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Isolation and characterization of cholesterol degrading microbes from the human gut

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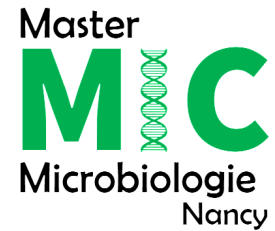
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***Isolation and characterization of cholesterol
degrading microbes from the human gut***

Thesis presented by

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Internship January 10th to July 8th

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Abbreviations

CMESS: Center for Microbiology and Environmental Systems Science

DOME: Division Of Microbial Ecology

VLDL: Very low density lipoprotein

LDL: Low density lipoprotein

IDL: Intermediate density lipoprotein

HDL: High density lipoprotein

ACVD: Atherosclerotic cardiovascular diseases

WHO: World Health Organization

BONCAT: Bio-orthogonal non-canonical amino acid tagging

AHA: Azidohomoalanine

Cy5: Sulfo-Cyanine5

PCR: Polymerase chain reaction

BLAST: Basic Local Alignment Search Tool

FACS: Fluorescence-Activated Cell Sorting

FISH: Fluorescence In Situ Hybridization

GC TOF MS: Gas chromatography time of flight mass spectrometry

457chl medium: DSMZ 457 medium + 2 mM cholesterol

BHI: Brain heart infusion

PBS: Phosphate-buffered saline

chl: cholesterol amended samples

glc: glucose amended samples

Abc: abiotic samples, without fecal slurry

NoAmd: no amendment samples

NoAHA: no Azidohomoalanine samples

DSP: Dithio-bis succinimidyl propionate

DAPI: 4,6-Diamidino-2-phenylindole

MTBE: Methyl tert-butyl ether

I. Introduction

In the gut microbiota, microorganisms compete with each other in the use of various substrates, or on the contrary, cooperate to utilize them. These interactions are influenced by the modification of the intestinal environment at the physical and chemical level. Moreover, these modifications can impact the composition of the intestinal microbiota which can be associated with diseases, such as inflammatory bowel disease, cancer and intestinal infections.

These observations prove the link between diet and health that has long been established. The consumption of specific dietary products can impact parameters of the human body such as blood pressure, blood sugar level or blood cholesterol level (Lamichhane et al. 2021). Cholesterol is a lipid biosynthesized by all animal cells as a structural component. It is the precursor of e.g., steroid hormones, vitamin D, bile acids or fat-soluble vitamins in the body. It is produced by every animal cell and can have several roles such as facilitating the digestion of fats. The quantity of absorbed dietary cholesterol is therefore correlated with the amount of consumed animal products. The blood cholesterol levels are influenced by the consumption of dietary cholesterol as well as endogenous cholesterol produced by human cells. The concentration of circulating endogenous cholesterol is controlled by the liver, the main regulator of cholesterol production due to its capacity to transform it (Kriaa et al. 2019). The cholesterol production rate of the liver depends on the amount of dietary cholesterol ingested. The liver, thus, has a reserve of cholesterol which is packed into lipoproteins along triglycerides. When needed, the lipids are then disseminated in every part of the body through the bloodstream (Kriaa et al. 2019). In the bloodstream, cholesterol circulates in the form of lipoproteins. In the human body, they are present in four different forms: The very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL) (Kumar et al. 2012). The cholesterol secreted by the liver is also transported to the small intestine via the biliary system in its free form or, more often, as bile acids. There, the endogenous cholesterol from the liver and the ingested dietary cholesterol are combined. A small fraction of these bile acids will be removed from the body as a waste product, but most will be returned to the liver and used again for digestion. The enterocytes reabsorb remaining cholesterol and

transport it back to the liver (Schade, Shey, and Eaton 2020). This process is called enterohepatic circulation (Figure 1). During these steps, the gut microbiota gets in contact with cholesterol, giving it the opportunity to modify it.

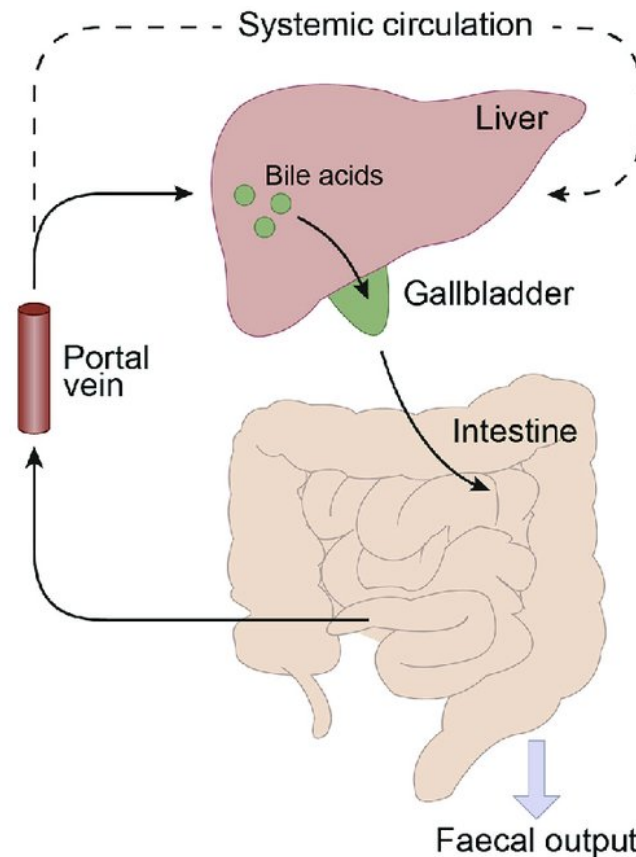


Figure 1: Figure from Mertens et al. 2017, Enterohepatic circulation from the liver is the main regulator of endogenous cholesterol production. Cholesterol is secreted into the small intestine via the biliary system. In the small intestine, the cholesterol supports the digestion of fats and thereby also gets in touch with the intestinal microbiota. Enterocytes (re-)absorb cholesterol and transport it back to the liver.

Despite the body's complex regulatory mechanisms, blood cholesterol level can be elevated above physiological levels, causing high cholesterol, also called hypercholesterolemia. It is defined as blood cholesterol levels above 6.2 mmol per L (Durrington 2003). The cause of hypercholesterolemia is a combination of genetic and environmental factors like weight, stress or diet. Thus, increased cholesterol levels can e.g. occur due to conditions such as anorexia, hypothyroidism, type 2 diabetes, familial hypercholesterolemia (caused by a genetic mutation in the LDL cholesterol receptor gene) (Akioyamen et al. 2017). According to the lipid

hypothesis, a higher blood cholesterol level could be linked to atherosclerosis (Ahrens 1976). This hypothesis, acknowledged by most of the medical community, postulates a link between blood cholesterol levels and cardiovascular diseases. These diseases can be caused by atherosclerosis, which manifests by the narrowing of the artery due to an abnormal accumulation of material in the inner layer of an artery wall, also called atheromatous plaque. This accumulation of plaque can for instance result in a heart attack. In the same way, the obstruction of an artery supplying the brain can cause a stroke. Knowing this cause-and-effect relationship, the rise of hypercholesterolemia is a world health concern. Indeed, atherosclerotic cardiovascular diseases (ACVD) (Feingold 2000; Kazemian et al. 2020) are predicted to be the leading cause of death in the world by 2030 according to the World Health Organization (WHO) (Villette et al. 2020). The conversion of cholesterol before its re-absorption could be a solution to limit the increase of blood cholesterol levels and prevent ACVDs in advanced cases of hypercholesterolemia.

Reactions involving cholesterol take place during digestion in the small intestine. Indeed, both endogenous and dietary cholesterol are present in the intestine. It offers the opportunity for the gut microbiota to come into play with circulating cholesterol. There are different mechanisms by which the gut microbiota can influence gut lipid metabolism (Bourgin et al. 2020; Villette et al. 2020). The microbiota produces small metabolites that influence host metabolic pathways and act as signaling molecules (Gérard 2013; Allayee and Hazen 2015). For example, short-chain fatty acids affect the regulation of cholesterol synthesis or the production of bile acids (Ishimwe et al. 2015; Zhang et al. 2020). Furthermore, bacteria with bile salt hydrolase activity can modify bile salts into secondary bile salts which can act as signaling molecules (Lamichhane et al. 2021). Due to the decreased reabsorption efficiency of secondary bile acids in the small intestine, they are mainly removed from the enterohepatic recycling and directly defecated, thereby decreasing the general cholesterol levels in the body (Bourgin et al. 2020; Horáčková, Plocková, and Demnerová 2018). Additionally, there are several mechanisms for microbial cholesterol entrapment such as its binding onto the bacteria's cellular surface (Kumar et al. 2012; Kriaa et al. 2019), its incorporation into the bacteria's cell membrane (Lye, Rusul, and Liong 2010), the precipitation of cholesterol or the conversion of cholesterol to coprostanol (Kriaa et al. 2019; Lamichhane et al. 2021).

The conversion of cholesterol to coprostanol is an indirect reduction mechanism following a three-step process (Figure 2). Cholesterol (1) is first oxidized to cholestenone (2). Then the $\Delta_{4,5}$ double bond of cholestenone (2) is further reduced to form coprostanone (3), from which coprostanol (4) is produced by oxidation (Kenny et al. 2020; Björkhem and Gustafsson 1971) The absorption of coprostanol by the intestinal cells is minimal and most of the product is excreted. As the amount of absorbable cholesterol reaching the bloodstream is reduced, this conversion is of particular interest (Horáčková, Plocková, and Demnerová 2018).

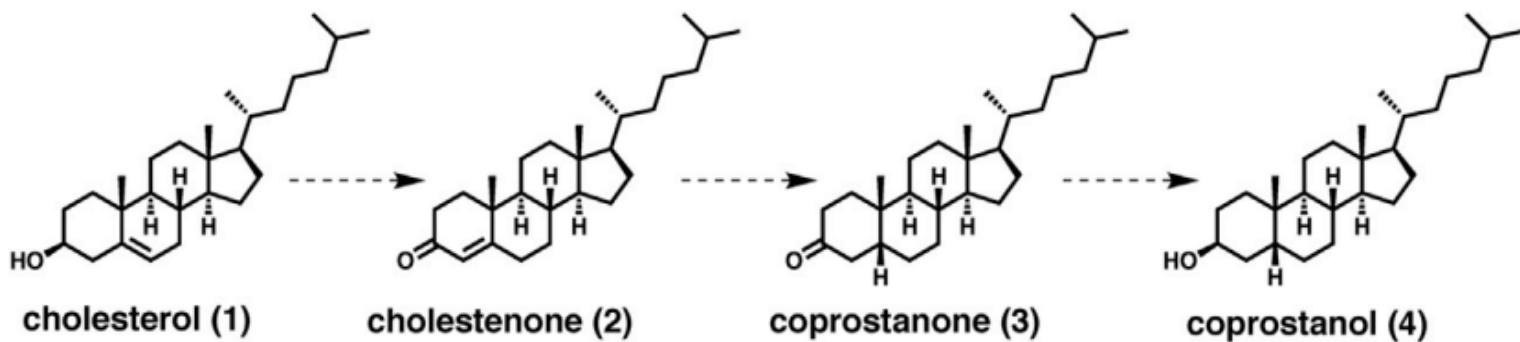


Figure 2: Microbial conversion of cholesterol (1) to coprostanol (4) with the intermediates cholestenone (2) and coprostanone (3) (Figure adapted from Kenny et al. 2020).

Although this reaction has already been studied for decades (Björkhem and Gustafsson 1971), a 2020 study by Kenny et al. identified a microbial key enzyme in this conversion, catalyzing the first and last step of the conversion from cholesterol to coprostanol (Kenny et al. 2020). In this study, the researchers integrated from previous studies available metagenomic and metabolomics data on cholesterol altering microbes from other environments. In the first step, they tried to find putative cholesterol metabolizing enzymes inside the human gut microbiota. They used a multi-disciplinary analysis consisting of the large-scale integration of general microbiome data as well as metabolomics of human stool and explored the genomes of coprostanol-producing microorganisms. A biochemical analysis was carried out to prioritize the enzymes with the best catalytic capacities for the metabolization of cholesterol. In a next step, the identified enzymes were characterized with regards to their capability to oxidize cholesterol to cholestenone. With this, Kenny et al. were able to link coprostanol formation from cholesterol to a group of cholesterol dehydrogenases, called IsmA. They showed that the microorganisms encoding the

ismA gene may belong to a phylogenetic clade of uncultured microorganisms found in geographically diverse human cohorts in the neighbourhood of Clostridium cluster IV. Kenny et al. also showed that individuals bearing ismA coding bacteria presented lower fecal cholesterol levels and lower serum total cholesterol compared to individuals that did not show the presence of these bacteria. Although there are already known and isolated bacteria interfering with the metabolism of cholesterol (e.g. due to bile salt hydrolase activity and cholesterol scavenging) no cultivated isolate of the human gut microbiome carrying ismA exists at the moment. The cultivation of such an isolate could eventually lead to the development of a probiotic with cholesterol lowering effects.

Analyzing the community present in fecal samples regarding their bacterial composition especially bacteria that can use cholesterol for growth could also help this goal. A method called BONCAT (Bio Orthogonal Non Canonical Amino-acid Tagging) (Dieterich et al. 2006) was developed in order to recognize metabolically-active cells. The principle of BONCAT is based on the higher sensitivity of small bioorthogonal chemoselective groups. In a first step, the recently translated proteins are tagged with a modified methionine-derived azide-bearing artificial amino acid azidohomoalanine (AHA). While azides are non-toxic and stable in the biological systems, they are not biologically synthesized in most animal systems (the only exception being unicellular cultures). In the second step, the AHA-containing proteins are tagged with a reactive dye such as Sulfo-Cyanine5 (Cy5) thanks to a copper-catalyzed click labeling. This incubation could lead to the quantification and identification of active cells in samples incubated with cholesterol. In this project, the BONCAT approach is a method that can help identify bacteria that are activated during incubation with cholesterol in a faster way than doing single-colony isolations.

Those hypotheses are the basis of my internship project. The project was first started at DOME in 2021 by the master student Florentina Drochter. During her internship, she designed a suitable medium in order to cultivate cholesterol-utilizing bacteria. She also cultured and analyzed single colonies from complex human fecal samples with a 16S rRNA gene-targeted PCR (Polymerase Chain Reaction), Sanger sequencing and subsequent BLAST (Basic Local Alignment Search Tool). She incubated human fecal slurries containing cholesterol, glucose or no additional carbon source. The incubations were additionally amended with AHA for later

BONCAT-FACS (Fluorescence-Activated Cell Sorting) and sampled 5 times within 24 hours in order to create a complete sample library.

As part of my masters thesis, I was given the opportunity to continue this project with the goal to isolate and characterize cholesterol-utilizing bacteria from the human gut. My project contained three work packages. The entry phase focused on the isolation of cholesterol-utilizing bacteria from human stool samples and aimed to collect yet uncultured bacterial isolates. The entry phase was followed by a community analysis step. This phase aimed to identify metabolically-active bacteria in cholesterol-amended fecal microcosms. To do so, metabolically-active cells in the culture were labeled using BONCAT. This labeling was followed by the characterization of active cells with BONCAT-FISH (Fluorescence In Situ Hybridization) and the sorting of the metabolically-active cells using FACS. The community analysis of the human fecal microcosms led to the isolation of one microorganism from the human gut that grows on a cholesterol amended media. The third phase consisted in the study of cholesterol and coprostanol content of the culture supernatants of the BONCAT incubations.

By answering the questions inherent to this project, it could be possible to have a better understanding of the reaction transforming cholesterol to coprostanol and potentially promote it. This transformation could influence the blood cholesterol levels by lowering the reabsorption of cholesterol and coprostanol. This project is therefore in line with the efforts to combat hypercholesterolemia and by extension atherosclerotic cardiovascular diseases.

For this project I had the chance to intern at the Center for Microbiology and Environmental Systems Science (CMESS) which is part of the scientific organizational units of the University of Vienna (Figure 3). The center is composed of two departments, the Department of Microbiology and Ecosystem Science as well as the Department of Environmental Geosciences. These departments are divided into 4 divisions, CUBE (focusing on computational systems biology), EDGE (focusing on environmental geosciences), TER (focusing on terrestrial ecosystems) and DOME (focusing on microbial ecology). In total, the Center consists of 14 groups. The two scientific fields on which the CMESS focuses on, microbiology and environmental

systems, are two major challenges of our time. The Center offers many points of collaboration in those fields, which results in the transdisciplinarity of the research. The Division of Microbial Ecology (DOME) covers mainly three research areas: biodiversity, symbiotic association as well as animal and human microbiota. The biodiversity research at DOME focuses on uncovering evolutionary history and understanding the function of microbes catalyzing the global cycles of carbon, sulfur, and nitrogen. The research on symbiotic association concentrates on the evolution of intracellular symbiotic associations as well as their mechanisms of interaction. Their main focus is amoebae and their bacterial endosymbionts. The animal and human microbiota research concentrates mainly on the impact of how host diets impact physiological interactions between intestinal microorganisms.

Centre for Microbiology and
Environmental Systems Science

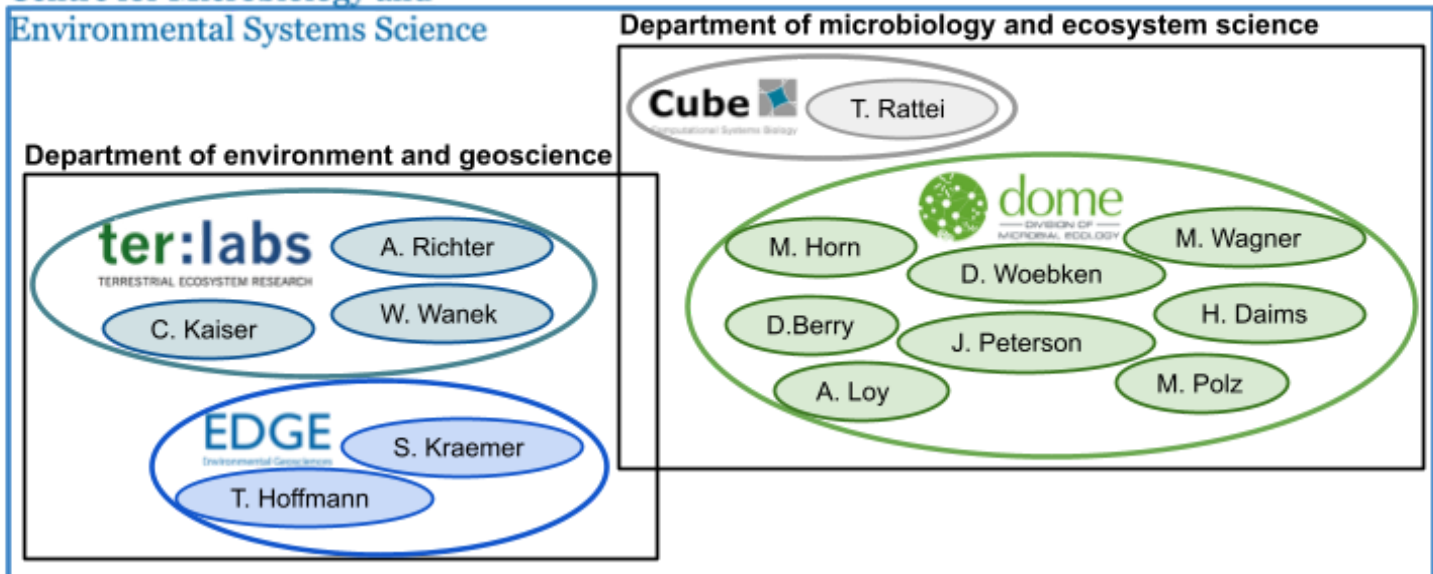


Figure 3: Organization of the Center for Microbiology and Environmental Systems Science (CMESS), including the four divisions (CUBE, EDGE, TER and DOME) with the respective group leaders.

One of the teams of the DOME is the Loy team that focuses on two topics: a part of the team studies the role of sulfur and its metabolization in microorganisms. These studies concentrate in particular on the ecology and biogeography of microorganisms of the sulfur cycle in globally relevant anoxic ecosystems such as the terrestrial wetlands and the ocean floor. The other part of the team investigates

the topic of human and animal microbiota. Their research relies on the observation that the dysbiosis of the gut microbiota can impact the health of the host. Two questions result from this observation: How can host diets lead to the modification of the microbial community and what are the interactions between intestinal microorganisms in health and diseases.

II. Material and methods

All steps involving living fecal bacteria were carried out in an anaerobic tent (Coy Laboratory Products, IncGrass Lake, US) with an atmosphere of 85 % nitrogen (N₂), 10 % carbon dioxide (CO₂) and 5 % hydrogen (H₂).

1. Media

a. *DSMZ 457 medium (+ 2mM cholesterol)*

DSMZ 457 medium + 2 mM cholesterol (Braunschweig, Germany) (457chl medium) was designed to selectively enhance the growth of cholesterol-utilizing bacteria from the human gut and, thus, supplemented with 2mM cholesterol as the sole carbon source. This media was prepared in liquid and solid form (precise composition of the Media in appendix 1).

b. *BHI medium*

Brain Heart Infusion (BHI) medium was prepared from a dehydrated premix (Thermo Scientific, Brain Heart Infusion Broth, Dehydrated). This rich medium was used to grow isolates more efficiently than in the specific selective media. The medium was prepared in liquid and solid form.

2. Plated samples from fresh and glycerol stock fecal samples

In order to isolate new cultures, the plating of fecal samples was performed twice in total, the first time was from fresh fecal samples. The samples were suspended in a bicarbonate buffer (3.8 g of feces per 100 ml). After settling of particles for 5 minutes, 100 µl of the 3.8 % fecal slurry was plated out on 457chl medium agar plates (according to Fernández de Las Heras et al. 2009). The second culturing was performed from glycerol stocks with validated monocultures generated prior to this project. 100 µl of the glycerol stock was plated out on 457chl medium agar plates (according to Fernández de Las Heras et al. 2009).

a. *Single colony identification*

After plating of fecal slurry, growth on plates was followed closely. For single colony identification using the 16S rRNA gene, PCR reactions (composition of PCR reaction in appendix 2) were prepared in a PCR workstation. Single colonies were then picked, streaked out on fresh 457chl medium agar plates and furthermore added to the PCR reaction with the 1492r (5'-GGTTACCTTGTTACGACTT-3') and

616v (5'-AGAGTTTGATYMTGGCTC-3') primers. Each PCR included a negative control of PCR water and a positive control containing pre-validated 16S rRNA gene DNA. DNA fragments were amplified in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Feldkirchen, Germany) (settings in appendix 3). Successful amplification of 16S rRNA gene fragments was confirmed using gel electrophoresis. A 1% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) was prepared using a TBE buffer (Biotium, Fremont, US). Bands were visualized by adding 0.016 μ L per mL GelRed (Biotium, Fremont, US) to the cooled liquid gel. Validated PCR products were purified using the innuPREP PCRpure Kit (Analytik Jena GmbH, Jena, Germany). DNA concentrations of the purified PCR reactions were then determined using a NanoDrop ND-1000 Spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Finally, the samples were submitted for Sanger sequencing with Microsynth (Microsynth AG, Balgach, Swiss). Generated sequences were analyzed using NCBI BLAST (Basic Local Alignment Search Tool) to identify the isolates.

b. Liquid cultures of isolates

Validated single colonies were cultivated in 10 mL BHI medium. Culture growth was followed hourly using an optical density measurement (Biochrom Ultrospec 10). Once the culture reached the exponential phase, 1 mL of culture was added to 14 mL of liquid 457chl medium. After inoculation, optical density was measured and 1mL of culture was sampled daily and stored at -20 °C for subsequent metabolite analysis.

c. Long-term storage of the validated isolates

Validated isolates were anaerobically stored at -80 °C in 20 % glycerol. Identity and viability of the isolate were again checked by re-culturing 100 μ L of the glycerol stock sample on a fresh BHI plate. After another identity check using 16S rRNA gene PCR, fresh glycerol stocks were prepared and deposited in the group's culture collection.

3. Bioorthogonal Non Canonical Amino acid Tagging (BONCAT)

Prior to the start of this project, a BONCAT incubation was performed. For this, fresh fecal samples were collected from volunteers who had not received antibiotics in the three months before sample collection and did not suffer from any

digestive disease. The BONCAT samples (sample library: table appendix 4) were obtained from fresh fecal slurry composed of a mixture of Phosphate-buffered saline (PBS) and fecal matter (1mL of 10% fecal slurry in 400µl PBS). Cholesterol samples (chl) were prepared with 100 µM cholesterol in ethanol as the only carbon source added. The samples were tagged with 50 µM AHA. This tagging was performed in order to locate the active cells in which recently translated proteins integrated the AHA. Those AHA bearing cells would then be tagged with a Cy5 probe thanks to a copper-catalyzed click labeling.

The following controls were also prepared: a glucose (glc) positive control containing 100 µM glucose as the only carbon source and 50 µM AHA, an abiotic control (Abc) without fecal slurry but with 100 µM glucose and 50 µM AHA, a negative control (NoAmd) without any carbon source added but with 50 µM AHA, and a negative control without AHA (NoAHA) and with 100 µM glucose as a carbon source, making the BONCAT click reaction impossible. Samples were taken 0, 3, 6, 10 and 24 hours after the start of the incubation. Samples underwent reversible dithio-bis succinimidyl propionate (DSP) fixation according to standard protocol (Xiang et al. 2004) and were stored at -20 °C.

BONCAT on slide: To perform BONCAT-FISH, the click reaction was performed on a slide Hatzenpichler et al (2016) . For this analysis, BONCAT samples (chl), positive control (glc), negative no amendment control (NoAmd) and negative no AHA control (NoAHA) from three different timepoints (0, 6 and 24 hours) were used. 20 µL of the fixed BONCAT samples were added on a microscope glass slide with 10 reaction wells (Marinefield, 10 x 6 mm Ø numbered), dried and dehydrated with an increasing ethanol series (50, 80 and 100%). The Cy5 probe was diluted 1: 20 in Ultrapure water in order to perform a copper-catalyzed click labeling of the AHA bearing cells. To do so, 1 µL of the diluted probe was added to every well. The slide was hybridized for 3 hours with a 10% hybridization buffer [180 µL 5M NaCl, 20 µL 1 M Tris/HCL, 699 µL Ultrapure water, 100 µL Formaldehyde and 1 µL of 10% SDS] and then washed in a 10% washing buffer [4.5 µL 5M NaCl, 1 µL 1M Tris/HCL and 44.5 µL Ultrapure water] incubated in a 48 °C water bath for 10 minutes. Slides were then dipped in ice-cold distilled water and dried with compressed air. Subsequently, the slides were stained with DAPI, a general bacterial dye used as a positive control. To do so, 15 µL of 10 µg per mL DAPI in 1x Ultrapure water was added to each well. After 5 minutes, the DAPI solution was removed and the slide was dipped into

ice-cold Ultrapure water. The slides were then mounted with drops of an antifadent (citifluor) and a cover slip.

BONCAT in solution: In order to perform BONCAT-FACS, click reactions were performed in solution. For this analysis, BONCAT samples (chl), positive control (glc), and negative no amendment control (NoAmd) from three different timepoints (0, 6 and 24 hours) were used. A copper-catalyzed click labeling of the chemically fixed cells was performed. To do so, 200 μ L of each sample was dyed with Syto9 and Cy5 in order to stain all bacterial cells and specifically metabolically-active cells, respectively. Four different controls were also prepared in order to set the gating (Mistry and Chou 2019).

4. Fluorescent microscopy:

Slides were analyzed using fluorescent microscopy with thunder imaging technology (Leica THUNDER Imager Live Cell & 3D Assay) to optimize the images. Samples were examined with two different incident lights. The first one was used to light up the Cy5 probe linked to the AHA (651 nm), in order to see the metabolically-active cells. The second one led to the visualization of the DAPI stained cells (359 nm), acting as a general positive control to attest the presence of bacterias in the samples.

5. DAIME analysis

The BONCAT click-reaction allowed the visualization of metabolically-active cells in our incubations. The time points of 0, 6 and 24 hour of incubation were selected in order to have an overview of the activity of the cells through time. 20 pictures were taken from all of the samples and controls to have a representative view of the entire sample. The pictures were then analyzed with the DAIME software (Daims, Lückner, and Wagner 2006). DAIME is a digital image analysis tool used in microbial ecology. It is used to analyze 2D and 3D images to quantify microbial populations as well as to analyze the structure-function relationships in microbial communities. In this project, the software was used to quantify the proportion of metabolically-active cells in the selected samples. To do so, the cell count of each signal was estimated and were both submitted to the segmentation tool. The tool relies on the brightness of the different bacteria in the pictures and counts every dot of light. Then, the biovolume fraction tool of the software was used to compare the two segmentation counts in the two groups of pictures, giving back the

metabolically-active cells to general cells ratio in percentage. Subsequent data visualization was performed using Ggplot2 (v3.3.3 Villanueva and Chen 2019).

6. Fluorescence-Activated Cell Sorting (FACS)

The analysis of the samples was performed on anhe BD FACSMelody cell Sorter and BD FACSCorus software (BD Bioscience).

Gating: Gating is the first step of FACS analysis that is used to locate the signal of the two dyes and to minimize background activity. Four controls were treated using a classical gating protocol (Mistry and Chou 2019). With these controls, the location of the Syto9 and Cy5 signal was selected in the two scatter plots.

Cell sorting: The gating step allowed the separation of the metabolically-active cells which are Cy5 and Syto9 positive from the non metabolically-active cells which are Cy5 negative and Syto9 positive. 50000 cells were sorted in each sample.

Conservation of the samples: 750 μ L DNA extraction buffer [10mM Tris hydrochloride 1mM Ethylenediaminetetraacetic acid] was added to the sorted samples. Samples were subsequently stored at -80 °C.

4. Gas Chromatography Time Of Flight Mass Spectrometry (GC TOF MS)

a. Coprostanol and cholesterol standards

The isolates were characterized regarding their capacity to convert cholesterol to coprostanol. To do so, standards of 10 mg per ml of cholesterol and coprostanol were prepared in chloroform. They were later diluted in 200 μ L of iso-octane and analyzed to make sure that the signals of those two molecules are distinguishable.

b. Chromatography on cholesterol amended culture supernatant

For GC TOF MS analysis of culture supernatants, triplicated samples incubated with cholesterol (100 μ M) sampled after 0, 10 and 24 hours were selected. A lipid extraction protocol was designed for this project according to Erben et al. (2021) and Singh and Ecker (2018). To perform this extraction, 200 μ L of 75% Ethanol was added to 500 μ L of the samples. They were then vortexed for 2 minutes and 500 μ L of Methyl tert-butyl ether (MTBE) was added. Samples were then incubated for an hour in a shaker at room temperature, vortexed again for 2 minutes and incubated at room temperature for 10 additional minutes. Samples were subsequently centrifuged at full speed at 4 °C for 15 minutes, the supernatant was discarded and dried at 37 °C until complete evaporation of the supernatant. Finally,

samples were resuspended in 10 μ L of chloroform. A positive control was also prepared, consisting of 2.5 mg of cholesterol in 1 ml of chloroform.

Extracted samples and standards were dissolved in 100 μ l iso-octane and analyzed with a Hewlett-Packard HP 6890 gas chromatograph equipped with an autosampler and a mass selective detector (MSD; Hewlett-Packard 5973). They were injected in a split-splitless injector (heated at 280 °C) with split turned on at ratio 1:5, a helium mobile phase and with a constant flow of 1.5 ml per minute. Compounds were then separated on a DB-5 column (60 m length, Agilent). The GC temperature was programmed to be held at 160 °C upon injection for 1 min, and then increased 20 °C per minute in order to reach 300 °C, where it was held for 20 min. At the end of each injection, the column was baked-out at 310 °C for 10 minutes in order to decrease column bleed in subsequent samples. As the cholesterol peaks were not distinct enough in the first analysis of the samples, they were concentrated in a nitrogen drying step with an electric dry nitrogen blower (Termovap) and resuspended in 50 μ L of iso-octane in order to intensify the signals.

III. Results

1. Identification of the isolates

Nineteen isolates were identified, validated and preserved in glycerol stocked at -20 °C by the previous intern. During the course of the project, one new isolate was isolated and added to this library. The identity of this isolate was validated regularly using 16S rRNA gene PCR followed by Sanger sequencing and NCBI BLAST. The colony was isolated from long-term stored fecal slurry in 20 % glycerol (3.8 g of dried stool in 100 ml of PBS) (Material and methods 1. a.). The isolate was validated before and after being stored in 20 % glycerol at -80 °C and cultures of the isolate were validated regularly. The best match obtained for this isolate using NCBI BLAST was an *Enterocloster clostridioformis* strain with 98.86 % of identity. Finally the new isolate was added also to the DOME culture collections.

2. Characterisation of the samples

a. Counting of metabolically-active cells using BONCAT-FISH

BONCAT-FISH was used to quantify metabolically-active cells in relation to all general bacterial cells. For this, 20 pictures per sample were taken for both the DAPI and the Cy5 channel (Material and methods 3. b.). Figure 4 shows four of them, with pictures 4A and C displaying the DAPI signal for visualization of general bacterial cells. Pictures 4B and D show the metabolically-active cells using the Cy5 click reaction. The pictures used for this count correspond to two color channels from the same area of the sample.

4A and B are pictures from the Glc1 T24 sample (100µM Glucose and 50µM AHA, incubated for 24h) which is a positive control where a high number of cells are expected to be visible in both the DAPI and the Cy5 channel. On the contrary, pictures 4C and D were taken with the NoAHA T24 sample (100µM Glucose No AHA 0h), a negative control. This sample was incubated without any AHA making the BONCAT click reaction impossible even though unspecific binding could still be possible. The DAPI channel shows the cells that utilize glucose as a carbon source whereas the Cy5 channel does not show any signal corresponding to active cells.

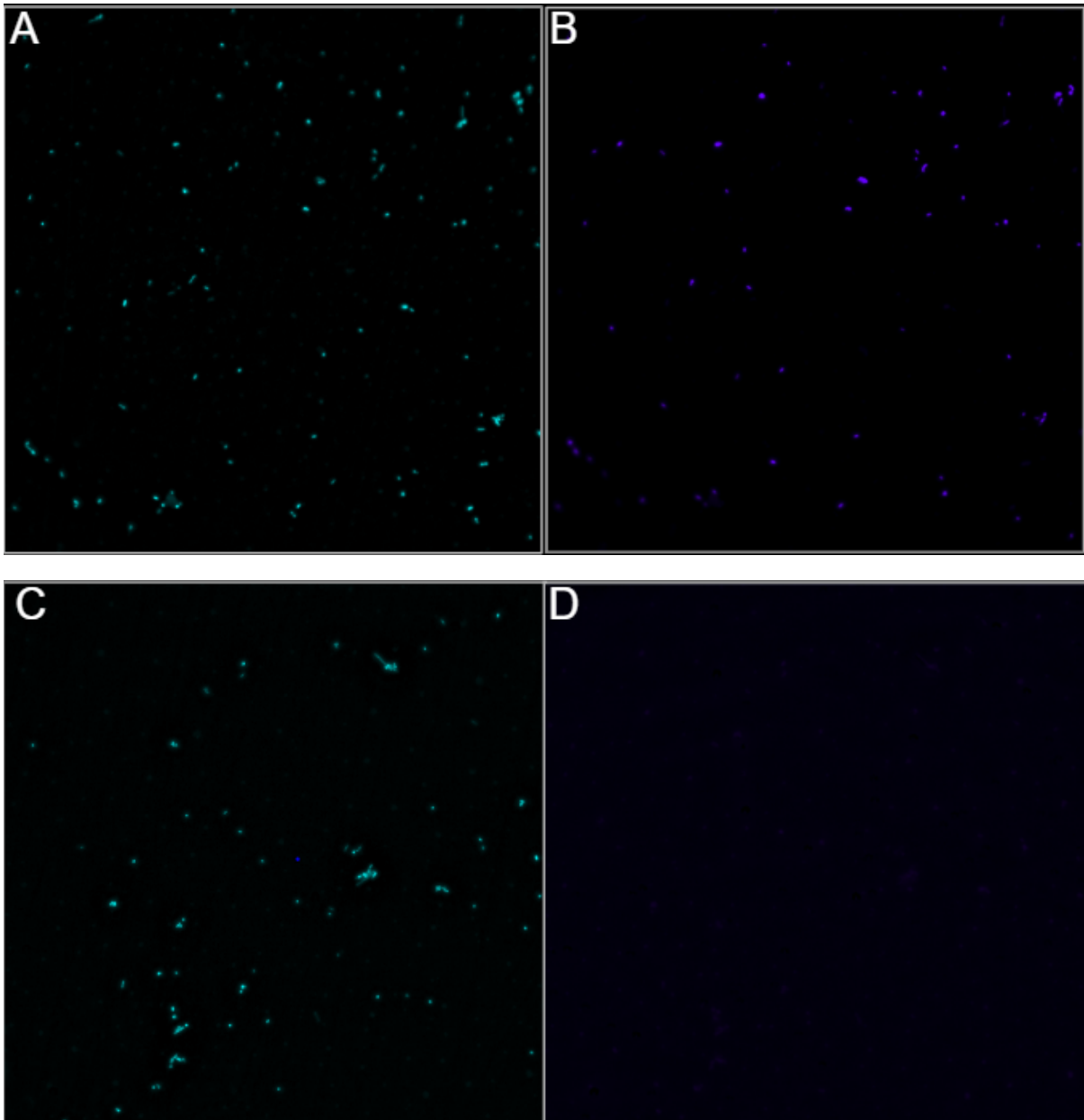


Figure 4: Thunder microscopy pictures from samples of BONCAT on slide 4A and B: Glc1 T24 sample (100µM Glucose AHA 50µM incubated for 24h) with A, DAPI channel displaying general bacterial cells and B: Cy5 channel, showing metabolically-active cells visualized using the BONCAT click reaction and Cy5 staining. 4C and D: NoAHA T24 sample (100µM Glucose No AHA 0h) with C, DAPI channel displaying general bacterial cells and D: Cy5 channel showing metabolically-active cells visualized using the BONCAT click reaction and Cy5 click reaction.

All of the pictures were then analyzed using daime (Material and methods 3. c.), resulting in the collection of the proportion of metabolically-active cells in the general bacterial population for each sample (Appendix 5). Using Ggplot2 in R studio, the results of the quantification were visualized in a bar graph (Figure 5). In this figure, three time points that were analyzed (0, 10 and 24 hours) are shown. Along with the cholesterol amended samples, the glucose samples were analyzed as a positive control. No amendment (NoAmd) samples were analyzed as a negative control in order to assess the number of cells active without any external source of carbon added. They could stay active with the leftover food particle present in the sample. The last control is the no AHA (NoAHA) negative control to verify possible autofluorescence due to unspecific binding of the probe, causing background noise. All samples were analyzed in triplicates except for cholesterol T24 which was just available in duplicates. The value displayed in the graph is a percentage of metabolically-active cells of the general bacterial population calculated using the DAIME software from 20 pictures taken of each sample.

In this experiment, the average expression level of the NoAHA samples varied from 14% to 26.06%. The proportion of active cells counted for the NoAmd samples stayed between 19.23% and 29.43% with a small increase after 6 hours of incubation. Samples incubated with glucose showed the biggest increase of average activity (from 23.5% to 44.9% after 6 hours of incubation and 72.86% after 24 hours). The average activity level of the cholesterol samples (chl) is increasing as well from 10.7% in the beginning of the incubation to 33.8% and 66.4% after 6 and 24 hours of incubation, respectively. The standard deviation (3.477 to 29.712) for each group of samples was also calculated from the triplicate of the samples.

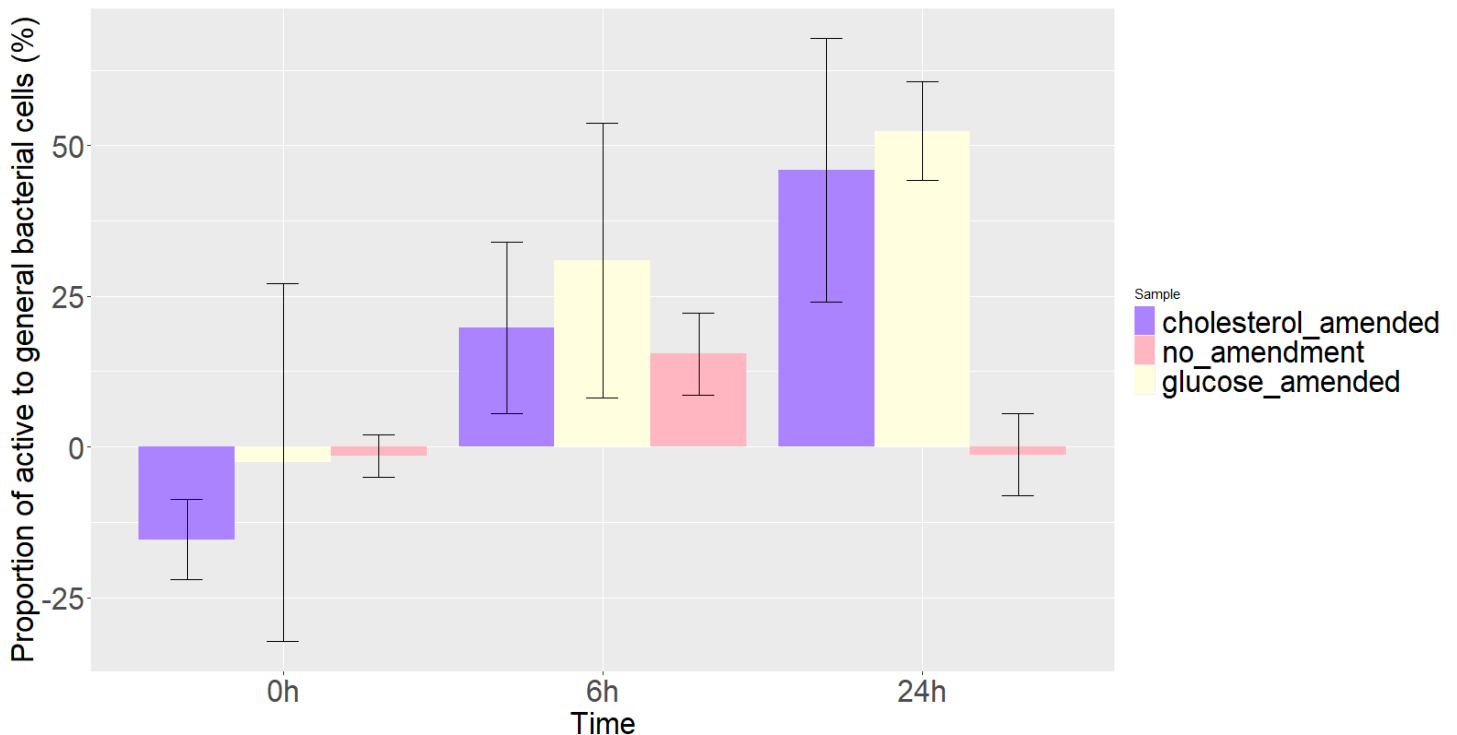


Figure 5: Proportion of metabolically-active cells calculated using the DAIME software from 20 pictures of each BONCAT-FISH sample. The average activity level of each sample (cholesterol and controls) is displayed including their standard deviation according to three timepoints (0, 6 and 24 hours of incubation). Error bars result from the standard deviation calculated from the triplicate results. All of the results were normalized for unspecific binding by subtracting the NoAHA value for each timepoint.

b. Active cell sorting with BONCAT-FACS

The sorting of the cells with FACS also allowed the estimation of the number of active cells in the different samples using the BONCAT specific (Cy5) probe and a general (Syto9) probe to target general bacterial cells (Material and methods 3. d.). This experiment enabled the count and sorting of the BONCAT positive and BONCAT negative cells in each sample. It led to the calculation of the percentage of active cells (Appendix 6) as well as sorted samples to later perform a 16S rRNA gene amplicon sequencing in order to identify metabolically-active cells present in the BONCAT positive fraction. To do so samples from three time points were analyzed (0, 6 and 24 hours). Along with the chl samples, the glc samples and NoAmd samples were analyzed. The average activity value of the samples was graphically displayed in Rstudio using Ggplot2 (Figure 6). All of the sorted samples were stored in a DNA extraction buffer at -80 °C.

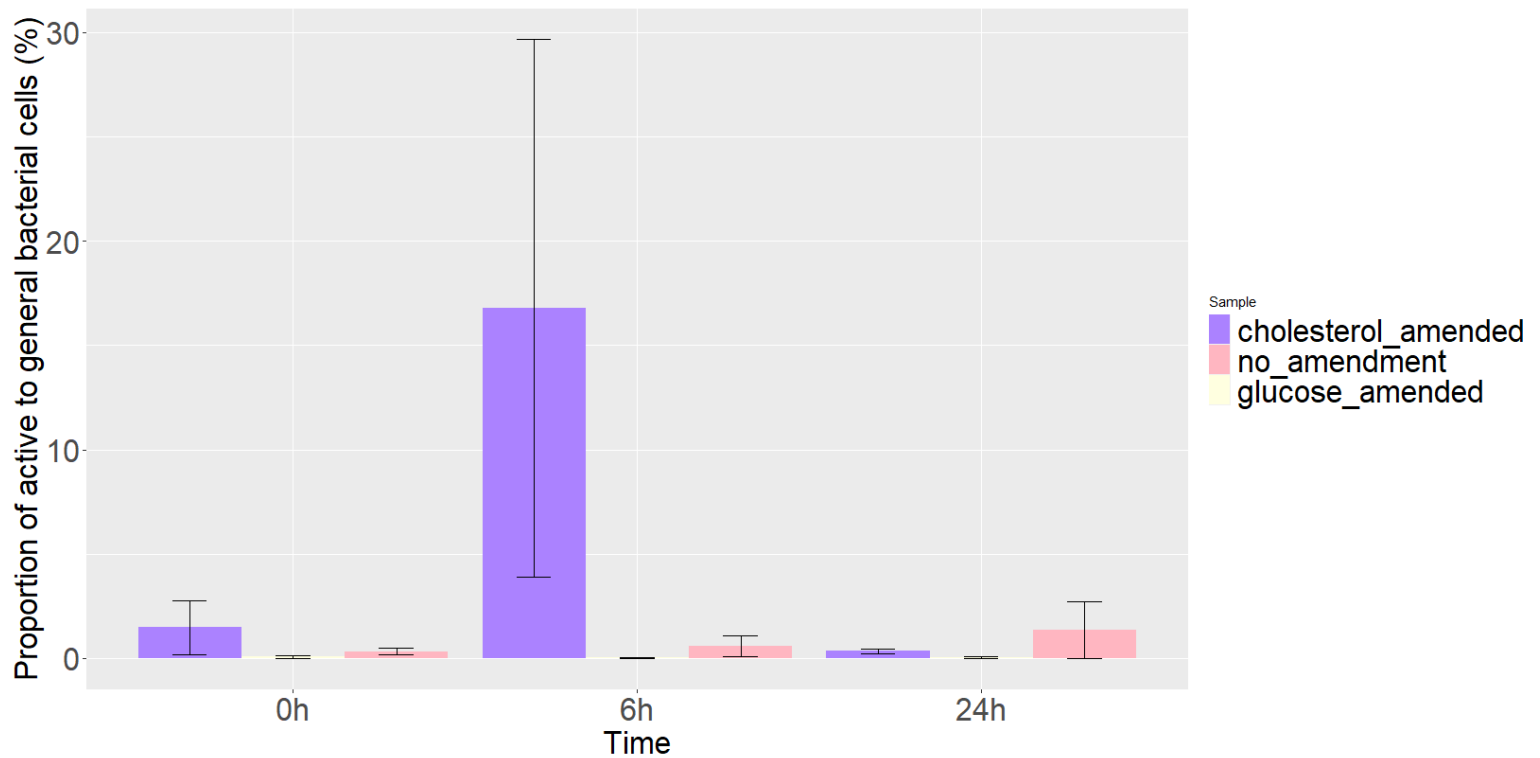


Figure 6: Graphical representation of the metabolically-active cells proportion calculated using FACS cell sorting. The average activity level of each sample (cholesterol), positive control (glucose) and negative control (NoAmd) including their standard deviation is displayed according to three timepoints (0, 6 and 24 hours of incubation).

The negative control active cell proportion was on average 0.318 % in the beginning of the incubation, 0.573% after 6 hours of incubation and 1.353% after 24 hours of incubation. The positive control showed an active to general bacterial cell proportion of 0.034% on average in the beginning of the experiment, 0.026% after 6 hours of incubation and after 24 hours, 0.074% were sorted as BONCAT positive cells by the FACS. Concerning the samples incubated with cholesterol, they showed an increase in metabolically-active cells at the time point 6 (with on average 1.481% of active cells in the beginning of the experiment, 16.777% after 6 hours of incubation and 0.348% after 24 hours).

c. Cholesterol metabolism in the samples (GC TOF MS)

The goal of this experiment was to detect cholesterol and coprostanol in the supernatant of the samples using the area under the curve of the specific peaks (Material and methods 4.). The first step of this experiment was to ensure that the peaks obtained for a cholesterol and coprostanol standard (Material and methods 4. a.) were distinguishable and their retention time was assessed. These results are displayed in Figure 7. The chromatogram of the two molecules showed two distinct peaks. Retention times were 18 minutes with a peak around 3.5×10^8 arbitrary unit (AU) for coprostanol and 19 minutes for cholesterol with a peak around 3.5×10^8 AU.

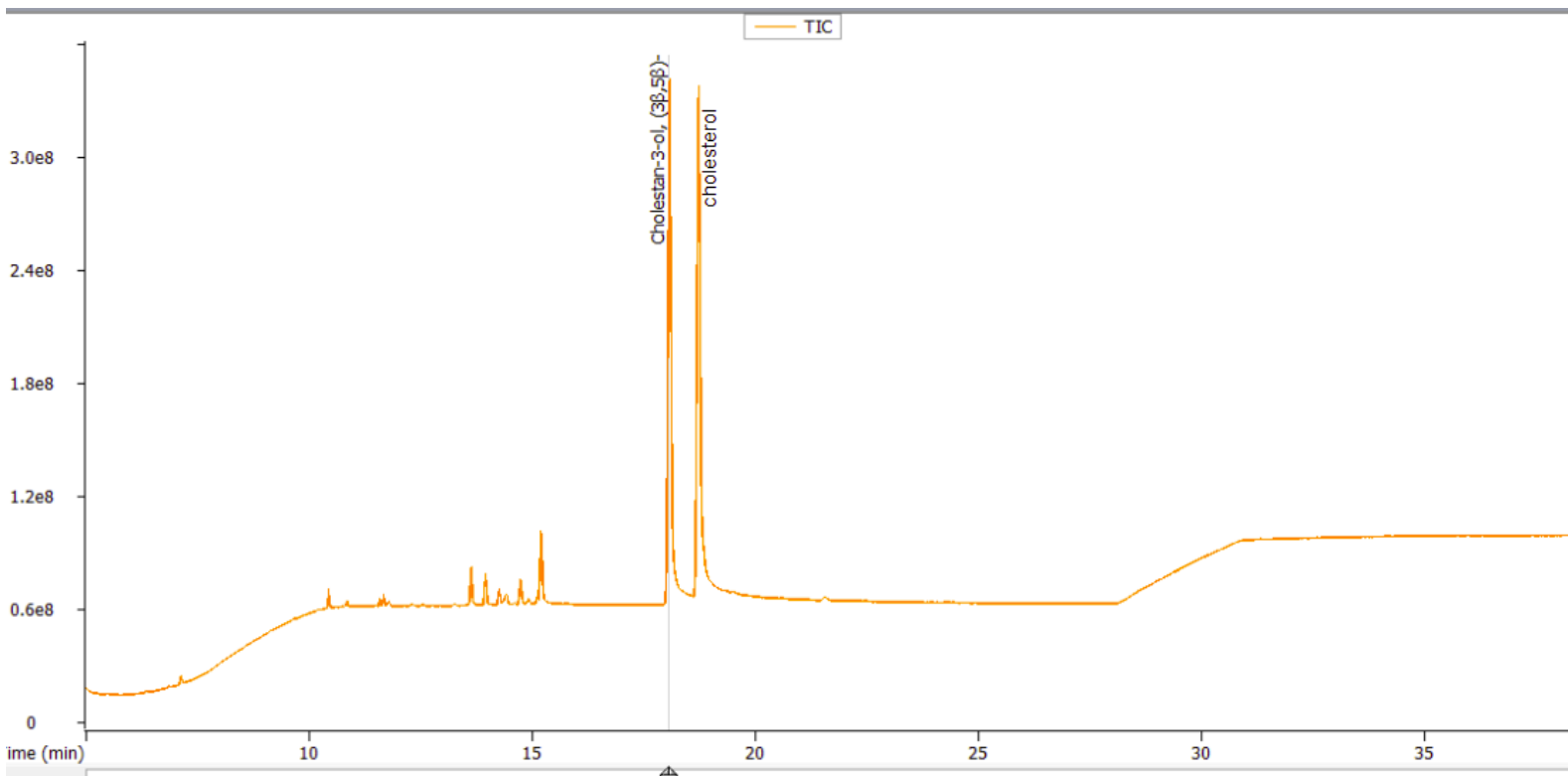


Figure 7: GC TOF MS chromatogram of a 10 mg per ml cholesterol and coprostanol chloroform solution diluted in 200 μ L of iso-octane.

The culture supernatant from fecal slurry incubated with cholesterol were then analyzed along with a pure cholesterol positive control (Material and methods 4) b.). The chromatograms of three of those samples incubated for 0, 10 and 24 hours are displayed in Figure 8.

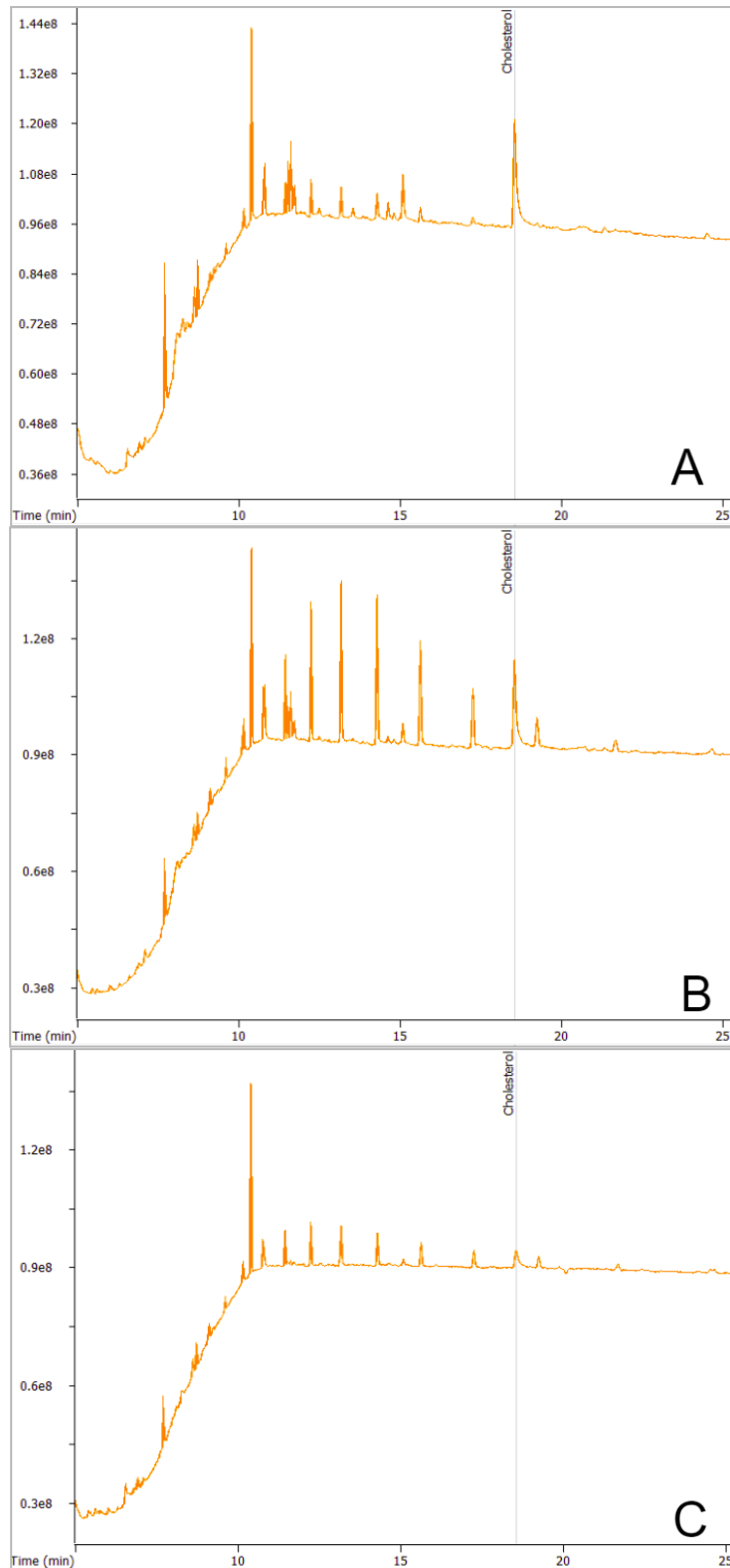


Figure 8: GC TOF MS chromatogram of three culture supernatants of fecal slurries incubated with 50 μ M cholesterol for 0 hours (A), 10 hours (B) and 24 hours (C). After sterol extraction and subsequent resuspension in 200 μ L of iso-octane (A) or 50 μ L of iso-octane (B and C).

In this figure, the cholesterol peak is visible in all three chromatograms (retention time of around 19 minutes). The peak intensity is 1.2×10^8 AU for Figure 8A, displaying a supernatant sample after 0 hours of incubation with cholesterol, in 200 μ L of iso-octane. The chromatogram 8B shows a peak around 19 minutes for cholesterol as well, with a peak intensity around 1.2×10^8 AU. This signal displays the chromatogram of a supernatant sample after 10 hours of incubation with cholesterol, resuspended in 50 μ L iso-octane. The last signal (Figure 8C) shows the chromatogram of the supernatant after 24 hours of incubation with cholesterol. Here, the samples were resuspended as well in 50 μ L iso-octane. The 19 minutes peak intensity is around 1×10^8 AU.

The GC TOF MS analysis of these samples can lead to an approximate comparison of the concentration of sterol contained in them using the area under the peak. However, these results alone cannot be quantitatively interpreted without any further experiment.

IV. Discussion

The goal of this thesis was to identify and characterize gut bacteria performing the cholesterol to coprostanol transformation as well as the identification of human gut microorganisms that become metabolically active upon exposure to cholesterol. The first step is to investigate the metabolic capabilities of stool samples regarding cholesterol and identify relevant bacteria. Several experiments of this project showed results regarding this goal.

The first experiment confirming this utilization is the isolation of colonies on the selective cholesterol medium containing 2mM of cholesterol as the only carbon source. The fact that bacteria can grow and regrow after being picked from the selective medium, indicates that they can utilize cholesterol as a carbon source. However, the plated fecal slurry can contain diluted residual nutrients, such as carbohydrates, fiber, protein and fat, that are plated out along the fecal bacteria (Umu et al. 2020). Cholesterol-utilizing bacteria were also isolated using solid cholesterol amended media in environmental studies (Wali et al. 2019; Pino-Otín et al. 2021) showing that cholesterol-degrading bacteria can be isolated from various environments. Brinkley et al. already designed a brain agar supplemented with cholesterol to grow and isolate cholesterol-degrading bacteria from the gut, in their 1982 experiment, showing that bacterial growth is possible on a solid cholesterol medium. Using a rich medium might thus be a valid option instead of a selective medium when trying to isolate cholesterol-utilizing bacteria. However, concerning the cholesterol to coprostanol transformation, only one bacterium (*Eubacterium coprostanoligenes*) that performs this reaction has been isolated for the moment (Ren et al. 1996). This bacterium was isolated from environmental samples (hog sewage lagoon) (Freier et al. 1994) and not from the human gut microbiota. This also shows that the isolation and characterization of bacteria with such a precise focus as trying to isolate bacteria using one amendment source only is a long and uncertain process (Sadzikowski, Sperry, and Wilkins 1977; Ren et al. 1996), especially concerning cholesterol to coprostanol transformation, as the coprostanol-forming bacteria do not use cholesterol as a carbon source. Indeed, the conversion of cholesterol to coprostanol releases no carbon atoms and coprostanol-producing bacteria are hypothesized to use cholesterol as a terminal electron acceptor in this conversion (Kriaa et al. 2019). In a 1973 study, Eyssen et al.

isolated coprostanol-producing gut bacteria (Eyssen et al. 1973). To do so, they used a complex media containing brain powder. However, later on Mott and Brinkley came across the fact that this media contained other components (e.i: lipids containing an alkenyl ether residue), along with cholesterol, which could induce the growth of bacteria by interfering in the cholesterol metabolism (Mott and Brinkley 1979). Therefore, these reports suggest that bacteria found different ways to utilize cholesterol.

In the course of this project, an *Enterocloster clostridioformis* strain could be isolated from the plated fecal slurry (Result 1). Bacteria from this strain were already isolated recently from human fecal samples by Boran et al. (Seo et al. 2021). In this study, they state that those isolates are unable to utilize most carbon sources except d-glucose and l-arabinose. This project indicated that they could also utilize cholesterol as their only carbon source, which was never discussed in any other study.

The BONCAT-FISH preparation of the samples allowed the estimation of the proportion of active cells in the samples using a subsequent DAIME analysis. In the FISH pictures taken (Figure 4), the positive control is validated because both pictures show lots of bacteria (general and metabolically-active) in both of the pictures meaning that cells are active when incubated with glucose. The negative control was validated as well because no active cells are visible in the Cy5 channel. This was expected because no AHA was present in this sample making the only possible Cy5 probe binding unspecific (e.g: to food particles). Nevertheless, unspecific probe binding is possible, reducing the active cell count after the subtraction of the background signal detected with the NoAHA samples. The number of active cells in the NoAmd samples stayed constant. This result matches our expectations as without an external source of carbon added the activity level should stay constant. The increase of the count after 6 hours could be caused by the utilization of dead cells by living cells as well as the utilization of remaining fecal debris present in the samples (Rose et al. 2015). Concerning the positive control of this experience, glucose incubated samples, the increase in the active cell count through time shows that a simple source of carbon such as glucose activates the cells well. Moreover, it also shows that activation is detectable by FISH and analysable by the DAIME software.

According to the DAIME software count, the proportion of metabolically-active cells compared to general cells incubated with cholesterol also increased over time. This increase indicates that the cells in the cholesterol incubations can utilize cholesterol as the only additional carbon source. However, the high standard deviation of the samples has to be put into account in this experiment. The variation can be explained by the low cell density of the samples as well as the noise caused by non-bacterial signals detected by DAIME (e.g., food residue particles) (Rose et al. 2015). The fact that some signal was detectable in the NoAHA samples (Results 2 a.) also support this explanation. A study using the same process to count active cells (FISH, thunder and DAIME) was also done in 2020 (Riva et al. 2020) and showed potential noise signals in the no AHA pictures as well. In this study, Riva *et al.* also mentioned that the active cell proportion never got higher than 50%, such as in this experiment.

The potential cholesterol utilization by the bacterial cells in these BONCAT treated fecal samples cannot be directly linked to a transformation to coprostanol. Ren et al proved in 1996 the transformation occurring in *Eubacterium coprostanoligenes* in vitro (Ren et al. 1996). Moreover, Sadzikowski *et al.* also showed that in vivo, the increase of the cholesterol in the diet of the rat could not directly be linked to an increase in the coprostanol level found in their feces (Sadzikowski, Sperry, and Wilkins 1977). In addition, a Li et al. experiment done in 1998 proved that feeding of *E. coprostanoligenes*, to germ-free mice may decrease plasma and blood cholesterol concentration, however they also proved that the colonization of the gastro-intestinal tract of the mice depends on its association with other bacteria, making it impossible to induct an efficient colonization of *E. coprostanoligenes* on its own (Li et al. 1998).

Concerning BONCAT-FACS, the results obtained were not the one expected and differing from BONCAT-FISH. Indeed, the active cell proportion showed no logical increase or decrease through time. Concerning the glucose incubated positive control (glc), the proportion of active cells was expected to rise according to time with the utilization of this carbon source by the cells. Only the negative control could be validated for this experiment because the proportion of active cells in those samples seemed to stay low and constant through time. However, the results showed a constant low number of active cells compared to general cells. The standard deviations concerning this experiment are low in this experiment

nevertheless. All of these results must be seen in the context of the fact that most of the cell metabolically-active cell counts in this experiment are unexpectedly low. 17 out of the 27 total counts performed during the sorting showed a proportion of active cells lower than 1%. Concerning the samples that were incubated with cholesterol as their only supplementary carbon source, their activity levels were expected to increase through time. In this study, the increase in the count of their metabolically-active cells after 6 hours of incubation (Result 2) b.) cannot be interpreted reliably due to lack of reliability in the positive control. Furthermore, another BONCAT-FACS study was performed in 2019 by Couradeau et al., on soil microbiomes where they also showed surprising cell count with this technique (Couradeau et al. 2019). In this study, these surprising results were explained by technical biases or the loss of bacteria during the incubation time interfering with certain taxonomic units and leading to the non-activation of specific cell groups. In the case of this project, those results can firstly be explained by the fact that the metabolically-active cell count results were obtained using the sorting function of the FACS machine, leading to a low cell count ('BD-FACSMelody-FACSCorus-Quick-Reference-Guide.' page 11). Although this first bias is probably not the main cause of the results obtained, a manual bias during the gating step could have also led to them as the gating was set conservatively to avoid sorting fecal particles (Bruder et al. 2016). However, this strict selection used for the gating might have been too stringent and might have led to missing BONCAT positive cells. In all cases, the results obtained by this technique can be complemented by the sequencing of the sorted samples, in order to characterize their bacterial composition. If the sorting step is performed again, the composition of general and BONCAT positive sorted cells could be a major information regarding the goal of this project in order to characterize the bacteria using cholesterol. In general, this study showed a significantly different number of metabolically-active cells with BONCAT-FISH compared to BONCAT-FACS. Concerning the BONCAT incubation, the BONCAT-FISH active cell count showed a high number of Cy5 stained cells with FISH, showing that the BONCAT click reaction seems to be successful. In 2016, Hatzenpichler also used those two techniques in their study but with FACS counting instead of sorting as we did (Hatzenpichler et al. 2016) and did not seem to have as much difference in their results. However, their conclusion seems to indicate that those techniques should be complementary.

Finally, the cell activation in presence of cholesterol shown by the BONCAT results is also to be linked with the GC TOF MS experiment results. The preliminary verification performed with cholesterol and coprostanol standard showed two separate peaks with close but distinct retention time. This verification was necessary in order to carry on with the cholesterol amended supernatant. This last experiment showed large peaks with short retention time in the beginning of the signal are due to the rubber septum. Also, in these chromatograms (Figure 8) the cholesterol related peaks got smaller through time (Result 2) c.), which might indicate that the level of cholesterol present in the fecal samples decreases over time showing that the activated cells indeed use this lipid. The presence of sterols in culture supernatants of fecal slurries can also indicate that the extraction process designed and used for this study is valid. Nevertheless, this experiment did not show results concerning coprostanol. Indeed, none of the chromatograms attested the presence of coprostanol, even after 24 hours of incubation with cholesterol. However, this transformation goes through two other intermediaries (cholestenone and coprostanone). The absence of coprostanol in the analyzed supernatant samples can then be explained by the time of incubation. In 24 hours, the transformation might not have the time to happen fully inside the cells and the coprostanol might not be excreted yet. This hypothesis could be verified by measuring the level of the two intermediary molecules (cholestenone and coprostanone), even though in the chromatography obtained with this experiment, no other peaks than cholesterol were visible. In a rat experiment, Björkhem and Gustafsson tried to unravel the mechanism of cholesterol into coprostanol microbial transformation using a radioactivity assay and Gas chromatography mass spectrometry (Björkhem and Gustafsson 1971). In their experimental setup, they incubated rat stools with deuterium water for 4 days at 37 °C and showed a conversion of 50% of the cholesterol to coprostanol. Moreover, several studies focusing on human fecal samples reported a bimodal rate of microbial cholesterol-to-coprostanol conversion in human populations, with high converters (almost complete conversion of cholesterol to coprostanol) and low or inefficient converters (coprostanol representing $\frac{1}{3}$ of the total sterol content) (Veiga et al. 2005). Moreover, a quick, general FISH analysis on the DSP-fixed samples from the BONCAT incubation was performed to investigate the distribution of relevant bacterial taxa in the human gut in these samples. A probe was used in order to target *Clostridia* (Erec 482, details in Appendix 7). This experiment did not show

an important proportion of *Clostridia*, impacting the general cholesterol metabolism seen with these samples. Indeed, most studies reporting a cholesterol to coprostanol conversion linked it with the Clostridia class (e.i: *Eubacterium coprostanoligenes*) (Ren et al. 1996). Moreover, the colony isolated in this study (*Enterocloster clostridioformis*) (Seo et al. 2021) is part of the *Clostridia* class as well.

The BONCAT approach could also not be the right approach concerning cholesterol to coprostanol transformation. Indeed with coprostanol formation, cholesterol could also not be used as the carbon source. It could also be interesting to get a closer look into the cholesterol utilization of the isolates. They could be used to perform another incubation leading to a GC TOF MS analysis of their cholesterol and coprostanol content. The low cell concentration should also be a factor to consider for future incubations of that kind as cell density might be a limiting factor to the results obtained here (e.g: FISH cell count, FACS cell sorting...). For this reason, it could be interesting to dilute the samples less if the incubation is performed once again. In conclusion, this study showed clear signs of cholesterol utilization by human gut microorganisms but failed to confirm that this utilization is linked to coprostanol formation.

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Appendix

Appendix 1: DSMZ 457 medium + 2 mM cholesterol (Braunschweig, Germany)

Reagent	Amount
Cholesterol 2 mM	0.78 g
Na ₂ HPO ₄	2.44 g
KH ₂ PO ₄	1.52 g
(NH ₄) ₂ SO ₄	0.50 g
MgSO ₄ x 7 H ₂ O	0.20 g
CaCl ₂ x 2 H ₂ O	0.05 g
Trace element solution SL-4 (see below)	10.00 ml
Distilled water	ad. 1000.00 ml

Adjust pH to 6.9.

Prepare a separate solution for phosphate compounds and autoclave separately. Combine the two solutions after cooling. Rehydrate and cultivate lyophilized cells in complex medium (e.g. medium 1, 220 or 535). After this reactivation, cultivate in mineral medium 457 with the appropriate carbon source.

Trace element solution SL-4:

Reagent	Amount
EDTA	0.50 g
FeSO ₄ x 7 H ₂ O	0.20 g
SL-6 (see below)	100.00 ml
Distilled water	ad. 1000 ml

Trace element solution SL-6:

Reagent	Amount
ZnSO ₄ x 7 H ₂ O	0.10 g
MnCl ₂ x 4 H ₂ O	0.03 g
H ₃ BO ₃	0.30 g
CoCl ₂ x 6 H ₂ O	0.20 g
CuCl ₂ x 2 H ₂ O	0.01 g
NiCl ₂ x 6 H ₂ O	0.02 g
Na ₂ MoO ₄ x 2 H ₂ O	0.03 g
Distilled water	ad. 1000.00 ml

Appendix 2: Dreamtaq PCR (colony PCR) mastermix

Reagent	Amount for one PCR reaction (µl)
10 x Dream taq buffer	5
dNTPs 2 mM	5
Primer forward (616f) 50 µM	1
Primer reverse (1429r) 50 µM	1
BSA 20 mg/mL	0.5
Taq Polymerase 5 U/µl	0.5
PCR water	37
Total volume	50

Appendix 3: PCR cyclers settings

Temperature in °C	Time	
95	3 min	Initial denaturation
95	30 s	
52	30 s	30 cycles
72	90 s	
72	10 min	Final elongation
4	∞	Cooling

Appendix 4: BONCAT samples library

Sampling time Type of incubation	0	3	6	10	24
Cholesterol (Chl)	Chl1 T0	Chl1 T3	Chl1 T6	Chl1 T10	Chl1 T24
	Chl2 T0	Chl2 T3	Chl2 T6	Chl2 T10	Chl2 T24
	Chl3 T0	Chl3 T3	Chl3 T6	Chl3 T10	Chl3 T24
Glucose (Glc)	Glc1 T0	Glc1 T3	Glc1 T6	Glc1 T10	Glc1 T24
	Glc2 T0	Glc2 T3	Glc2 T6	Glc2 T10	Glc2 T24
	Glc3 T0	Glc3 T3	Glc3 T6	Glc3 T10	Glc3 T24
No amendment (NoAmd)	NoAmd1 T0	NoAmd1 T3	NoAmd1 T6	NoAmd1 T10	NoAmd1 T24
	NoAmd2 T0	NoAmd2 T3	NoAmd2 T6	NoAmd2 T10	NoAmd2 T24
	NoAmd3 T0	NoAmd3 T3	NoAmd3 T6	NoAmd3 T10	NoAmd3 T24
No AHA (NoAHA)	NoAHA T0	NoAHA T3	NoAHA T6	NoAHA T10	NoAHA T24
No fecal slurry (Abc)	Abc1 T0	Abc1 T3	Abc1 T6	Abc1 T10	Abc1 T24

Appendix 5: Proportion of active cells calculated from BONCAT-FISH Daime analysis

Sample	Active cells proportion (%)	Average active cells proportion per sample type (%)
T0 chl1	5	10,7
T0 chl2	20,1	
T0 chl3	7	
T0 glc1	7	23,5
T0 glc2	5,7	
T0 glc3	57,8	
T0NoAmd1	20,2	24,6
T0NoAmd2	28,7	
T0NoAmd3	24,9	
T0 NoAHA	26,06	26,06
T6 chl1	15	33,8
T6 chl2	49,4	
T6 chl3	37	
T6 glc1	57,8	44,9
T6 glc2	64	
T6 glc3	12,9	
T6NoAmd1	21,4	29,43333333
T6NoAmd2	38	
T6NoAmd3	28,9	
T6 NoAHA	14	14
T24 chl2	44,6	66,4
T24 chl3	88,2	
T24 glc1	83,1	72,86666667
T24 glc2	72,5	
T24 glc3	63	
T24NoAmd1	19,8	19,23333333
T24NoAmd2	10,6	
T24NoAmd3	27,3	
T24 NoAHA	20,5	20,5

Appendix 6: Proportion of active cells from BONCAT-FACS analysis

Sample	Boncat +	Boncat -	Active cells proportion (%)	Average active cells proportion (%)
T0 chl1	50	50000	0,1	1,481333333
T0 chl2	1614	50000	3,228	
T0 chl3	558	50000	1,116	
T0 glc1	75	50000	0,15	0,07403851274
T0 glc2	1	50000	0,002	
T0 glc3	23	32803	0,07011553821	
T0NoAmd1	66	50000	0,132	0,318
T0NoAmd2	201	50000	0,402	
T0NoAmd3	210	50000	0,42	
T6 chl1	869	50000	1,738	16,77733333
T6 chl2	7703	50000	15,406	
T6 chl3	16594	50000	33,188	
T6 glc2	23	32803	0,07011553821	0,03403851274
T6 glc3	16	50000	0,032	
T6NoAmd1	216	50000	0,432	
T6NoAmd2	563	50000	1,126	0,573333333
T6NoAmd3	81	50000	0,162	
T24 chl1	64	25260	0,253365004	0,3484550013
T24 chl2	163	50000	0,326	
T24 chl3	233	50000	0,466	
T24 glc1	0	8199	0	0,02619542756
T24 glc2	2	2639	0,07578628268	
T24 glc3	14	500000	0,0028	
T24NoAmd1	36	7706	0,4671684402	1,353722813
T24NoAmd2	331	50000	0,662	
T24NoAmd3	1466	50000	2.932	

Appendix 7: Erec 482 probe description

Name of the probe: Erec482

Specificity: Most of the Clostridium coccoides-Eubacterium rectale group

Probe sequence: 5' GCTTCTTAGTCARGTACCG

Fluorophore: 2x Cy3

Abstract:

A high blood cholesterol level can result in Atherosclerotic cardiovascular diseases. In humans, circulating cholesterol gets in contact with the gut microbiota that can transform it to the poorly absorbable coprostanol, resulting in lower blood cholesterol levels. In this study, the number of bacterial cells from the human gut that become metabolically active upon exposure to cholesterol was assessed using BONCAT. Metabolically active cells were sorted using FACS for later identification via 16S rRNA gene amplicon sequencing. Furthermore, a protocol to extract sterols and measure cholesterol and coprostanol via GC TOF MS was developed. Lastly, the isolation of a bacterium growing on a cholesterol-amended, selective medium was completed. Getting a closer look at the human gut transforming cholesterol to coprostanol could lead to alternative therapeutics.

Keywords: Gut microbiota, blood cholesterol, coprostanol, BONCAT, FISH, FACS, GC TOF MS, *Enterocloster clostridioformis*, isolation, lipid extraction.

Résumé:

Un taux de cholestérol sanguin élevé peut entraîner des maladies cardiovasculaires athérosclérotiques. Chez l'homme, le cholestérol circulant entre en contact avec le microbiote intestinal qui peut le transformer en coprostanol peu absorbable, ce qui entraîne une baisse du taux de cholestérol sanguin. Dans cette étude, le nombre de cellules bactériennes de l'intestin humain devenant métaboliquement actives lors de leur exposition au cholestérol a été évalué à l'aide de la technique BONCAT. Les cellules métaboliquement actives ont été triées par FACS pour une identification ultérieure par séquençage 16S nouvelle génération. En outre, un protocole d'extraction de stérols et de mesure du cholestérol et coprostanol par GC TOF MS a été développé. Enfin, l'isolement d'une bactérie se développant sur un milieu sélectif contenant du cholestérol a été réalisé. Le fait d'observer de plus près la transformation du cholestérol en coprostanol par l'intestin humain pourrait conduire à des thérapies alternatives.

Mots clés: Microbiote intestinal, cholestérol sanguin, coprostanol, BONCAT, FISH, FACS, GC TOF MS, *Enterocloster clostridioformis*, isolation, extraction de lipides.