Dissertation

Influence of Bacteriocins and the Supernatant of a Co-culture (OMNi BiOTiC® AAD10) in vitro and on the Fecal Microbiome in Mice

submitted by

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for the Academic Degree of

Doctor of Medical Science

(Dr. scient. med.)

at the

Medical University of Graz

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2022

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all individuals and organisations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

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- Castellani C, Obermüller B, Kienesberger B, Singer G, Peterbauer C, Grabherr R, Mayrhofer S, Klymiuk I, Horvath A, Stadlbauer V, Russmayer H, Miekisch W, Fuchs P, Till H, Heinl S. Production, Storage Stability, and Susceptibility Testing of Reuterin and Its Impact on the Murine Fecal Microbiome and Volatile Organic Compound Profile, accepted manuscript, Frontiers in Microbiology 2021, vol 12, pp 699858f, DOI: 10.3389/fmicb.2021.699858

- Kienesberger B, Obermüller B, Singer G, Mittl B, Grabherr R, Mayrhofer S, Heinl S, Stadlbauer V, Horvath A, Miekisch W, Fuchs P, Klymiuk I, Till H, Castellani C. (S) - Reutericyclin: Susceptibility Testing and In Vivo Effect on Murine Fecal Microbiome and Volatile Organic Compounds, accepted manuscript, International Journal of Molecular Sciences 2021, vol 22, pp 6424f, DOI: 10.3390/ijms22126424

- Kienesberger B, Obermüller B, Singer G, Arneitz C, Gasparella P, Klymiuk I, Horvath A, Stadlbauer V, Magnes C, Zügner E, López-García P, Trajanoski S, Miekisch W, Fuchs P, Till H, Castellani C. Insights into the Composition of a Co-Culture of 10 Probiotic Strains (OMNi BiOTiC® AAD10) and Effects of Its Postbiotic Culture Supernatant, accepted manuscript, Nutrients 2022, vol 14, pp 1194f, DOI: 10.3390/nu14061194

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Dr. Bernhard Kienesberger, eh

Klagenfurt am Wörthersee, 05.12.2022

Acknowledgements

The present work has been conducted in association with the Doctoral School “Molecular Medicine and Inflammation” at the Medical University of Graz, Austria.

The research was funded by The Austrian Research Promoting Agency (Österreichische Forschungsförderungsgesellschaft, FFG), grant number 867991 (to Christoph Castellani) and by funds from the Institute Allergosan, Gmeinstraße, 8052 Graz, Austria (to Bernhard Kienesberger).

Animal studies were approved by the Austrian Veterinary Board (BMBWF 66.010/0153-V/3b/2019).

Doctoral student Bernhard Kienesberger received funding from the Medical University of Graz through the Doctoral School Molecular Medicine and Inflammation (MMI).

The funders had no role in the design of the study, in the collection, analyses or interpretation of data, writing of the manuscripts or in the decision to publish the results.

Danksagung

Mein besonderer Dank gilt meinem Erstbetreuer und Doktorvater Assoz. Prof. Priv.-Doz. Dr. med. univ. Christoph Castellani, der mich in allen Phasen dieser Arbeit und des Projekts stets aufopferungsvoll unterstützt und gefördert hat, durchwegs zur Hilfe war und dies alles erst ermöglicht hat. Vielen lieben Dank!

Weiters möchte ich mich auch bei meinen Betreuern Assoz. Prof. Priv.-Doz. Dr. med. univ. Georg Singer und Priv.-Doz.in Dr.in med. univ. Daniela Sperl herzlichst bedanken, die mir stets zur Seite standen und mich unterstützen. Auch für die ständige Unterstützung und Hilfe seitens Mag.a rer. nat. Dr.in scient. med. Beate Obermüller möchte ich mich hier aufrichtig bedanken.

Zusätzlich bin ich Prof. Holger Till sehr dankbar, der stets ermutigend zur Seite stand.

Großer Dank gilt auch sämtlichen Koautoren, die bei diesem Projekt höchst professionell und hilfreich mitgewirkt haben.

Für die finanzielle Unterstützung möchte ich mich bei der Doc-School Molecular Medicine and Inflammation der Medizinischen Universität Graz und bei Allergosan aufrichtig bedanken.

Ganz besonderer Dank gilt meinen Freunden, insbesondere Prof. Johannes Schalamon und DDr. Christoph Arneitz, die immer, sowohl in stressigen Zeiten als auch in entspannten Zeiten, für mich da waren und mich gefördert haben.

Zu guter Letzt möchte ich natürlich auch meiner Familie großen Dank aussprechen, die in allen Belangen an meiner Seite stehen.

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Abbreviations and Definitions

µg	Microgram
µl	Microliter
1,3-PDO	1,3 – Propandiol
16S	RNA part of the 30S subunit of a prokaryotic ribosome
3-HPA	3-Hydroxypropionaldehyde
515F	5'-GTGYCAGCMGCCGCGGTAA-3'
806R	5'-GGACTACNVGGGTWTCTAAT-3'
AAD	Antibiotic Associated Diarrhoea
ANCOM	Analysis of Composition of Microbiomes
ANOVA	Analysis of Variance
AQ	Aqua
ATCC	American Type Culture Collection
AUC	Area Under the Curve
B.	*Bifidobacterium*
BALB/c	Concatenation "Bagg and Albino" (laboratory mouse strain)
BMBWF	"Bundesministerium für Bildung, Wissenschaft, Forschung"
C.	*Clostridium*
CFS	Cell Free Supernatants
CFU	Colony Forming Unit
cm	Centimetre
Da	Dalton
DADA	Divisive Amplicon Denoising Algorithm
DNA	Desoxyribonucleic Acid
E. coli	*Escherichia coli*

E. faecium	*Enterococcus faecium*
F	F Statistic Value (in combination with p-value; ANOVA)
FASTQ	Text-based Format for Biological Sequences and Scores
FDR	False Discovery Rate
FXR	Farnesoid X Receptor
G	Gauge
g	Gram
GALT	Gut Associated Lymphoid Tissue
GC	Gas Chromatograph / Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GMP	Good Manufacturing Practice
GPCR	G-Protein-Coupled Receptor
h	Hour/hours
H_2SO_4	Sulfuric acid
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HPC	High-Performance Computing
HPLC	High Pressure / Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
IATA	International Air Transport Association
IEC	Intestinal Epithelial Cells
IL-6/10	Interleukin 6/10
INF	Interferones
IQR	Interquartile Range
l	Litre

L.	*Lactobacillus*
LAB	Lactic Acid producing Bacteria
LC	Liquid Chromatograph / Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Effect Size
Log10	Logarithm with base 10
M	Molar
MAMPs	Microbe-Associated Molecular Patterns
m/q	m/q Ratio
ME	Methylester
min	Minute/minutes
MiSeq	Sequencer by Illumina, Inc.
ml	Millilitre
mm	Millimetre
MRS	Culture medium by De Man, Rogosa and Sharpe
MS	Mass Spectrometry
MSD	Mass Selective Detector
MVA	Multivariate Analysis
mzXML	Data format for MS-based proteomics data collection
n	Number
N_2	Molecular nitrogen
ng	Nanogram
NH_3	Ammonia
NIST	National Institute of Standards and Technology

nm	Nanometre
nM	Nanomolar
NOD	Nucleotide-binding and oligomerisation domains
NSF/ANSI	National Sanitation Foundation/American National Standards Institute
O_2	Molecular oxygen
OD600	Optical Density measured at a wavelength of 600nm
OTU	Operational Taxonomic Unit
P.	*Pseudomonas*
PBS	Phosphate-Buffered Saline
PCoA	Principal Component Analysis (plot)
PCR	Polymerase Chain Reaction
PE	Pentylester
pH	“Potential of hydrogen” (pH scale)
Pr.	*Propionibacterium*
PrE	Propylester
PRR	Pattern Recognition Receptor
QC	Quality Control
Qexactive	Quadrupole Orbitrap (mass spectrometer)
QIIME	Quantitative Insights Into Microbial Ecology
R	Correlation coefficient R
R	Reuterin
RA	Room Air / Relative Abundanz (Abundance)
RDA	Redundancy Analysis
RM	Reuterin system Medium
RNA	Ribonucleic Acid

rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
RSD	Relative Standard Deviation
RZ	Reutericyclin
S	Svedberg unit (sedimentation rate)
S.	*Staphylococcus*
S48	OMNi BiOTiC® AAD10 supernatant after 48 h culture time
S196	OMNi BiOTiC® AAD10 supernatant after 196 h culture time
S/SN	Supernatant
SCFAs	Short Chained Fatty Acids
SD	Standard Deviation
SHIP	SH_2 domain-containing Inositolpolyphosphate 5-phosphatase
sL/min	Standard litre per minute
SM	Supernatant Medium
SPME	Solid Phase Micro Extraction
SPF	Specific pathogen free
Str.	*Streptococcus*
TGR5	Takeda G protein receptor 5
TGF	Transforming Growth Factor
TIBCO	The Information Bus Company
TJs	Tigh Junctions
TNF-α	Tumour Necrosis Factor α
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UHPLC	Ultra High Pressure / Performance Liquid Chromatography

UVA	Univariate Analysis
Var	Variance
VOC	Volatile Organic Compound

List of Devices/Manufacturers

1,2-PDO	Sigma Aldrich Handels GesmbH, Vienna, Austria
Agar-Agar	Kobe I, no5210-2, Carl Roth GmbH, Karlsruhe, Germany; TSA
Aminex HPX-87H column	Bio-rad Laboratories, Feldkirchen, Germany
ammonium citrate tribasic	Sigma Aldrich Handels GesmbH, Vienna, Austria
Amphotericin B	Ampho-Moronal® Suspension 100 g/ml, Dermapharm AG, Grünwald, Deutschland
Aurosan GmbH	Essen, Germany
BioCrick Biotech Co., Ltd	Sichuan, PRC
Beef extract powder	no B4888-50G, Sigma-Aldrich Handels GmbH, Vienna, Austria
Brain-Heart-Infusion	no X915.1, Carl Roth GmbH, Karlsruhe, Germany
Breeder mice	Centre for Biomedical Research of the Medical University of Vienna, Austria
Calypso online software	Calypso 8.84®, accessible through http://cgenome.net/wiki/index.php/Calypso
Dipotassium hydrogen phosphate	Sigma Aldrich Handels GesmbH, Vienna, Austria
Disks for resistance testing	BD Sensi-Disc™, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
Dual index primers	New England BioLabs, Frankfurt, Germany
Excel 2016®	Microsoft Corporation, Redmond, WA, USA
Excella E24	Incubator Shaker Series, New Brunswick Scientific, Canada
FastStart™ High Fidelity PCR-System	Sigma, Darmstadt, Germany
Galaxy instance	https://galaxy.medunigraz.at
Gas chromatograph	Agilent 7890 A
Glass vials	Gerstel GmbH, Germany

Glucose	Sigma Aldrich Handels GesmbH, Vienna, Austria
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
gplots package version 3.1.1	gplot2, Elegant Graphics for Data Analysis, New York, NY, USA, https://ggplot2/tidyverse.org, last accessed January 31st 2022
GraphPad Prism 9®	GraphPad, San Diego, CA, USA
HPLC analysis	Shimadzu, Korneuburg, Austria
HUMAnN2	https://github.com/biobakery/humann
ImageJ 2.0.0-rc-69/1.52p	ImageJ open-source image processing software, http://imagej.net/Contributors
Magna Lyzer Green bead Tubes	Roche, Mannheim, Germany
Magna Lyzer instrument	Roche, Mannheim, Germany
Magna Pure LC DNA III Isolation Kit (Bacteria, Fungi)	Roche, Mannheim, Germany
Magnesium sulphate	Merck, Darmstadt, Germany
Manganese sulfate	Sigma Aldrich Handels GesmbH, Vienna, Austria
Mass selective detector	Agilent 5975 C inert XL
Mass spectral library	National Institute of Standards and Technology 2005; NIST 2005, Gatesburg, PA, USA
Mass spectrometer	Qexactive, Thermo Fisher Scientific
Meat extract	VWR, Radnor, Pennsylvania
MetaPhlAn2	https://github.com/biobakery/metaphlan2
MetaCyc	https://metacyc.org; latest access on March 2nd 2022
Micro-Guard Cation H Cartridge	Bio-rad Laboratories, Feldkirchen, Germany
MiSeq desktop sequencer	Illumina, Eindhoven, Netherlands

MRS	Merck, Austria
mzXML	msConvert, ProteoWizard Toolkit v3.0.5
NEBNext Ultra II FS DNA	Library Prep Kit for Illumina, New England BioLabs, Frankfurt, Germany
O_2 absorber	Thermo Fisher Scientific, Waldham, MA, USA
OMNi BiOTiC® AAD10	distributor Institut AllergoSan Graz, Austria producer Winclove Probiotics B.V. Amsterdam, The Netherlands
Orange Data Mining Toolbox v3.31.0	Orange, The University of Lubljana, Lubljana, Slovenia, https://orangedatamining.com/, last accessed January 25th 2022
Oxid Anaerogen 2.5 l	Thermo Fisher Scientific, Waldham, MA, USA
Parallel fermentation system	Eppendorf, Hamburg, Germany
Photometer 2	WPA Biowave, CO8000 Cell Density Meter; WPA, Cambridge, UK
Peptone	Merck, Darmstadt, Germany
PeakScout	developeer Joanneum Research, Graz, Austria
Peptone	no P0431-250G, Sigma-Aldrich, Handels GmbH, Vienna, Austria
Piperacillin/Tazobactam	PIPeracillin/TAZobactam Kabi 4 g/0.5g, Fresenius Kabi, Graz, Austria
Proteinase K	Carl Roth, Germany
Rstudio® version 1.4.1106	R foundation for statistical computing, Vienna, Austria, https://R-project.org/, last accessed January 31st 2022
Sodium acetate	Sigma Aldrich Handels GesmbH, Vienna, Austria
SPSS 26.0®	IBM Corporation, Armonk, NY, USA
SpectraMax Plus 384	Molecular devices, San Jose, USA

SPME fibre	Sulpeco, Bellefonte, PA, USA
Struktol® SB2121 solution	Schill Seilbacher, Hamburg, Germany
SuperPure Water	Milli-Q Direct 8, Merck Millipore, Germany
Thermomixer	HLC, Germany
TIBCO Spotfire	v7.5.0, TIBCO, Palo Alto, CA, USA
TSB	Fluka Analytical, no T8907-500G, Honeywell, Charlotte, NC, USA
Trim Galore	https://github.com/FelixKrueger/TrimGalore
Tween 80	Sigma Aldrich Handels GesmbH, Vienna, Austria
Vancomycin	Hikma® 500 mg, Hikma Pharma, Planegg, Germany
Vitamin B12	Sigma Aldrich Handels GesmbH, Vienna, Austria
Vsearch	https://github.com/torognes/vsearch
Yeast extract	Fluka, Buchs, Switzerland

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Zusammenfassung

Einleitung: Postbiotika als Alternativen zu Probiotika zur unterstützenden Behandlung einer Dysbiose können für bestimmte Risikopatient*Innen, wie zum Beispiel Immunsupprimierte, Frühgeborene oder sehr alte Patient*Innen, von Interesse sein. Unser Ziel war es, Einblicke in die Produktion, Zusammensetzung, antimikrobielle Aktivität und den Effekt auf das fäkale Mikrobiom von verschiedenen Postbiotika zu erlangen.

Methoden: Als Postbiotika wurde ein Kulturüberstand von OMNi BiOTiC® AAD10, das Reuterin System als Kulturüberstand von *Lentilactobacillus diolivorans* und kommerziell bezogenes (S) - Reuterizyklin gewählt. Die Charakterisierung der Überstände erfolgte mittels HPLC (Reuterin) beziehungsweise Shotgun Analyse, Flüssigchromatographie-Massenspektroskopie (LC-MS) und Gaschromatographie-Massenspektroskopie (GC-MS) (OMNi BiOTiC® AAD10). Der antimikrobielle Effekt aller 3 Postbiotika wurde in Kulturen Gram positiver und Gram negativer Bakterien sowie *Candida albicans* untersucht. Zur Bestimmung des Effektes auf das fäkale Mikrobiom wurden die 3 verschiedenen Postbiotika im Vergleich zu Kontrollmedien bei BALB/c Mäusen gavagiert. Nach einem Zeitraum von 4-8 Wochen (je nach Postbiotikum) wurden der Stuhl gewonnen und die Mäuse euthanasiert. Aus dem Stuhl wurden eine Mikrobiombestimmung (16S Sequenzierung für Region V3) sowie eine Analyse der volatilen organischen Substanzen (VOSs, mittels GC-MS) durchgeführt.

Ergebnisse: Charakterisierung der 3 Supernatants: Die 10 probiotischen Stämme von OMNi BiOTiC® AAD10 zeigten ein sehr heterogenes Wachstum: die relative Abundanz (RA) von *L. salivarius*, *L. paracasei*, *E. faecium* und *B. longum/lactis* nahm über die Kulturzeit ab. Die RA von *L. plantarum* nahm zu, während *L. rhamnosus* nach initialer Zunahme bis 96h Kulturzeit im weiteren Verlauf wieder abfiel. Die Analyse der Genexpressionen zeigte eine Dominanz von Genen der Nucleotid und Aminosäuren Biosynthese, vereinbar mit dem Bakterienwachstum, wobei die Zeitpunkte der maximalen Genaktivität der verschiedenen Stämme mit dem Peak ihrer RA zusammenfielen. Die Metabolomics Analyse des Kulturüberstandes zeigte vorwiegend Veränderungen in den Gruppen Aminosäuren/Peptide und Nukleotiden, was den Ergebnissen der Genanalyse entspricht. Fumar-, Pantothen-, Apfel-, 9,3-Methyl-2-oxovalerian- und Asparaginsäure, Cytidinmonophosphat, Kreatin, Tryptophan, Orotidin und Phosphoserin korrelierten dabei mit der Kulturzeit. Für die Resistenztestung und die murine Applikation wurde zellfreier Kulturüberstand nach 48h (Frühphase) und nach 196h (Spätphase der Kultur) gewonnen.

Bei der Fermenterkultur von *L. diolivorans* konnte ein Supernatant mit 13,4g/l HPA (Reuterin) und 6.9g/l Glycerol produziert werden. Aufgrund des potentiellen kommerziellen Interesses in diese Substanz wurden auch Lagerungstests bei -20°C durchgeführt und zeigte eine gute Stabilität über 35 Tage.

(S)-Reuterizyklin wurde als Reinsubstanz bezogen und deshalb nicht weiter charakterisiert.

Bei der Resistenztestung zeigte der AAD10 Überstand abhängig von der Kulturzeit des Überstandes Aktivität gegen *S. epidermidis, L. monocytogenes, P. aeruginosae, E. faecium* und *C. albicans* (48h), beziehungsweise gegen *Str. agalactiae* und *S. epidermidis* (196h). Das Reuterin System zeigte Aktivität gegen *S. aureus, S. agalactiae* und *S. epidermidis* und (S)-Reuterizyklin nur gegen *S. epidermidis*.

Bei der *in vivo* Applikation zeigten sich bei der alpha- und beta-Diversität lediglich bei (S) - Reuterizyklin eine reduzierte Betadiversität. Auf Spezieslevel führte der AAD10 Überstand zu einer Zunahme von *Faecalibacterium prausnitzii* und *Anaeroplasma*. Reuterin verursachte einen Anstieg von *Ruminococcae* und *Eubacterium xylanophilum* und einen Abfall von *Lachnospiraceae* und *Ruminoclostridium*. (S) - Reuterizyklin führte zu einer Zunahme von *Muribaculum* und *Streptococcus* sowie einer Abnahme von Butyrat produzierenden *Roseburia*, *Ruminoclostridium* und *Eubacterium.* Die volatilen organischen Substanzen im Stuhl wurden nur für die Versuchsgruppen mit Reuterin und (S) - Reuterizyklin Applikation gemessen. Bei Reuterin konnte eine signifikante Abnahme von Heptan und eine Zunahme von 3-Methylbutanal nachgewiesen werden. Bei (S) - Reuterizyklin zeigten sich signifikante Anstiege von Heptan und Pentan und Abfall von 2-Heptanon und 2,3-Butandion.

Schlussfolgerungen: Alle 3 postbiotischen Kulturüberstände zeigten unterschiedliche antimikrobielle Aktivitäten im aeroben Bereich. Dieser Wirkung könnte in Zukunft eine Bedeutung als selektives Antibiotikum zum Beispiel in der Therapie von atopischen Hautkrankheiten (Wirksamkeit von Reuterin gegen Staphylokokken und Streptokokken), Nahrungsmittelstabilisator (Wirksamkeit von AAD10 Supernatant gegen *L. monocytogenes*) oder gezieltes Antibiotikum (Wirksamkeit von AAD10 Supernatant gegen *E. faecium*, *L. monocytogenes* und *P. aeruginosae*) zukommen. Während (S) - Reuterizyklin mit reduzierter Betadiversität und Rückgang der relativen Abundanz von Butyratproduzenten eher negative Effekte auf das murine fäkale Mikrobiom hatte, war die Wirkung vom Reuterin und AAD10 Überstand unspezifisch. Die Bedeutung der beobachteten Veränderungen der volatilen Stuhlgase als Marker des bakteriellen Stoffwechsels bedarf noch weiterer Forschung.

Abstract

Introduction: Postbiotics as alternatives to probiotics for the supportive treatment of dysbiosis may be of interest for certain high-risk patients (as neonates and immunocompromised or elderly patients). This study aimed to gain insights into the production, composition, antimicrobial activity and effect on the fecal microbiome of three different postbiotics.

Methods: Culture supernatants from OMNi BiOTiC® AAD10, the reuterin system as supernatant from *Lentilactobacillus diolivorans* and commercially available (S) - Reutericyclin were chosen as postbiotics. The supernatants were characterized by HPLC (Reuterin) or shotgun analysis, liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectroscopy (GC-MS) (OMNi BiOTiC® AAD10). The antimicrobial effect was examined in cultures of Gram-positive and Gram-negative bacteria and *Candida albicans*. To identify the effect on the fecal microbiome a murine model with male BALBc mice was chosen. Mice were gavage fed with either postbiotic substance or control medium for 4-8 weeks (depending on the postbiotic). At the end stool was harvested and mice were euthanized. Stool samples were used for microbiome analysis (16S analysis of V3 region) and determination of volatile organic compounds (VOCs) (GC-MS).

Results: Characterization of the 3 different supernatants: The 10 probiotic bacteria of the AAD10 showed different growth patterns during the observation period of 196h. The relative abundance (RA) of *B. longum/lactis, E. faecium. L. salivarius* and *L. paracasei* decreased over time. The RA of *L. plantarum* increased while *L. rhamnosus* showed an initial increase to 96h of culture time followed by a decrease towards the end of the observation period. Gene expression analysis revealed a dominance of gene activity in the group's nucleotide and amino-acid biosynthesis corresponding with cell growth. The peaks of the gene expressions of the different probiotic strains coincided with their peak RA. The metabolomics analysis confirmed these findings with a dominance of amino acids, peptides and nucleotides in the cell free AAD10 supernatant. Fumaric, pantothenic, malic, 9,3-methyl-2-oxovaleric and aspartic acid, cytidine monophosphate, creatine, tryptophan, orotidine and phosphoserine correlated with culture time. For susceptibility testing and murine application cell free supernatant was harvested after 48h (early phase) and 196h (late culture phase).

The fermenter culture of *L. diolivorans* allowed production of the supernatant containing 13.4g/l 3-HPA (Reuterin) and 6.9g/l glycerol. Due to a potential commercial interest storage stability was tested for this supernatant and revealed a good stability over 35 days at -20°C.

As (S) - Reutericyclin was obtained as pure substance no further characterizations were performed for this postbiotic.

In susceptibility testing the AAD10 supernatant showed activity against *E. faecium, L. monocytogenes, P. aeruginosae, S. epidermidis* and *C. albicans* (48h), or against *S. agalactiae* and *S. epidermidis* (196h). The Reuterin system showed activity against *S. aureus, S. agalactiae* and *S. epidermidis* and (S) - Reutericyclin against *S. epidermidis* only.

The murine application revealed a significant decrease of beta diversity in the fecal microbiome of mice receiving (S) – Reutericyclin. Neither AAD10 Supernatant nor the Reuterin system had effects on alpha or beta diversity. At species level, AAD10 supernatant increased *Faecalibacterium prausnitzii* and *Anaeroplasma*. Reuterin caused a rise in *Ruminococcae* and *Eubacterium xylanophilum* and a depletion in *Lachnospiraceae* and *Ruminoclostridium*. (S) - Reutericyclin caused an increase in *Muribaculum* and *Streptococcus* and a depletion in butyrate-producing *Roseburia, Ruminoclostridium* and *Eubacterium xylanophilum*. The VOC testing in the stool was only performed for Reuterin and (S) - Reutericyclin groups. A significant depletion in heptane and a growth in 3-methylbutanal could be demonstrated for Reuterin. Significant increases in heptane and pentane and decreases in 2-heptanone and 2,3-butanedione were found for (S) - Reutericyclin.

Conclusions: The 3 different postbiotic culture supernatants showed different antimicrobial profiles against aerobic bacteria. These effects could be used as “selective” antibiotic in the future – for instance in the therapy of atopic dermatitis (effect of Reuterin against staphylococci and streptococci), food stabilizer (effect of AAD10 supernatant against *L. monocytogenes*) or targeted antibiotic (effect of AAD10 supernatant against *E. faecium, L. monocytogenes* and *P. aeruginosae*). While (S) – Reutericyclin with reduced beta diversity in the microbiome analysis and a decreasing relative abundance of butyrate producers had rather negative effects on the murine fecal microbiome, the effects of Reuterin and AAD10 supernatant were rather unspecific. The importance of the observed changes of volatile stool gases as a marker of bacterial metabolism remains to be addressed in future research projects.

1. Introduction

1.1. The Microbiome

The term microbiome defines the total set of genes present in certain body organs (for instance the intestine). The human microbiome contains more than 100 trillion microbial cells (1) and is composed of bacteria, archaea, protists and fungi (2-4). Approximately 90% of all human cells are in some association with microbial cells demonstrating, that each organ has its specific flora (5, 6). Mainly, it is essential to note, that each human and animal individual houses a complex microbiome in diverse body sites greatly differing in arrangement and function (7-9).

The initial scientific interest focused on the intestinal microbiome. Consequently, its importance has won broad scientific interest in health and disease. The literature concerning this topic has grown to a vast extent over the last years (2). While the intestine was once considered as a digestive organ only, technologies as 16S rRNA sequencing and in the later years whole genome sequencing have helped to gain a more detailed view on the intestinal microbiome and its activity.

Studies in mammals and humans have revealed that the gut microbiota is involved in many physiological processes essential to the hosts health, including digestion of certain nutrients (xenobiotic degradation), energy homeostasis, metabolism, brain development and behaviour, gut epithelial health and immune activity (2, 3, 7, 9). This makes the gut microbiota an active part in the physiology of the host. Furthermore, the composition of the microbiome, containing beneficial and potentially harmful microbes, seems to be crucial for certain disease developments (10). Consequently, modifications of the fecal microbiome have been described in chronic diseases like the chronic inflammatory bowel disease, type two diabetes, obesity or tumour-associated cachexia (11). The idea of dysbiosis as essential part in the pathology of certain diseases is intriguing as the intestinal microbiome can easily be modified by diet, antibiotics, pre-, pro- or synbiotics conferring health benefits for the patients.

1.1.1. Development and Composition of the Intestinal Microbiome

In the past years there were many speculations about the bacterial colonisation of the meconium. A recent study, however, could prove the sterility of the meconium in utero (12). Consequently, the baby's intestine is colonised for the first-time during delivery. As such the composition of the microbiome depends on time and mode of delivery (13). The colonisation process then continues by feeding and skin contact to the surroundings. Initially, the gastrointestinal tract is colonised by facultatives as *E. coli*, *Enterococcus spp.*, *Strepto-* and *Staphylococci* (14). These bacteria consume oxygen creating an anaerobic condition in the intestine. Coupled with the exposition to maternal milk oligosaccharides, this results into a shift towards an anaerobic environment. This environment consists of anaerobic bacteria including *Bacteroidetes*, *Bifidobacterium* and *Clostridium spp*. (5). The establishment and development of the gut microbiome seems to take place in the first year of age significantly. Breastfeeding, for instance, significantly influences the gut microbiome of infants, especially through the prebiotic effect of human milk oligosaccharides (15). The fermentation of these oligosaccharides stimulates the growth of *Lactobacillus* and *Bacteroides* within the intestine of the infant and promotes the fermentation of short-chained fatty acids and modulates the immune system to express IgG (16). By the age of one year, the core human microbiome has been developed, but is continuously evolving from children to adolescents, adults and elderly (17).

Overall, the phyla with the highest abundances in the adult human intestine are Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria with the most detected genera being *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Eubacterium*, *Fecalibacterium*, *Ruminococcus, Peptococcus* and *Peptostreptococcus* (18). *Bacteroides* are the most abundant with a relative frequency of approximately 30%, proposing them playing a pivotal role in the operating of the organism of the host (19).

In detail however, the intestinal microbiome varies greatly in composition and abundance throughout the different levels of the intestine (Figure 1).

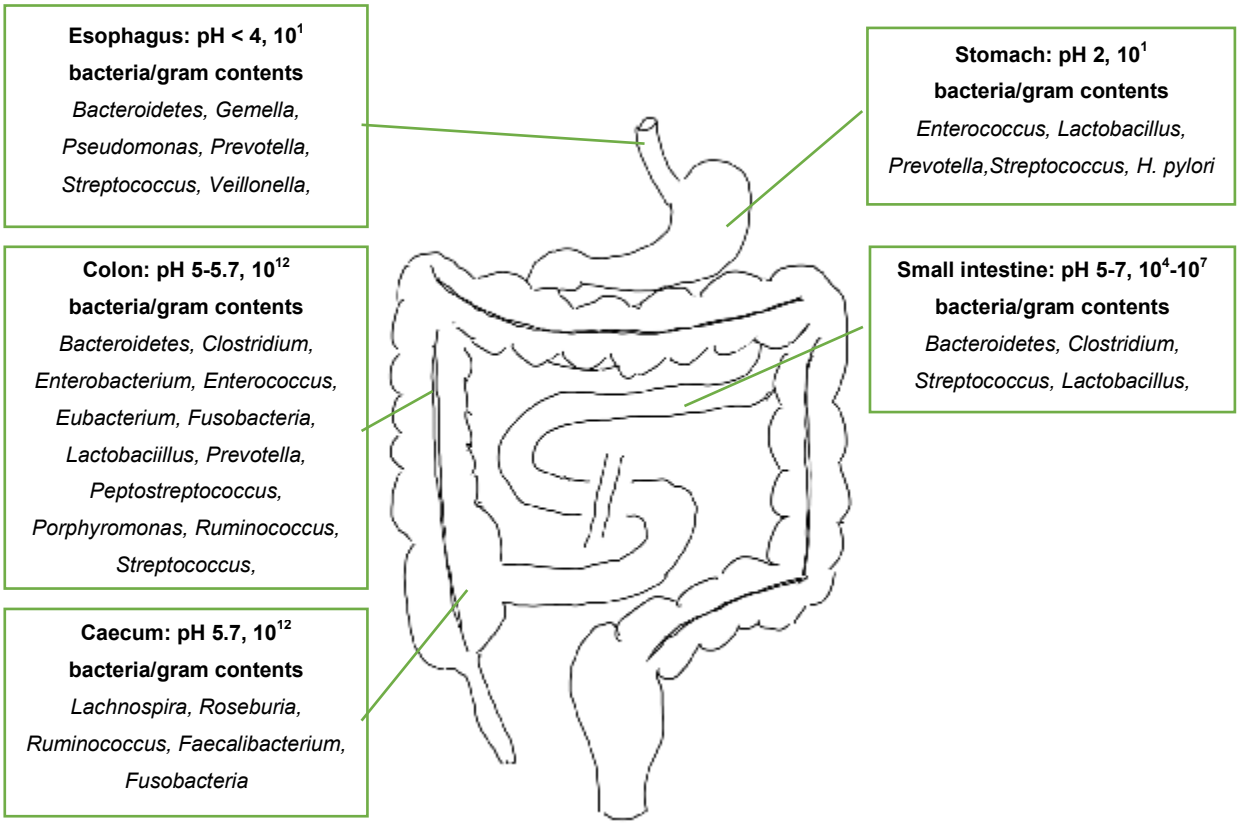


Figure 1: pH and bacteria colonising different levels of the intestinal tract (20)(image created with KingDraw®).

1.1.2. Function of the Intestinal Microbiome

The phyla Firmicutes and Bacteroidetes followed by Actinobacteria and Verrucomicrobia mainly compose an intact and well-functioning gut microbiome (20). The whole gut microbiome is enormously important for nutrient metabolism, antimicrobial protection, immunomodulation and the integrity of the gut barrier (20).

Intestinal microbiota are responsible for “digesting” dietary compounds into bioactive food components. They can metabolise indigestible oligosaccharides like cellulose and starch into short-chained fatty acids (SCFAs) such as propionic, acetic and butyric acids (21). Colonic germs like *Bacteroidetes*, *Enterobacteria*, *Bifidobacterium*, *Faecalibacterium* and *Roseburia* are predominantly responsible for SCFA production (22, 23). SCFAs responsibly influence the progress of the immune system, dietary processing and energy homeostasis. They are essential nutrients for the intestinal epithelial cells of the host and thus have

beneficial impacts on intestinal motility and bowel wall integrity (24). Furthermore, they may be metabolised to Acetyl-Coenzyme A in the liver, serving as an energy source for the host. SCFAs influence immune cell function by interacting with G-protein-coupled receptors (GPCR) 41 and 43. These receptors are expressed on mostly every immune cell like epithelial cells, neutrophils and macrophages for example (25). Maslowski et al., for instance, found GPCR-deficient mice to reveal severe inflammation in mouse models of arthritis, asthma and dextran-sulphate-sodium-induced colitis (26, 27). Furthermore, anti-inflammatory and chemo-preventive qualities of SCFAs have been reported, identifying SCFAs as potential tumour suppressors (28). In this regard, the anti-cancerogenic and anti-inflammatory effects of butyrate and propionate has been previously reported (29, 30).

Furthermore, the microbiota play a beneficial role in vitamin synthesis like biotin, riboflavin, vitamin B and K (31). Gut microbiota took also part in the synthesis of conjugated fatty acids, bile acids and cholesterol (32). The liver generates bile acids from cholesterol and these are further metabolised by intestinal microbiota. They are conjugated to glycine or taurine for better solubility in water (33). First and foremost bile acids like cholic acid and chenodeoxycholic acid are secreted to the gut and serve important functions in fat metabolism. They may be further metabolised to secondary bile acids like lithocholic acid or deoxycholic acid by certain intestinal bacteria (34). Both primary and secondary bile acids also have hormonal functions, as they can work as signalling molecules to control lipid, glucose and energy metabolism. They bind to the nuclear hormone farnesoid X receptor (FXR) and Takeda G protein receptor 5 (TGR5) in diverse organs of the host (35). These conversions seem to modulate and regulate diverse metabolic pathways.

The intestinal immune system is confronted with the challenging need to tolerate beneficial and to fight pathogenic bacteria (20). A simple mechanism against pathogens is the capability of competitive exclusion suppressing the growth of pathogens by nutritional competition (36). The mucus layer is another defensive mechanism and one of the most important factors protecting intestinal epithelia from luminal microbial contact (37, 38). It is produced by colonic goblet cells and consists of a denser, germ free epithelial layer and a more dynamic, loser luminal layer which also provides glycans as nutritional source for microorganisms (39). Additionally, goblet cells can secrete trefoil-factor and resistin-like molecule beta helping in the stabilization of mucin polymers thus maintaining the barrier integrity (40). In contrast to the colon, antimicrobial proteins are very important for the antimicrobial protection of the small intestine (20). Consequently, the gut microbiota and the Paneth cells of the intestinal wall interact closely via pattern recognition receptor (PRR)

mediated mechanisms (41). PRR family includes toll like receptors (TLRs), C-type lectin receptors, and nucleotide-binding and oligomerisation domains (NOD) (42). PRR are turned on by microbe-associated molecular patterns (MAMPs) successively as peptidoglycan, lipopolysaccharide and flagellin activating various (pro-inflammatory) signalling pathways essential for barrier function, mucin production and IgA secretion. Although a healthy microbiome seems to be a premise for AMP production *Lactobacillus* and *Bacteroides* seem to play a key role driving this production (43). Lactic acid producing bacteria (LAB) as *Lactobacillus* generate lactic acid as other important mechanism for antimicrobial protection. By interfering the outer bacterial cell wall lactic acid is able to multiply the antimicrobial activity of the hosts lysozyme (44). Finally, especially Gram-negative bacteria as *Bacteroidetes* were shown to activate dendritic cells in the intestinal submucosa triggering the secretion of secretory IgA (45). The essential role of bile acids and SCFAs has already been discussed above.

Besides the antimicrobial protection, gut microbiota contribute to intestinal immunomodulation. This is accomplished by influencing the gut associated lymphoid tissue (GALT), regulatory and effector T-cells, IgA producing B-cells, resident macrophages and dendritic cells in the lamina propria (20).

Regarding the intestinal wall, the intestinal epithelial cells (IECs) form the inner layer as barrier between luminal contents and host (46). An intact epithelium with competent tight junctions (TJs) is regarded as most important component of this barrier (47). An increased intestinal permeability can result from either loosening of the TJs (either by increased nanoporosity due to altered TJ composition or by distraction of the TJ through myosin contraction) or epithelial defects (apoptosis or necrosis of IECs) (48). Claudins are TJ components responsible for the nanoporosity of TJs (47). Depending on the type of claudin implemented in the TJ its porosity can increase (claudins 2 or 10) or decrease (claudins 1, 3, 4, 8 or 15) (47). These changes are mediated by either anti-inflammatory (TGF and IL-10 leading to decreased porosity) or pro-inflammatory (IL6, TNF, INF and others leading to increased porosity) cytokines (49). This displays the close interaction between intestinal microbiome, microbial products, intestinal wall, metabolism and inflammation.

1.1.3. Examples of Lifestyle Impacts on the Intestinal Microbiome

Nutrition is substantial in the development and change of the intestinal microbiome. For instance, a dominance of *Bacteroidetes* has been previously reported in vegetarians' microbial composition (50). Non-digestible fibres are essential substrates for the production of SCFAs. Consequently, fibre consumption is essential for keeping up the integrity of the intestinal gut barrier (51). In contrast, consuming a high fat and high protein Western diet, the abundance of bile tolerant organisms like *Alistipes* and *Bilophila* increases while members of the phylum Firmicutes decrease (52, 53). This type of diet is known to cause negative effects on the immune system rising the risk of infections and the development of metabolic diseases (7) and intestinal cancer.

Next to its direct impacts on the human body, smoking has also been revealed to influence the intestinal microbiota. Alterations of the fecal microbiome composition were noted with an improvement of the relative abundance of Actinobacteria and Firmicutes and a reduced occurrence of Proteobacteria and Bacteroides (7, 54).

1.1.4. Possibilities to Modify the Intestinal Microbiome

An attractive preventive or even supportive therapy approach is represented in the modification of the bacterial composition of the intestine towards a "healthier" microbiome for certain diseases (55). This could be achieved by dietary modifications, antibiotic therapy, stool transplantation which is not covered in this thesis or food supplementation with either pre-, pro- or synbiotics.

The use of antibiotics aims to either destroy pathogens or in limiting their growth rate (bactericidal or bacteriostatic antibiotics). While on the one hand the destruction or reduction of pathogens is a desired effect, most antibiotics are rather unselective and also take impact on beneficial symbionts potentially causing dysbiosis (56). This mechanism may lead to increased growth of pathogens as *Clostridium difficile* or *Salmonella spp.* during or shortly after antibiotic therapy (57). Importantly, the diminished diversity caused by broad-band antibiotics can lead to a long-time reduction with persistent non-recovery of Bacteroidetes after clindamycin intake up to 2 years after administration (58). Similarly, *H. pylori* eradication with clarithromycin caused reduced diversity of *Actinobacteria* with increased resistance gene expressions even 4 years after antibiotic intake (59). Next to a direct impact on

microbial diversity, there are major concerns about the horizontal transfer of resistance genes, which may have major negative impact on antibiotic resistances in the future (60). Consequently, the use of antibiotics should be restricted to severe pathologic health conditions. Special probiotics as OMNi BiOTiC® AAD (antibiotic associated diarrhoea)10 have been specially designed to mitigate negative effects of antibiotic treatment on the intestinal microbiome. Antibiotic therapy with conventional antibiotics aiming to modify the intestinal microbiome may be helpful in selected cases (for instance before stool transplantation).

Substrates selectively utilised by the microorganisms of the host with a health benefit are termed prebiotics (61), whereas probiotics are defined as administered alive microorganisms in adequate doses conferring a host health benefit (62). Synbiotics are a combination of those two in a mixture. They are comprising live microorganisms and adequate substrates. Both of them can be selectively used by host microorganisms to award one or even more benefits on the host (63). The probiotic effect of *Lactobacilli,* for example, has been examined in a variety of *in vivo* experiments revealing advantageous results in several diseases (64). Generally, probiotics are considered as safe. However, there is still a minimal risk for disadvantageous side effects particularly in cohorts of vulnerable patients like elderly, neonates, or immunocompromised patients (65). Fungemia, probiotic bacteremia, an increase of antimicrobial resistances and transformed long-term immune responses have been denoted in this regard (65-70). These patients need an alternative treatment for gut dysbiosis (71).

Inanimate microorganisms and/or their components are termed postbiotics per definition. Postbiotics impart the host´s health benefit (72). Any substance produced by the microorganisms or through their metabolism are included. Both a direct or indirect beneficial effect on their host is possible (73, 74). These substances can either be bacterial lysates, cell free supernatants (CFS), cell wall fragments, enzymes, exopolysaccharides or short-chained fatty acids (SCFAs) (74). CFS is composited of amino acids, bacterial toxins, carbohydrates, electrolytes, lactate and other known and even still unknown bacterial metabolites. Supernatants gained from several different bacterial strains contain various activities. This also includes anti-inflammatory and antioxidative effects on intestinal epithelial cells, neutrophils and macrophages. The effects are reached by the reduction of the levels of pro-inflammatory tumour necrosis factor α (TNF-α) and by the increase of anti-inflammatory interleukin 10 (IL-10) (74). Previous bacterial culture experiments have shown that the growth of pathogenic organisms like *Escherichia*, *Enterobacter, Klebsiella* and *Salmonella*

has been inhibited with success (75-77). In this regard, some gut bacteria can synthesise specific bacterial toxins based on various classes of antimicrobial compounds which are able to stunt the increment or inactivate other competitive bacterial strains defending their own habitat. Some of these antimicrobial compounds such as reutericin or reutericyclin are ribosomally synthesized, post-translationally modified short peptides named bacteriocins (78, 79). Other antimicrobial metabolites such as acetic acid, lactic acid or reuterin are small organic compounds (80, 81). Postbiotics containing bacteriocins and metabolites could be effective alternatives because of the antimicrobial activities to treat dysbiosis in immunocompromised patients for example. The most effective advantages of postbiotics are their inherent stability in industrial production processes and their simple storage. Another benefit is the intellectual property protection because no alive microorganisms can be gained from the postbiotic. Maybe the most important benefit for immunocompromised patients is their better safety profile compared to probiotics (72).

1.2. Reuterin

Reuterin (3-HPA; 3-hydroxypropionaldehyde), firstly mentioned in 1989 is an antimicrobial substance with a very broad spectrum produced by *Limosilactobacillus (L.) reuteri* (former *Lactobacillus reuteri*) (82). It was determined to be a complex fluent component system more recently. The reuterin complex is a fluent system which is generated by the dehydration of glycerol (Figure 2A) to 1,3-propanediol (1,3-PDO) (Figure 2F) (83). Excreted into an aqueous solution containing very low levels or even no glucose, the monomer 3-HPA (Figure 2B) comes across a reversible dimerization and hydration, forming three main components of an equilibrium. These components are 3-HPA (HPA-monomer), its dimer (2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane; Figure 2D) and its hydrate (1,1,3-trihydroxypropane/1,1,3-propanetriol; Figure 2C) (83). So HPA or Reuterin is no single substance but a very complex mixture (83). Even though the HPA-dimer was patented in 1988 as Reuterin, it was held accountable for the antibiotic effects of the complex system (83). The word Reuterin is now widely used synonymously for the complete system itself (84). As 3-HPA spontaneously dehydrates in an aqueous solution, it was proposed to include the resulting molecule acrolein (Figure 2E) defining the Reuterin system more accurately (85).

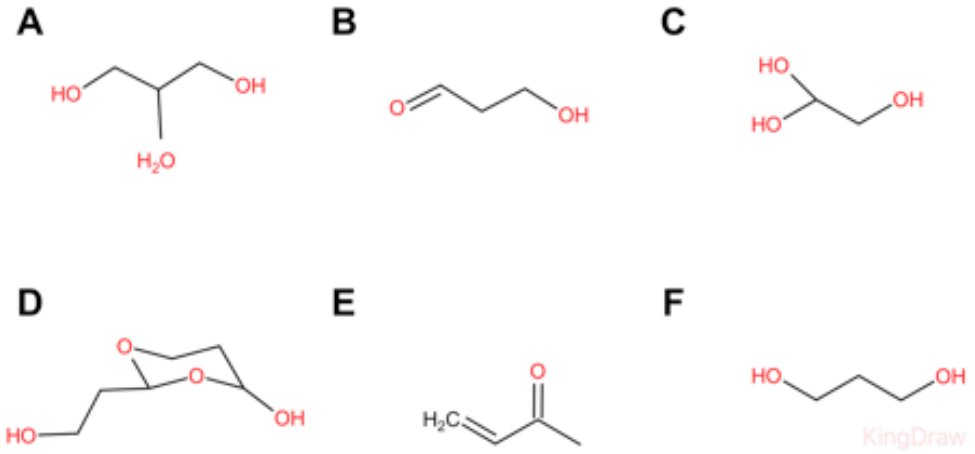


Figure 2: Reuterin System. Created with KingDraw®.

Members of the genera *Lactobacilli* were known to produce 3-HPA, However, in the meantime also *Bacillus*, *Clostridium*, *Citrobacter*, *Klebsiella* and *Enterobacter* (86) have been identified to be able to produce this substance. Nevertheless, *Lactobacilli* produce a plethora of bioactive molecules and therefore are one of the most common prokaryotes used in industry (87). Prior studies have additionally found *L. collinoides, L. reuteri* (88), *L. brevis* (89), *L. buchneri* (89), *L. coryniformis* (90), *L. diolivorans* (91), *L. hilgardii* (92) and *L. pentous* (93) to also metabolise glycerol to 1,3-PDO via 3-HPA. Usually, 3-HPA is an intracellular intermediate that is reduced to 1,3-PDO but does not accumulate. It is secreted into the extracellular space after all (93). Only a few strains of *L. reuteri*, *L. diolivorans* and *L. coryniformis* are known to be able to extracellularly accumulate 3-HPA (92). The mechanisms of production and secretion are still unknown. Interestingly enough, 3-HPA concentrations of 15-30 µg per ml were found to suppress the growth of Gram-positive and Gram-negative bacteria, protozoa, fungi and even yeasts *in vitro* (94). The probiotic impact of *Lactobacilli* has been examined in several *in vivo* experiments showing positive results in diverse diseases (64, 95, 96). The majority of these studies has concentrated on the effects of Reuterin as probiotic. The direct supplementation of live bacteria with its consequences was the main goal of these studies. The effect of the Reuterin system itself was an interesting by-product.

1.3. Reutericyclin

In the year 2000, Reutericyclin an amphiphilic molecule with a molecular mass of 349 Da was mentioned for the first time. It is a low molecular weight antibiotic produced by certain lactic acid bacteria (97). It is a typical 1,3-bisacyltetramic acid and contains 4 methyl and 7 methylene groups and 2 olefinic and 2 aliphatic methine groups. The corresponding HSQC spectrum visualizes the hydrophilic, negatively charged head group with two hydrophobic side chains (98, 99, 100). The weak acid gets more active the lower the pH level (99). At present, there are S- and R-forms of Reutericyclin (Figure 3) and many different chemical variations of Reutericyclin available on the market (101). It was identified as a proton ionophore which causes the antimicrobial effect of Reutericyclin on Gram-positive bacteria (100). Reutericyclin selectively disperses the transmembrane potential of bacteria as proton ionophore (102, 103). However, yeasts, fungi and Gram-negative bacteria have been shown to be resistant to Reutericyclin (100).

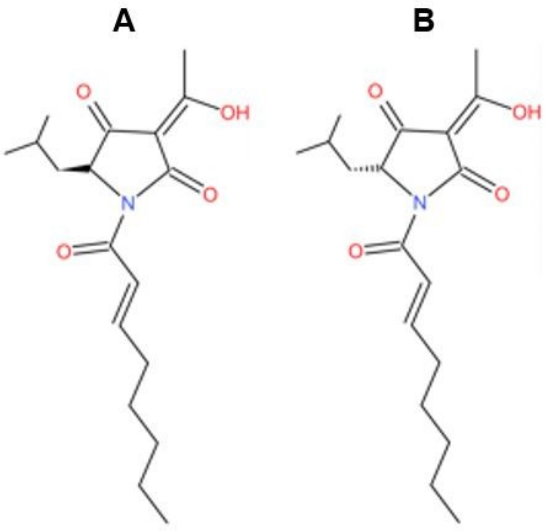


Figure 3: S-form (A) and R-form (B) of Reutericyclin. Created with KingDraw®.

1.4. Aims

The aims of this thesis were to produce and characterize postbiotic supernatants of probiotic bacteria including a co-culture of a probiotic containing 10 different strains, to assess their antimicrobial activity and their *in vivo* effect on the fecal microbiota in a murine model

Three different postbiotics were chosen for this thesis:

- Culture and cell free culture supernatant of OMNi BiOTiC® AAD10
- Culture supernatant of *Lentilactobacillus diolivorans* containing Reuterin
- Commercially available (S) - Reutericyclin

2. Materials and Methods

2.1. Production and Analysis of OMNi BiOTiC® AAD10 Supernatant

2.1.1. Production of AAD10 Supernatant

OMNi BiOTiC® AAD10 is a commercially available probiotic food supplement distributed by Institut AllergoSan and produced by Winclove Probiotics B.V.. Winclove Probiotics B.V. is a GMP faculty complying with NSF/ANSI standard 173-2008 and certified according to ISO 22000:2005. OMNi BiOTiC® AAD10 is composed of 10 probiotic strains:

- *Bifidobacterium (B.) bifidum* W23
- *B. lactis* W18
- *B. longum* W51
- *Enterococcus* (*E.*) *faecium* W54
- *Lactobacillus* (L.) *acidophilus* W55
- *L. acidophilus* W37
- *L. paracasei* W72
- *L. plantarum* W62
- *L. rhamnosus* W71
- *L. salivarius* W24

All of these strains are found exert beneficial effects in the host (reviewed in (65)). To test for the optimal habitat for the OMNi BiOTiC® AAD10 bacteria a preculture was performed in 125 ml glass falcons filled with 24 ml sterile fluid MRS broth under N_2 atmosphere. 1 g (equalling $4.5x10^{10}$ CFU) OMNi BiOTiC® AAD10 was gently mixed with 15 ml of filtered SuperPure Water. The solution was stored at room temperature in absolute darkness for 10 minutes of activation time. 400 µl OMNi BiOTiC® AAD10 solution was injected in each of a total of 12 glass falcons in three approaches. The falcons were incubated at 37°C and 120 rpm in the Excella E24. Directly after the injection with the OMNi BiOTiC® AAD10 solution the first 400 µl sample was taken for OD600 measurement and plate culture by disinfecting the cap and inserting a 23G needle through the rubber lid of the

glass falcon connected to a 1 ml syringe. For the OD600 measurement a 96 well plate was used and 100 µl of OMNi BiOTiC® AAD10 solution was placed in two free wells. The plate was immediately measured with the SpectraMax Plus 384 at an OD of 600 nm. The rest of the sample was put into an Eppendorf tube for dilution series in MRS broth until 10^6, 10^7 and 10^8 dilutions were reached. Between all steps, the respective sample was vortexed for 30 seconds. MRS Agar plates were brought to room temperature and labelled with time point, date and dilution. All plates were done as duplicates and OMNi BiOTiC® AAD10 solution was vortexed for 15 seconds before taking each sample. To distribute the OMNi BiOTiC® AAD10 solution evenly 5-6 sterile glass pearls were placed on the surface before adding 100µl OMNi BiOTiC® AAD10 solution to each plate. The plate was then gently shaken until the glass pearls evenly distributed the fluid on the agar plate but at least for one minute. The glass pearls were then removed and the plates were put up-side down in a plastic box with one bag of O_2 absorber and one O_2 control strip and the plastic box was closed immediately. The firmly closed plastic box was incubated at 37 °C for 48 hours. When the box was opened, a photo was taken of each plate with visible label and the number of colonies and colony creators were counted. The above-described procedure was done 4, 8,12, 24, 28, 32, 34, 36, 48, 50, 52, 54, 56, 58, 60, 72, 76, 80, 84, 96, 100, 168 and 192 hours after starting the culture at timepoint 0. After evaluation of these data as described below the production of the supernatant for the animal experiment was conducted as following: 500 ml MRS broth in a 1 l Duran glass bottle were sterilized. After cooling down the bottle was flooded with N_2. 8.3 ml of OMNi BiOTiC® AAD10 solution produced as described above were injected sterile through the rubber lid of the glass bottle. OD600 measurement and streaks were performed as described above at timepoint 0 and after 8, 12, 24, 36, 48, 96, 150, 166 and 196 hours. Production step 1 was harvested after 48 h. 170 ml of the supernatant were sterile filtrated, aliquoted and stored at -80 °C until the administration. The bottle with the left supernatant was further cultivated as described in detail above. It was harvested after 196 hours as production step 2, also sterile filtrated, aliquoted and stored at -80°C until the application. The growth data of the AAD10® culture are shown in Figure 4.

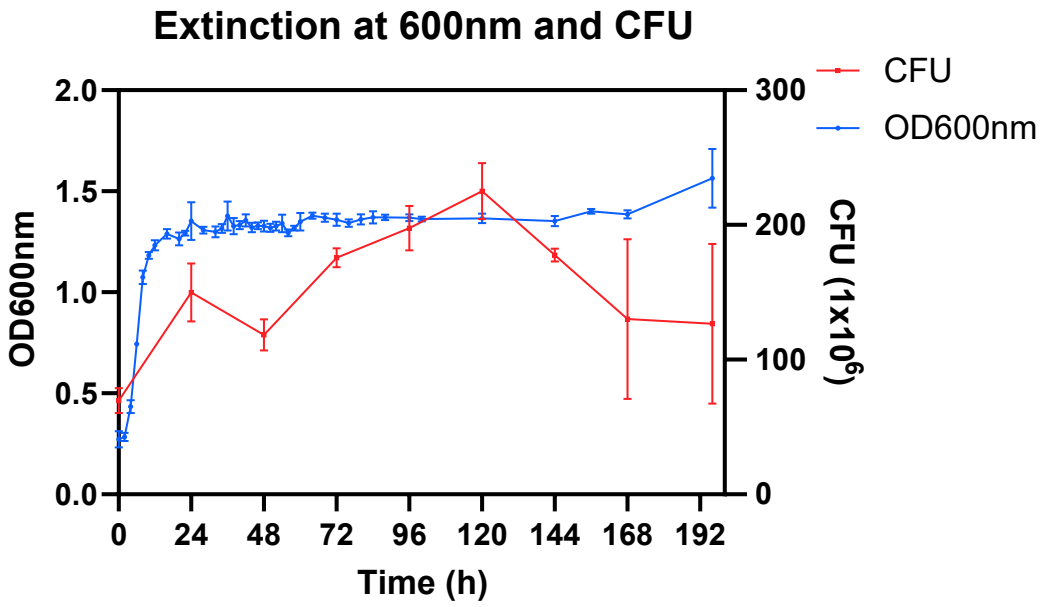


Figure 4: Growth curve of the AAD10® culture (retrieved from Figure 1 of the original publication (104); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

At defined time points (Table 1) supernatant samples were obtained for LC-MS metabolomic analysis (CFS), Shotgun analysis (whole culture) and volatile organic compound analysis (CFS) in the headspace of the culture supernatant.

	0h	8h	12h	24h	36h	48h	96h	168h	196h
VOC	1	1	1	1	1	1	1	1	1
Shotgun	0	1	1	1	0	1	1	0	1
Metabolomics	0	1	1	1	0	1	1	0	1

Table 1: Sampling time (0...no sample examined; 1...sample examined)

For these additional tests, samples of the culture were taken by disinfecting the cap and inserting a 23 G needle through the rubber lid of the glass bottle connected to a 10 ml syringe. For the evaluation of volatile organic compounds (VOCs) three glass vials were prepared for each time point. Two of them were filled with 2 ml centrifuged and filtered supernatant (CFS) each and the third one was opened in the room where the experiment

took place to serve as base line. The shotgun analysis required 1.5 ml of pure OMNi BiOTiC® AAD10 supernatant per time point in an Eppendorf tube immediately frozen and stored at -80°C. Two times 2 ml of CFS were also stored at -80 °C in Greiner Cryo Vials for LC-MS analysis per time point.

2.1.2. Analysis of Volatile Organic Compounds of the AAD10 Supernatant

Volatile organic compound (VOC) cell free supernatant samples were harvested into glass vials at time points described above and stored at 6 °C. To exclude possible contaminations, room air samples were taken at the same time. All samples were immediately packed according to the IATA guidelines and transported to the Rostock Medical Breath Analysis and Technologies Centre at the Medical University of Rostock via overnight express for analysis. The headspace of samples was used to analyse VOCs as previously described in literature (105-107). A solid phase micro extraction (SPME) fibre (carboxen/polymethylsiloxane) was used to pre-concentrate VOCs. The compounds were separated with a gas chromatograph (GC) coupled to a mass selective detector (MSD) and the desorbed VOCs were identified from the SPME device. The measured retention time was used to identify all detected marker substances using a mass spectral library. The contamination limit was set to 30% of the median of the room air samples. If the event of the median of the headspace samples exceeded this 30% a potential contamination was assumed. The so identified substances were excluded from any following analysis. Each substance has a defined retention time. The responses of a selected m/q ratio were recorded, integrated and used for group comparison.

2.1.3. Shotgun Analysis of the AAD10 Supernatant

All supernatant samples were harvested as described above. Total DNA was isolated according to standard procedures using a Quiagen extraction kit for shot gun sequencing of samples. The library preparation was performed with 200 ng of total DNA with the NEBNext Ultra II FS DNA Library Prep Kit according to the manufacturer’s guidelines. