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"Detecting choline degrading bacteria with BONCAT-FACS in the human gut"

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Contents

Abstract

Choline is a semi-essential nutrient, a major component in eukaryotic cell membranes, a precursor for neurotransmitters, and a methyl donor in the methylation pathway. It can be degraded to trimethylamine (TMA), which came under the suspicion to play a role in cardiovascular diseases and its oxidized form, trimethylamine N-oxide (TMAO), can promote microbial anaerobic respiration in the colon. The conversion from choline to TMA can be exclusively performed by bacteria. Genomic data suggest a range of possible candidates performing this step, but experimental validation is needed. In this thesis a general activity marker based method, Bioorthogonal noncanonical amino acid tagging (BONCAT), was used to identify key players that take part in choline degradation in the human gut microbiota. A workflow was developed combing BONCAT, fluorescence-activated cell sorting (FACS), and 16S rRNA gene profiling for investigating microbial substrate utilization from fecal samples. *Lachnoclostridium* sp. was identified as major player, which confirms previous findings where its abundance was correlated with increased TMA and decreased short-chain fatty acid (SCFA) levels.

Zusammenfassung

Cholin ist ein semi-essentieller Nährstoff, Hauptbestandteil in Eukaryotischen Zellen, eine Vorstufe for Neurotransmitter und Methyl Donor im Methylierungs Weg. Es kann zu Trimethylamin (TMA) degradiert werden, welches unter Verdacht steht eine Rolle in Kardiovaskulären Erkrankungen zu spielen und dessen oxidierte Form, Trimethylaminoxid (TMAO), kann mikrobielle anaerobische Atmung im Darm fördern. Die Umwandlung von choline zu TMA kann exklusive nur von Bakterien ausgeübt werden. Genomische Daten weisen auf ein Spektrum von möglichen Kandidaten, die diesen Schritt ausführen, hin. In dieser Arbeit wurde auf ein generellen Aktivitätsmarker basierende Methode, Bioorthogonal noncanonical amino acid tagging (BONCAT), verwendet um die Hauptakteure, die eine Rolle in der Degradation von Cholin im menschlichen Darm spielen, zu identifizieren. Ein Workflow wurde entwickelt, der BONCAT, fluorescence-activated cell sorting (FACS) und 16S rRNA Gen Profiling kombiniert, um mikrobiologische Substrat Nutzung in Fäkal Proben zu untersuchen. *Lachnoclostridium sp.* wurde als Hauptakteur identifiziert, was vorhergegangene Befunde bestätigt, welche dessen Häufigkeit mit gesteigerten (TMA) und verminderten kurzen Fettsäuren Niveaus korreliert.

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Abbreviations

AHA	L-azidohomoalaine
ASV	amplicon sequence variant
BONCAT	Bioorthogonal noncanonical amino acid tagging
CDV	cardiovascular disease
cutC	choline utilization cluster C
CutC	trimethylamine-lyase
Cy5	Cyanine-5
DEPC	diethyl pyrocarbonate
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FISH	fluorescence *in situ* hybridization
HPG	L-homopropargylglycine
Met	L-methionine
NA	no amendment
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PFA	formaldehyde
RT	room temperature
SCFA	short-chain fatty acid
THPTA	tris(3-hydroxypropyltriazolylmethyl)amine
TMA	trimethylamine

TMAO trimethylamine N-oxide

1. Introduction

1.1 Choline, trimethylamine, and the microbiota

The human intestine is home to trillions of microorganisms, the microbiota, which collective genomes exceeds the human genome by ≥ 100 times and thus its physiological capabilities are invaluable for the host. Indigestible compounds of the diet (e.g. fibers) can be turned into useful, even essential nutrients, like certain vitamins or short chain fatty acids which the host is not able to synthesize by itself. But on the other hand some members of the microbiota can also compete with the host for nutrients. This is the case with choline (Romano *et al.*, 2017), a semi-essential nutrient produced by the liver, but in insufficient quantities to maintain a healthy lifestyle. It is a major component in all eukaryotic cell membranes in form of sphingomyelin, choline plasmalogen, lysophosphatidylcholine, and phosphatidylcholine (Zeisel *et al.*, 1991). Furthermore it is precursor for the neurotransmitter acetylcholine (Tiwari *et al.*, 2013) and an important methyl donor in the methylation pathway (Mora-Ortiz and Claus, 2017). Choline can be found in a large variety of foods including eggs, beef liver, cod fish, cauliflower, and sunflower seeds. 90% of the US-Americans fail to consume the recommended amount (Wallace *et al.*, 2018),however. The impact of choline deficiency on humans needs still investigation but some results suggest that it may lead to development of non-alcoholic fatty liver disease (Corbin and Zeisel, 2012). In in adult mice and their offspring leads deficiency to adiposity, metabolic diseases, altered DNA methylation patterns, and anxious behavior (Romano *et al.*, 2017).

In contrast, a surplus of choline may also harm the host indirectly by the same organisms that compete with it. Choline that reaches the colon is metabolized exclusively by bacteria into TMA which is taken up by the colon and further reaches the liver via the circulatory system, where it is oxidized by hepatic flavin monooxygenase 3 into TMAO (Wang *et al.*, 2011). A recent study showed that TMAO is excreted back in the colon in a process termed metabolic

