difficult to identify from a larger picture. For example, we hypothesised that the difference between egg- and soy-derived L-α-phosphatidylcholine might be such a factor. The lower purity soy-based lecithin contains e.g. sphingomyelin not found in the egg-based one, but as conversion was observed in the *E. coprostanoligenes* cultures grown in egg BCM this difference does not seem crucial at least in the short-term. Furthermore, the different media preferences shown by *E. coprostanoligenes* and *Bacteroides* sp. strain D8 may indicate that different coprostanoligenic strains require different conditions to grow and/ or convert cholesterol. This would also obstruct both metagenomic and culturing studies into the necessary conditions, as potentially contradictory results would make it difficult to establish a clear, uniform picture.

While our failure to isolate coprostanoligenic bacteria across four media, three tube types and 1-10% inoculum is unfortunate, it is not particularly surprising. This project was intended as a part of the larger ongoing effort to close the 70% culturability gap between metagenomic identification of gut microbes and the cultivation and metabolic characterisation of those same fastidious bacteria. Even in this context – and keeping in mind that stochastic factors always pose a risk in isolation attempts – there are indications that coprostanoligenic bacteria count amongst the more fastidious gut microbes. Firstly, conversion is established relatively late in children, which is generally seen for other highly difficult-to-cultivate microbes such as the genera *Methanobrevibacter* and *Christensenella* [107]. Secondly, with the culturability gap in mind, microbiota research is not usually discussed on the timescale that applies to coprostanoligenic bacteria. For comparison, the genus *Christensenella* was discovered only 11 years ago [108], while there have been consistent attempts of isolating coprostanoligenic bacteria for at least 70 years. The team behind the isolation of *E. coprostanoligenes* conducted 'hundreds of trials' attempting to isolate cholesterol-converting bacteria from innumerable sources [109] with only one successful characterisation. All the same, with metagenomic studies starting to reveal health associations of coprostanoligenic bacteria and their metabolism, we remain interested in harnessing this functional potential in the form of e.g. next-generation probiotics or the establishment of prebiotics supporting cholesterol conversion. For such research to ever be possible, isolation attempts are necessary.

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Supplementary data

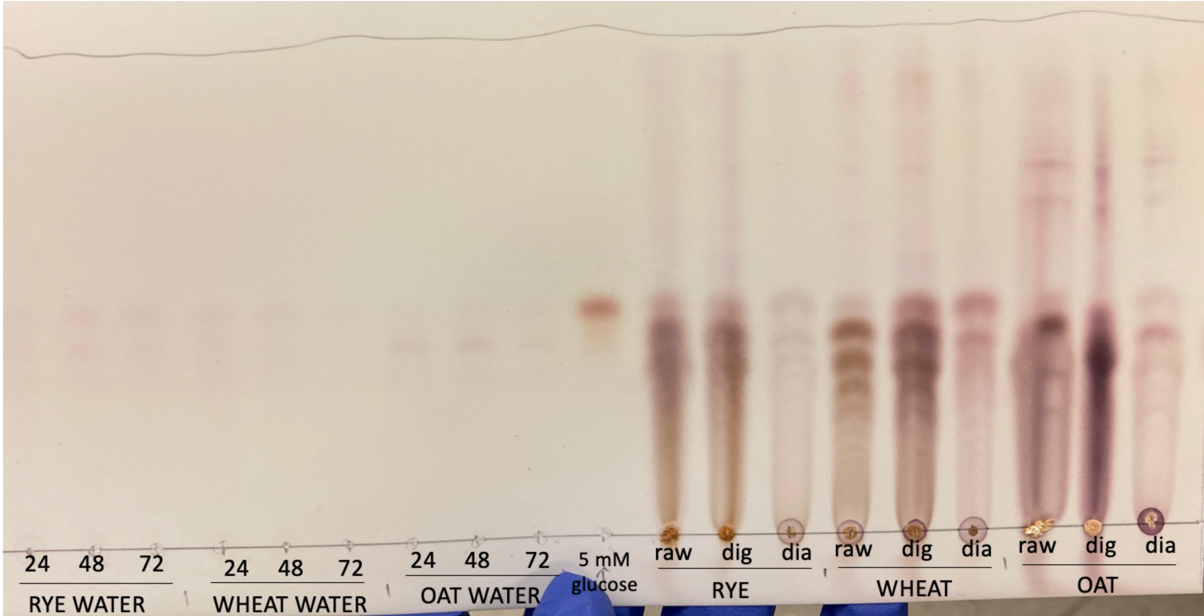

Figure S1. Polysaccharide thin layer chromatography of fibre samples. Lanes to the right of the glucose standard contain samples of milled rye, wheat, and oat bran, dissolved in water (raw), amylase-digested (dig), and dialysed against water for 72 h (dia). Lanes to the left of the standard contain water which was dialysed against, marked by which fibre was dialysed and at what timepoint (h) the sample was taken.

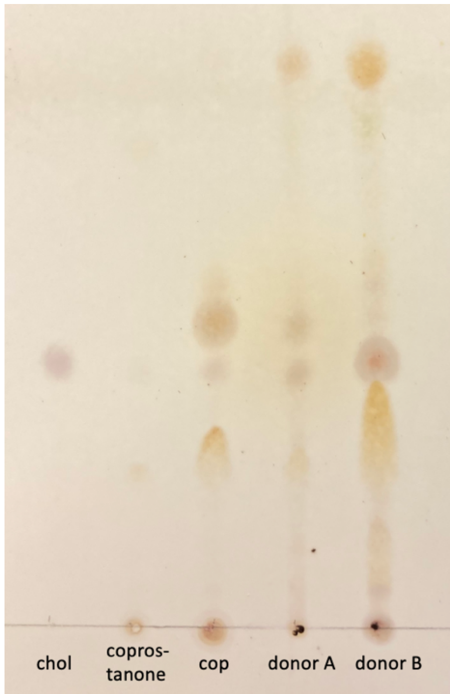

Figure S2. Thin layer chromatography of faecal samples for series I. Standards are denoted 'chol' (cholesterol), 'coprostanone', and 'cop' (coprostanol), while A and B are faecal samples from the two donors.

Table S1. Components of vitamin mix I for SHIRM medium.

Ingredient (manufacturer)	Amount per 100 ml
Biotin (Sigma-Aldrich)	1 mg
Cobalamin (Sigma-Aldrich)	1 mg

p-Aminobenzoic acid (Sigma-Aldrich)	3 mg
Folic acid (Sigma-Aldrich)	5 mg
Pyridoxamine (Sigma-Aldrich)	15 mg

Table S2. Components of vitamin mix II for SHIRM medium.

Ingredient (manufacturer)	**Amount per 100 ml**
Thiamine (Sigma-Aldrich)	5 mg
Riboflavin (Sigma-Aldrich)	5 mg
Mucin, type II (from porcine stomach, Sigma-Aldrich)	5 g
Agar (BD Difco)	2 g

Table S3. Mass-to-charge ratio of selected ions in sterol GC-MS.

Ion	**Mass-to-charge ratio (*m/z*)**
Cholesterol, target	329
Cholesterol, qualifier	368
Coprostanol, target	370
Coprostanol, qualifier	215
Cholesterol-d7, target	336
Cholesterol-d7, qualifier	375

Table S4. Running parameters for sterol GC-MS analsysis.

Parameter	**Value**
Flow	2 ml/min
Inlet temperature	280˚C
Auxiliary heater	280˚C
Mode	Split 50:1
Solvent delay	3 min
Gradient	200˚C, 30 s; 15˚C/min; 325˚C held 10 min
Total runtime	19 min

Table S5. Primer sequences for amplification of whole-length 16S rRNA gene.

Name	**Sequence**
27-F (forward)	5’-GTTTGATCCTGGCTCAG–3’
1492-R (reverse)	5’-CGGCTACCTTGTTACGAC-3’