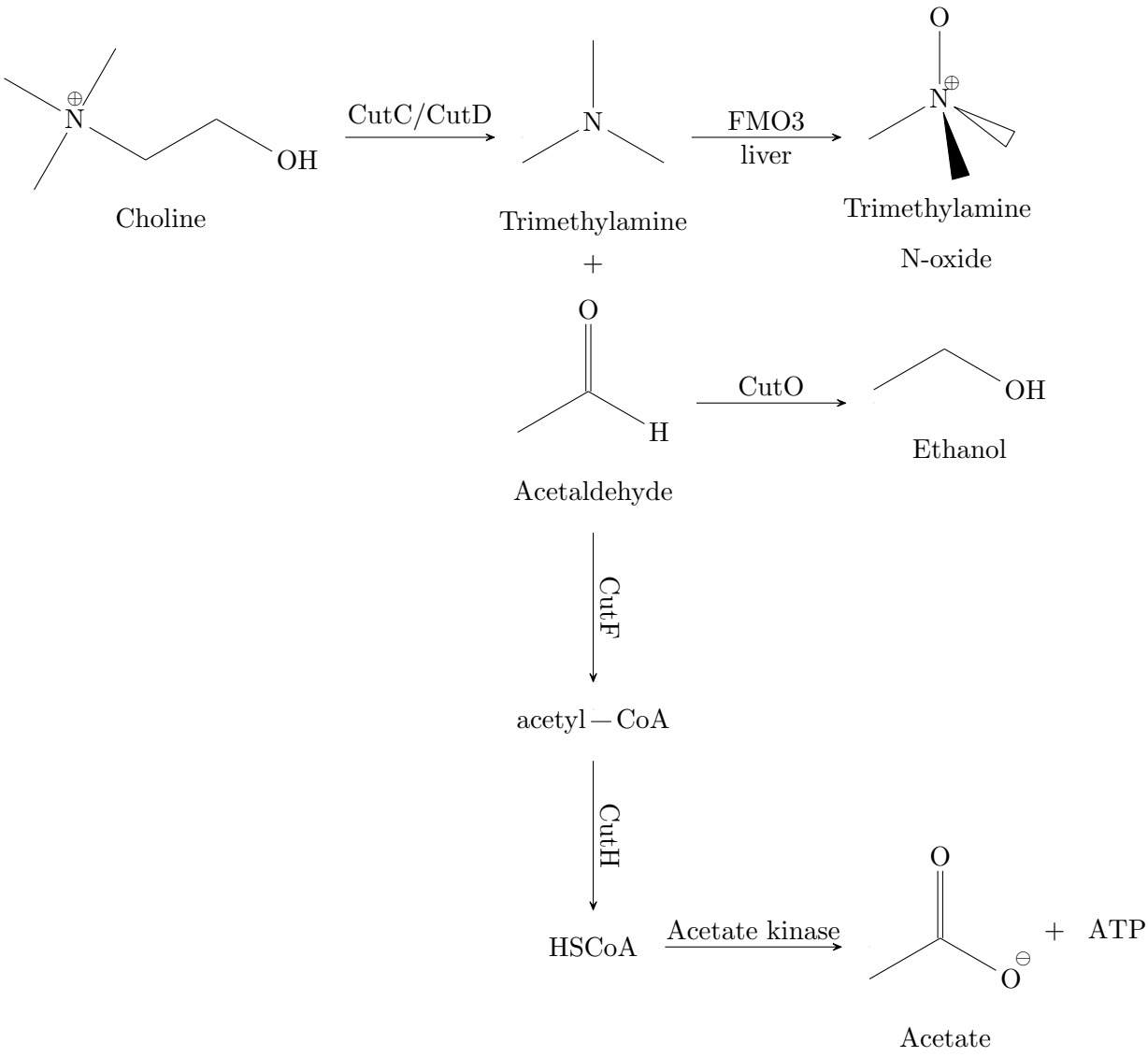


Figure 1: Microbial degradation of choline *Choline is cleaved by trimethylamine-lyase (CutC), into TMA and acetaldehyde. Flavin containing monooxygenase 3 (FMO3) oxidizes TMA into TMAO in the liver.*

retroversion and TMAO reductase is used as terminal electron acceptor for anaerobic respiration (Hoyles *et al.*, 2018). Several other studies found a correlation between high TMAO serum levels and cardiovascular diseases (CDVs) e.g. atherosclerosis (Koeth *et al.*, 2013; Stubbs *et al.*, 2016; Tang *et al.*, 2013; Trøseid *et al.*, 2015; Wang *et al.*, 2011).

The majority of TMA producing bacteria were identified by screening for the subunits of the functional genes choline TMA-lyase (cutC) and carnitine oxygenase (cntA) (Zhu *et al.*, 2014; Martinez-del Campo *et al.*, 2015; Rath *et al.*, 2017). Though TMA can be produced from carnitine, this thesis is focused on choline, because choline utilization cluster C (cutC) harboring bacteria can be found in almost all individuals, whereas cntA only in a fraction (Rath *et al.*, 2017).

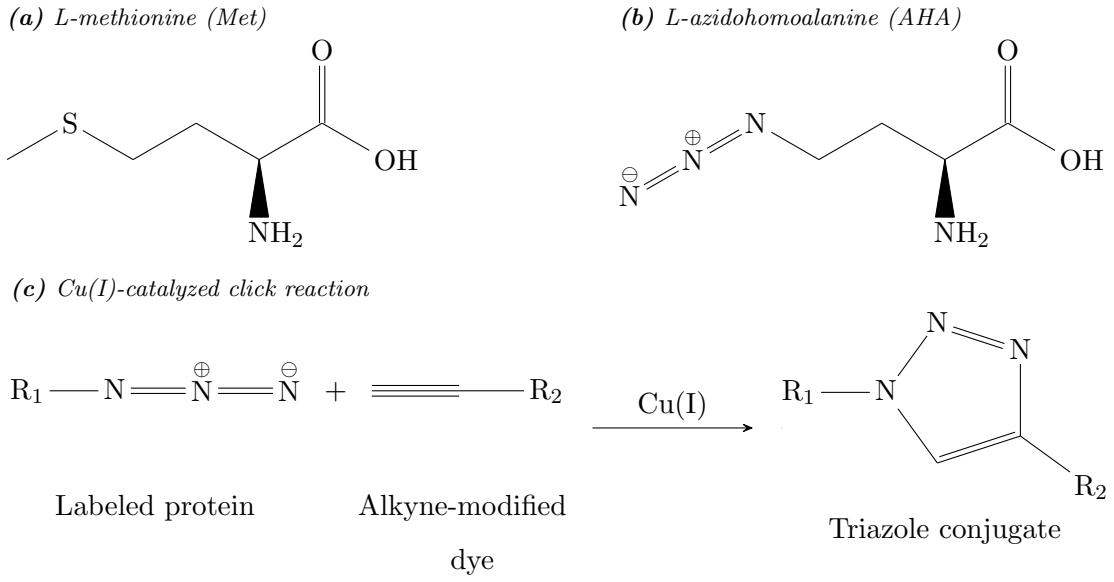


Figure 2: BONCAT components and click reaction (a) *Structure of L-methionine* ***(b)*** *Structure of L-azidohomoalanine* ***(c)*** *Cu(I)-catalyzed click reaction, where R_1 is a protein and R_2 is a fluorescent dye.*

In contrast to screen for cutC harboring bacteria via PCR to search for organisms that are able to utilize choline an activity based approach was used - BONCAT.

1.2 Bioorthogonal noncanonical amino acid tagging (BONCAT)

One of the big challenges in microbial ecology is linking phylogenetic information with function. As it turned out in the past sequencing and fluorescence *in situ* hybridization (FISH) are invaluable methods to assign affiliation of organisms to certain taxa, but phylogeny is in most cases not related with neither biological nor ecological function. There are several methods to address this issue, but most of them require expensive appliances (e.g. nanoSIMS, Raman microspectrometer) or specialized facilities, e.g. for handling radioactive substances for FISH-MAR. BONCAT, which utilizes bioorthogonal compounds coupled with click chemistry, on the other hand is a cheap and fast alternative for labeling and detecting microbial activity. Bioorthogonal compounds are artificial synthesized molecules that are analogous to compounds found in nature and that do not interfere with cell physiological processes (Hatzenpichler *et al.*, 2014). In case of BONCAT alternative amino acids, with a side group accessible to click chemistry, are used. The two most used noncanonical amino acids are the L-methionine (Met) (Figure 2.4 a) surrogates L-azidohomoalaine (AHA) (Figure 2.4 b) and L-homopropargylglycine (HPG), whereas AHA is

the more translational active one.

Translational active organisms are building the Met surrogate with a certain probability into their proteins during synthesis. A fluorescent dye can be "clicked" either by a Cu(I)-catalyzed or a strain-promoted reaction. In this work only the Cu(I)-catalyzed reaction was used, which fulfills a reaction between an azide labeled protein and an alkyne-modified dye that results into a triazole conjugate and a fluorescent labeled protein (Figure 2.4 c).

1.3 Application of BONCAT in gut samples

In this thesis general BONCAT features were investigated and a comparison was conducted between BONCAT and another general activity marker approach, D_2O-Raman. An improved incubation approach was developed for a more distinct BONCAT result. The fluorescent properties of BONCAT were used for FACS and subsequent 16S rRNA gene sequencing of the translational active community to investigate which members of the microbiota take part in the degradation of choline. It was found out that many members of the *Lachnoclostridium sp.* genus become active during incubation with choline.

2. Material and Methods

2.1 Fecal samples

Fecal samples were acquired the same day or the day before the experiment by the participants themselfs. In total there were 10 participants (5 male, 5 female), different nationalities, in the age ranging from 21 to 36, where 9 were omnivorous and one was vegetarian. Methods of collecting the samples and the methods of analyzing the data have been approved by the Ethics Committee of the University of Vienna.

2.2 Anaerobic Incubation

To maintain anaerobic conditions all steps were carried out in a vinyl anaerobic chamber (Coy laboratory products, USA), containing an atmosphere that consisted of 70% N_2, 20% CO_2, and 10% H_2. All solutions were made anaerobic by bubbling with a gas mixture consisting of 80% N_2 and 20% CO_2.

2.2.1 Preparation of the inoculum

To keep the basal activity of the organisms as low as possible it is necessary to remove dissolved and particular nutrients from the environmental sample. Fecal samples were homogenized by adding phosphate buffered saline (PBS) to the collection tube, resulting a 10% (w/v) dilution, and by shaking and vortexing. The fecal slurry was let to sit for a couple of minutes so that coarsely matter can sink to the bottom. From the top phase 1 mL was transferred into 9 mL fresh PBS and the whole solution was filtered through a 5 µm syringe filter. The filtered solution was transferred into centrifugation tubes, and washed two times in PBS resulting in a white yellowish pellet, which was used as inoculum for incubation.

2.2.2 Incubation with the activity marker L-azidohomoalanine

The inoculum of each sample was mixed with 30 mL PBS resulting in a dilution of 0.1% (w/v) of the original fecal sample and AHA was added to a final concentration of 10 µM. Two times 5 mL were aliquoted into hungate tubes to serve as no amendment control. Choline chloride was added to the remaining solution to a resulting concentration of 1 mM and 5 mL was aliquoted into each of four hungate tubes. Each tube was sealed with a rubber stopper and incubated at 37 °C for 20 hours. After incubation cells were harvested, washed two times in PBS, fixed with ethanol (EtOH), for FACS, and 1 mL was collected for DNA extraction.

2.3 Cell fixation

Cells were fixed either with just EtOH or 1% formaldehyde (PFA). Cells were washed twice in PBS by centrifugation at 14,000 g for 3 minutes at room temperature (RT) and replacing the supernatant with 1 mL PBS. For EtOH fixation the cell pellets were first resuspended in PBS and 1 volume EtOH was added. For PFA fixation the cell pellets were resuspended in 750 µL PBS, 250 µL 4% PFA was added and incubated for 2 hours at 4 °C. Afterwards the cells were again washed twice in PBS resuspended in PBS and 1 volume EtOH was added. Fixed cells were stored at −20 °C.

2.4 BONCAT - click reaction

The click reaction was carried out according to Hatzenpichler *et al.* (2014). Fixed cells were pelleted by centrifugation at 14,000 g for 3 minutes at RT and afterwards removing the supernatant. To dehydrate cells 1.5 mL EtOH was added, mixed by vortex, and incubated for 3 minutes at RT before pelleting again. Cells were resuspended in 884 µL PBS before 50 µL of 100 mM freshly prepared sodium ascorbate and 50 µL of 100 mM freshly prepared aminoguanidine hydrochloride was added and incubated for 3 minutes at RT. A dye mix was prepared as follows: 5 µL 20 mM $CuSO_4$, 10 µL of 50 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 1.2 µL of alkyne Cyanine-5 (Cy5) dye, and incubated for 3 minutes at RT before adding to the previous mixture. The solution was mixed by inversion and incubated for 30 minutes at RT. Samples were washed 3 times in PBS, as described in 2.3 and one time in 50% EtOH. Cells were resuspended in PBS and 1 volume EtOH was added. Samples were stored at −20 °C until further use but not longer than two weeks to avoid losing fluorescence signal.

2.5 Microscopy and Raman measurement

Image acquisition was performed on a Leica inverted fluorescent microscope using the Leica suite AF imaging software. Raman spectra were acquired on a Horiba raman spectrometer.

2.6 Comparison of BONCAT and Raman/D_2O

2.6.1 Testing AHA concentration

A *Bacillus subtilis* overnight culture was diluted 1:1 in MSgg medium and incubated with 10 µM or 100 µM AHA, respectively. Samples were taken after 30 minutes and two hours, EtOH fixed and stored at -20°C. Fresh human fecal samples were diluted in 1x PBS (10 mM Na_2HPO4, 10 mM NaH_2PO4, 130 mM NaCl) 10% w/v and used as inoculum.

2.6.2 Relationship between BONCAT fluorescent intensity and %CD

A fresh fecal sample, prepared as mentioned above, was added to solution consisting of 50 gL^{-1} glucose, 10 µM AHA and PBS:D_20, resulting in a final feacal dilution of 1%. The sample were incubated for 20 h and fixed with either ethanol or PFA.

2.7 Fluorescence-activated cell sorting

FACS was carried out at the Anna Spiegel Center of Translational Research of the Vienna General Hospital (Allgemeines Krankenhaus der Stadt Wien) by Ao.Univ.-Prof. Dr. Andreas Spittler on a MoFlo Astrios EQ™ (Beckman Coulter, United States) flow cytometry system.

Before cell sorting all samples were pelleted, resuspended in 1 mL PBS, and filtered through a 70 µm cell sieve to avoid clogging of the machine. A total of 100,000 to 1 million events of Cy5 positive cells were collected. After cell sorting the machine was flushed with PBS to collect a PBS-sheath fluid negative control.

For the initial FACS gating settings Cy5 BONCAT labeled gut bacteria from a feacal sample, which were incubated with glucose, were analyzed and sorted. It was endeavored to sort as conservative as possible, however the analysis of the sorted sample shows that a handful of cells fall outside the gate. Yet the vast majority of cells is inside the gate, which lies clear apart from the unlabeled cells (figure 3). An analysis of choline incubated or no amendment (NA) samples was not possible due to the relative small yield.

(a) Used gating *(b) Sorted sample analyzed* *(c) Unlabeled sample*

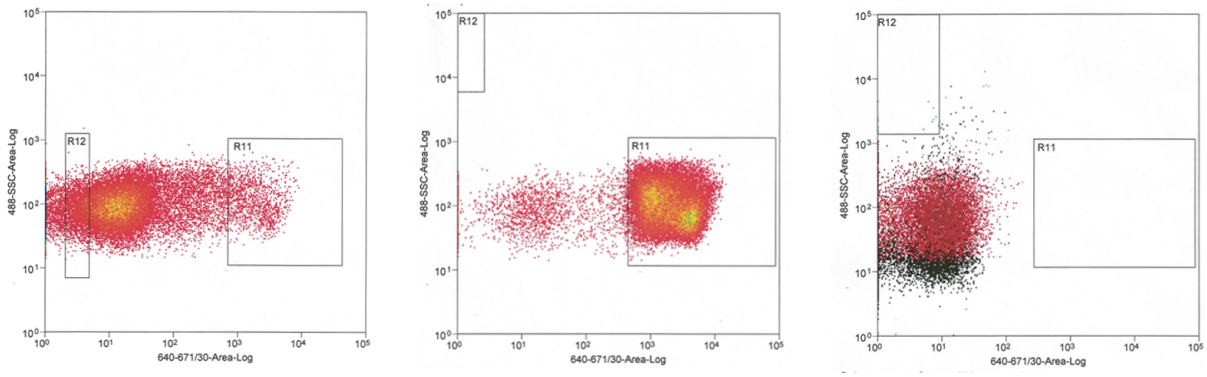


Figure 3: FACS gating scatter plots *Each dot represents an event. Each event that fell into the R11 gate was sorted.*

2.8 Nucleic acid extraction

A slightly modified protocol for DNA extraction with the DNA extraction DNeasy® Blood & Tissue Kit (Quigen, Germany) was used. Before following the manufacturer's instructions, an additional lysis step was performed. An enzymatic lysis buffer was prepared as follows: 20 mM Tris·Cl (pH 8.0), 2 mM sodium EDTA, 1.2 % Triton™X-100 (Sigma Aldrich, USA), and lysozyme 20 mg/ml. Before adding lysozyme, all enzyme free reagents were aliquoted and together with some centrifuge tubes UV-radiated for 1 hour.

Samples were pelleted, resuspended in 180 µL enzymatic lysis buffer, and incubated with lysozyme for 1 hour at 37 °C. The following steps were performed following the manufacturer's instructions. 25 µL proteinase K and 200 µL Buffer AL was added and incubated for 1 hour at 56 °C. The mixture was transferred into the DNeasy® Mini spin column and centrifuged for 1 minute at 6000 g. The flow-through was discarded, 500 µL Buffer AW1 was added, and centrifuged for 1 minute at 6000 g. The flow-through was discarded, 500 µL Buffer AW2 was added, and centrifuged for 3 minutes at 20,000 g. The spin column was let to sit open to for 1 minute to let any residual ethanol evaporate. The column was placed in a UV-sterilized 1.5 mL centrifuge tube, a respective volume ultra pure diethyl pyrocarbonate (DEPC) treated water was added, incubated for 1 minute at RT, and centrifuged for 1 minute at 6000 g to elute DNA. DNA samples were stored at −20 °C until further use.

2.9 16S rRNA gene amplification and sequencing

16S rRNA genes were amplified from genomic DNA from 2.8 in a two-step barcoding approach (Herbold *et al.*, 2015). As forward and reverse primer were S-D-Bact-0341-b-S-17 (5'-

CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013) chosen, respectively, with an expected amplicon size of 514 bp. Polymerase chain reaction (PCR) was carried out in 20 µL total volume using DreamTaq DNA polymerase kit (Thermo Fischer, United States). The reaction was set up as shown in table 1. The amplification products were purified with ZR-96 DNA Clean-up Kit (Zymo Research, USA), quantified with Quant-iTTM PicoGreen® dsDNA Assay (Invitrogen, USA), pooled to an equimolar library, and sent for sequencing on the Illumina MiSeq platform at Microsynth AG (Balgach, Switzerland).

2.10 Sequence analysis

An in-house pipeline was used to process barcoded reads from 2.9 (Herbold *et al.*, 2015). The R library DADA2 (Callahan *et al.*, 2016) was used to assign 16S sequences to amplicon sequence variants (ASVs).

2.10.1 Change in relative abundance

Analysis were performed in the R environment. Relative abundance was calculated by dividing reads of each ASV by the total number of reads of the respective sample. Rarefaction curves and Diversity indices were calculated using the vegan package (Dixon, 2003). For this sequence libraries were rarefied to the smallest library (n=670).

The fold change in relative abundance was calculated by dividing relative abundances of sorted ASVs by the unsorted ones. But some ASVs thate were represented in the sorted cohort where below detection limit in the unsorted one. To overcome this problem and to not divide by 0 a pseudo-count p was introduced as described in Ronda *et al.* (2019), which is calculated as follows:

$$p = 10^{\lfloor -\log_{10} n \rfloor}$$

where n is the read count in the sorted sample. Therefore the fold change in relative abundance FC is calculated as follows:

$$FC_i = \frac{RA_{i,sorted} + p_i}{p_i}$$

where RA is the relative abundance.

Table 1: PCR reaction mixtures

1st step PCR		
H_20	13.075	µL
10X DreamTaq buffer	2.000	µL
dNTPs	2.000	µL
forward primer	0.400	µL
reverse primer	0.400	µL
DreamTaq DNA polymerase	0.125	µL
DNA template	2.000	µL
2nd step PCR		
H_20	13.075	µL
10X DreamTaq buffer	2.000	µL
dNTPs	2.000	µL
barcode primer	1.600	µL
DreamTaq DNA polymerase	0.125	µL
DNA template	1.200	µL

3. Results

3.1 Comparison of BONCAT and Raman/D_2O

3.1.1 Testing AHA concentration

Two concentrations, 10 µM and 100 µM of AHA were tested on a *Bacillus subtilis* pure culture. Samples were taken after 30 min and 120 min. Figure 4 shows the fluorescent images. After 30 min all cells were sufficient labeled for both concentrations. According to Hatzenpichler *et al.* (2014) it usually takes less than one cell cycle to get sufficient labeling. Regarding that AHA has a 390 times lower chance to get incorporated than methionine using a higher AHA concentration would also increase the sensitivity of the method. Therefore, for all following experiments a final concentration of 10 µM AHA was used, which is the lower end of the concentration spectrum which can be used.

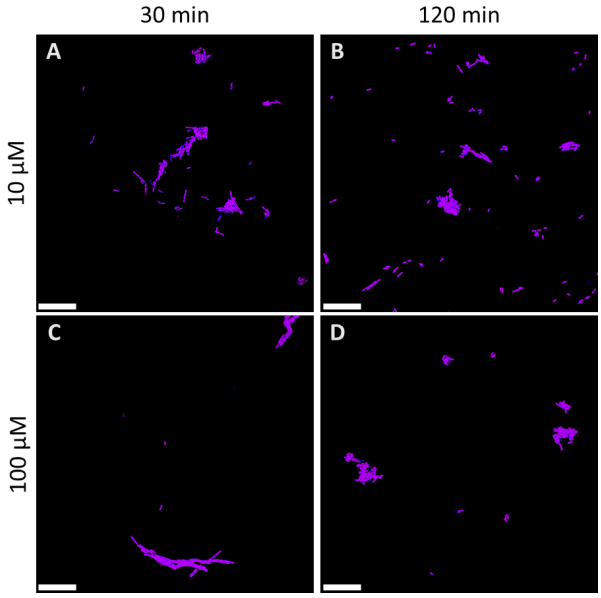


Figure 4: Testing AHA concentration *B. subtilis was incubated with two different concentration of AHA (10 µM and 100 µM) for 30 min and 120 min.*

3.1.2 Relationship between BONCAT fluorescent intensity and %CD

Figure 5 (A) shows a fluorescent image of a fecal sample with indication of labeled and non-labeled cells. There is no real correlation between the intensity of the CD-peak and the fluorescent signal (Figure 5 C), but a threshold of approximately 5%-CD (Figure 5 B) can be made for

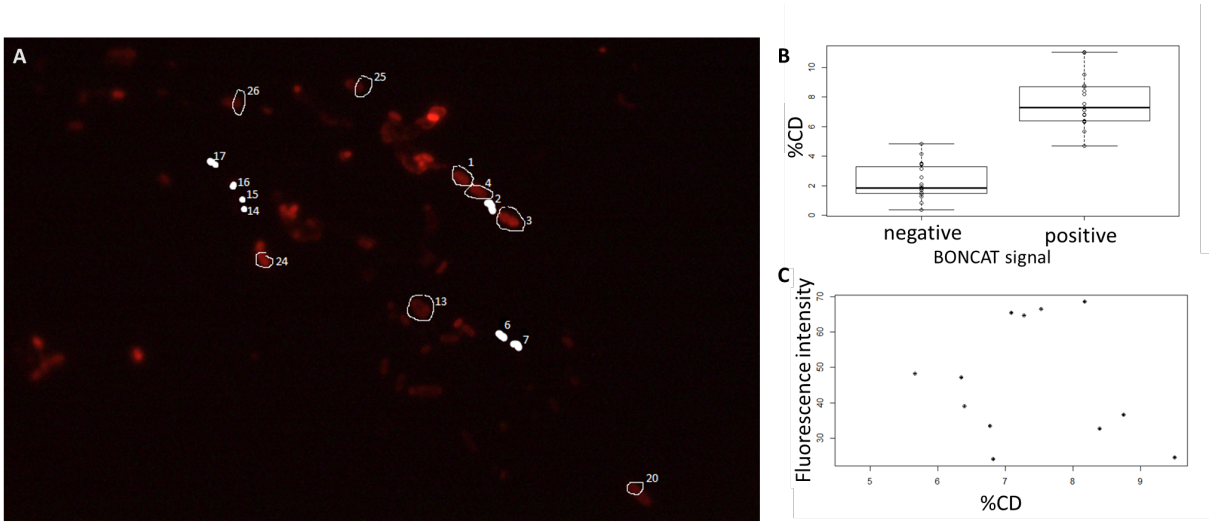


Figure 5: BONCAT vs RAMAN (a) *Fluorescent image of fecal bacteria after BONCAT. White circles indicate BONCAT positive cells. White dots indicate BONCAT negative cells.* ***(b)*** *Bar plot of BONCAT positive and negative cells compared to %CD.* ***(c)*** *Dot plot showing relation between %CD and fluorescence intensity.*

the specific instruments that have been used. It was found that some D_20-Raman positive cells were BONCAT negative, when cells were fixed with PFA. This was not the case with ethanol fixed cells. Therefore, an ethanol fixation was used for the following experiments.

3.2 BONCAT features

3.2.1 AHA is not triggering bacterial activity by itself

Even though AHA is not a canonical amino acid, it could be still used by organisms as a carbon or nitrogen source. Incubating fecal samples with just AHA shows no significant activity increase compared to a no amendment incubation (Figure 6). It can be assumed that no bias is introduced from AHA addition.

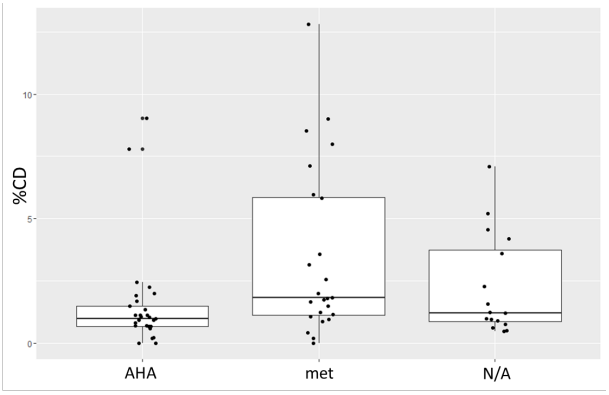


Figure 6: AHA activity *%CD of fecal bacteria incubated with AHA, methionine (met), and no amendment control.*

3.2.2 AHA and its competitor methionine

With increasing methionine to AHA ratio the fraction of labeled cells decrease in a negative

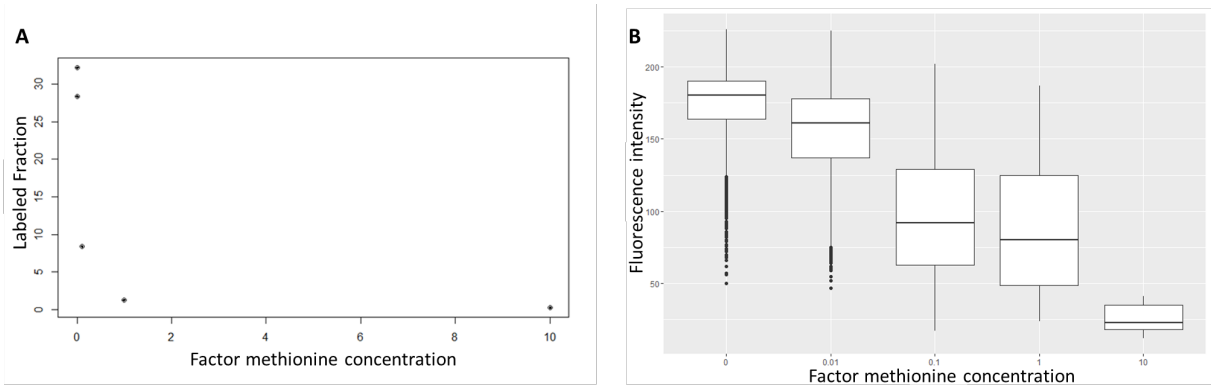


Figure 7: AHA vs met *Relation between methionine concentration and* ***(a)*** *labeled fraction,* ***(b)*** *fluorescence intensity.*

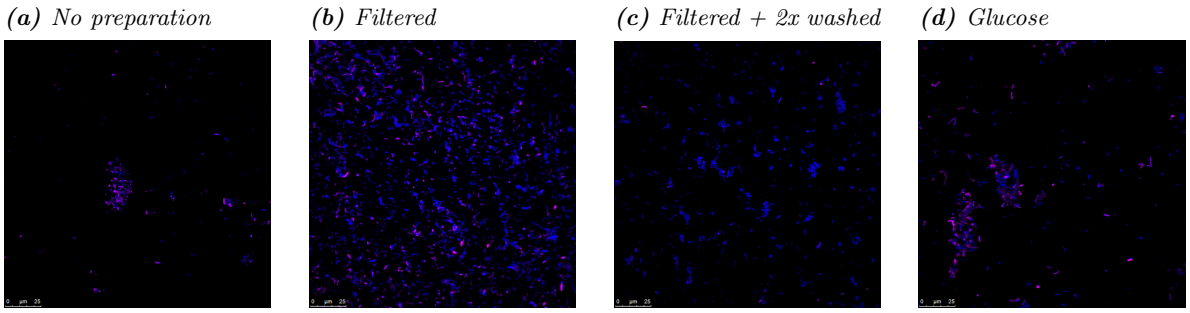


Figure 8: Optimizing the inoculum *Incubation effects of filtering and washing of fecal samples.* ***(a)*** *untreated sample* ***(b)*** 5 µm *filtered* ***(c)*** 5 µm *filtered and two times washed with PBS* ***(d)*** 5 µm *filtered, two times washed with PBS and added glucose.*

exponential way. The average fluorescence intensity of individual cells was also lower with higher methionine concentrations. This should be considered when dealing with nutrient (methionine) rich environments, like fecal matter.

3.2.3 Optimized inoculum

One big challenge working with general activity markers and environmental samples is, that it is hard to differentiate between activity triggered by the substrate of interest and activity that is triggered by residual environmental nutrients. An unprepared as well as a 5 µm filtered inoculum shows a high activity, whereas a filtered and washed one shows barely any activity. Upon addition of glucose again a high activity becomes visible (see Figure 8). Additionally, washing steps will reduce methionine concentration, which competes with AHA (see 3.2.2) in translational incorporation and reduces sensitivity.

Table 2: Normalized Richness *Richness values for unsorted (post incubation no amendment) and sorted (post incubation choline) samples. Percent values indicate the difference from unsorted to sorted.*

Sample	A	B	C	D	E	F	G	H	I	K
Unsorted	93	94	93	115	98	106	95	109	92	105
Sorted	47	47	49	71	102	90	85	98	75	93
%	50.54	50.00	52.69	61.74	104.08	84.91	89.47	89.91	81.52	88.57

3.3 FACS sorted samples

ASV richness is quite similar between the unsorted samples, whereas between the sorted samples it is more divergent. Overall richness decreased in all sorted samples except one (E), ranging from 50% to ∼90% of the unsorted samples, where samples A, B and C show the highest decrease as shown in Table 2. Also diversity dropped for all samples, but each in different magnitude ranging from -1.39 for sample I to -0.09 in sample E for Shannon Index (Fig. 9).

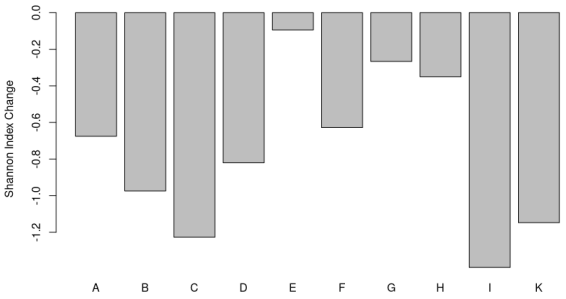
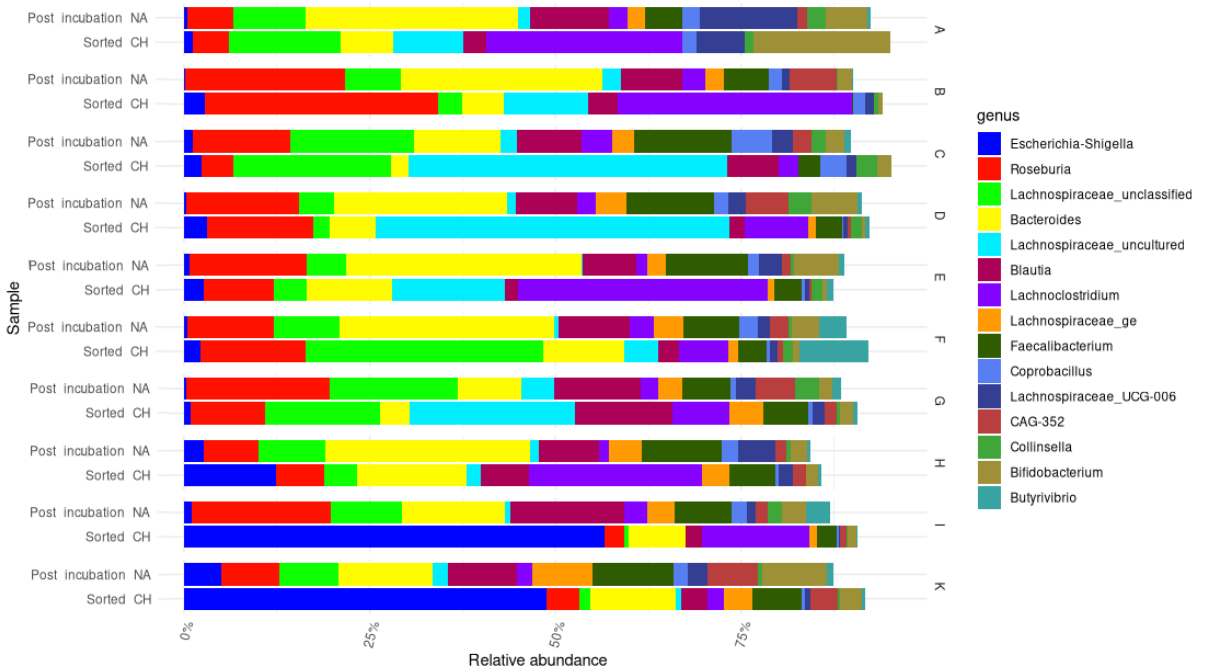


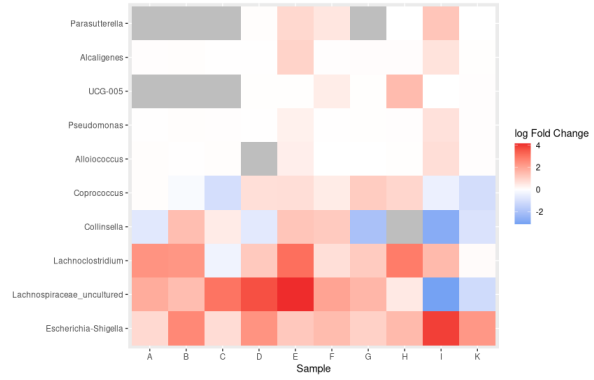
Figure 9: Change in Shannon Index *Diversity drop after FACS sorting.*

Figure 10a shows the 16S profile of the unsorted post incubation NA control community and sorted choline incubated one. A significant change in abundance can be observed in four taxa. A decrease in *Bacteroides sp.*, an increase in uncultured *Lachnospiraceae*, and *Lachnoclostridium*. Only in samples I and K the *Escherichia-Shigella* taxon was drastically increased. Which can be also seen on Figure 10b for better visualization on fold change.

(a) 16S relative abundance



(b) Genus with highest fold change



(c) Uncultured Lachnospiraceae sp. ASVs

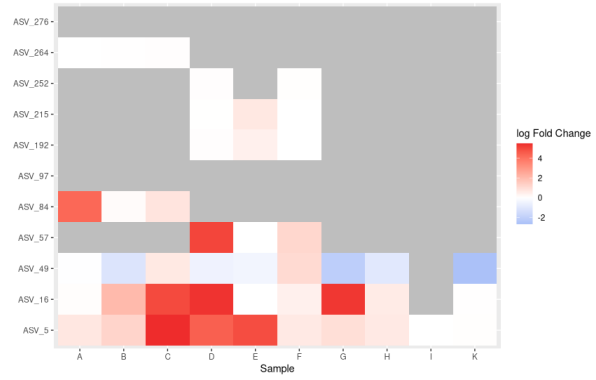


Figure 10: 16S relative abundance (a) *16S barplot of unsorted post incubation community without choline and sorted choline active community.* ***(b)*** *Heat map of logarithmic relative abundance change.* ***(c)*** *Blast best hits for uncultured Lachnospiraceae sp. ASV_5 ∼ Enterocloster bolteae, ASV_16 ∼ Enterocloster citroniae, ASV_49 ∼ Lachnoclostridium sp., ASV_57 ∼ bacterium NLAE-zl-H11 / Enterocloster bolteae, ASV_84 ∼ Anaerostipes hadrus, ASV_97 ∼ Clostridium fessum / Lachnoclostridium edouardi, ASV_192 ∼ Enterocloster bolteae, ASV_215 ∼ Enterocloster bolteae, ASV_252 ∼ bacterium NLAE-zl-H11 / Enterocloster bolteae, ASV_264 ∼ Lachnospiraceae bacterium / bacterium NLAE-zl-G417 / Faecalicatena contorta, ASV_276 ∼ Clostridium sp. C5-48*

4. Discussion

No real correlation was found between fluorescent intensity and %CD, except that a BONCAT fluorescent signal can be detected at approximately 5 %CD. It is not conclusive whether D_2O-Raman spectroscopy or BONCAT (in terms of fluorescent intensity) depicts more a quantitative approach in activity detection (see 3.1.2). Note that both methods work in different ways and even in pure cultures heterogeneous single cell activity may be observed. Nonetheless gives the imaging nature of BONCAT the advantage of accessing the activity of whole communities in one picture, and for example when combined with FISH, activity can be assigned taxa of interest. In this thesis BONCAT was combined with FACS, a well established method for high throughput cell sorting application, and 16S amplicon sequencing to access the identity of active microbial members, without the need of prior knowledge of community composition.

The big challenge when dealing with general activity markers in environmental samples is to differentiate whether the detected activity comes from remaining nutrients from the environment, especially when handling fecal samples, or from the substance of interest. To overcome this problem fecal samples where filtered and washed so that dissolved and undissolved nutrients where removed, basically extracting cells from the environmental samples (see 3.2.3). Unfortunately this introduces another bias, because the chemical environment is drastically changed for the bacterial community, which may lead in an underrepresentation of specific substrate utilizers. Additionally, to counteract high activity of gut microbiota, the concentration of the marker AHA was chosen on the lower end at 10 µM, which is sufficient for proper labeling (see 3.1.1), so that basic cell upkeep does not give the impression that detected activity was induced by substrate. One can also omit the cell extraction step and instead choose a high enough dilution factor, so that it is possible to distinguish between substrate amendment and baseline activity, provided that a mentionable portion of the community may utilize provided substrate, which is not given for choline (Rath *et al.*, 2017). It is to be kept in mind that AHA is a Met derivative,

hence both will compete for incorporation and therefore a high Met containing environment will lower the sensitivity (see 3.2.2). In total it is a trade off between nonspecific signal and a narrowed view of the substrate degrading community. Nonetheless it may give first insights in the big ecological question of "who is doing what", or in this case: What organisms are able to utilize choline in the human gut?

The presence of the functional genes cutC/D in the genome, either from a clone or binned from a metagenome, is a good indicator, but experimental validation is still needed to make sure that the function is really carried out. It would be a tedious task to screen every possible pure culture, so an activity based approach, like BONCAT, makes it possible to screen all organisms in an environmental sample with the advantage that it also covers uncultured organisms.

In vitro incubation, FACS sorting, and sequencing resulted in a drop in richness and alpha-diversity, like expected, as the FACS sorted community represents a subset of the unsorted community. However the change occurred to a different degree across the samples. This may come due to the heterogeneous character, like community composition or fecal consistency, of the samples, which each originates from a different individual, or sample handling. Nonetheless a clear shift in relative abundance with some dominating taxa can be observed in the sorted samples (see fig.10).

The most considerable taxa, that were enriched by sorting were *Escherichia-Shigella*, *Lachnoclostridium*, and ASV 5 and ASV 16 of the uncultured *Lachnospiracae* cluster, which the next relative belongs to the *Enterocloster* genus according to best BLAST hit results. It is known that all of the above mentioned genera have members that harbor the cutC/D genes.

In several studies it was shown that *Lachnoclostridium sp.* abundance correlated positive with TMA levels. Matsumoto *et al.* (2017) showed in a double-blind, placebo-controlled study, that supplementation with *Bifidobacterium animalis subsp. lactis* LKM512 probiotics reduced colonic TMA levels, as well as the abundance of *Lachnoclostridium sp.* in humans. Likewise Li *et al.* (2021) found out, that a fiber rich diet, which promotes SCFA producing bacteria, would have similar effects in mice. This fit well with the finding of Nogal *et al.* (2021), who showed that *Lachnoclostridium sp.* correlates negatively with circulating SCFA levels. A recent publication by Cai *et al.* (2022) identified by integrated metagenomics *Lachnoclostridium sp.* a major player in the production of TMA, which was also significantly abundant in patients suffering from atherosclerosis.

Even considering possible limitations of the experimental setup, it was possible to detect a major player of choline degradation with BONCAT-FACS and subsequent 16S rRNA gene sequencing - *Lachnoclostridium sp.*. This was shown before mostly by correlation and metage-

nomics in current literature, as mentioned above and this study is, to the author's knowledge, the first to give an experimental proof of its role in TMA production from choline.

Detecting translational active microorganisms via BONCAT in an environmental context is a powerful tool. In this study it was shown how organisms in an environmental sample can be detected that can utilize a certain substrate, but beyond that there are certainly more possibilities. For instance Taguer *et al.* (2021) used a similar BONCAT approach to track physiological changes on a community level in the course of a perturbation in dextran sodium sulfate mouse model for colitis. The authors could show changes in the translational activity profile of the community in the successive transition from a healthy to a disease state of the digestive tract, which would otherwise be missed with conventional 16S rRNA gene taxonomic profiling when comparing the different states. Furthermore, using the main feature of BONCAT, namely the labeling of newly synthesized proteins, not only as proxy for activity but for extracting such proteins for mass spectrometry based proteomics, may give a less noisy and time resolved data for protein expression in cells (Bagert *et al.*, 2014). Further development in experimental design and combination with other techniques may implement BONCAT as an indispensable method for microbial ecology in the future.

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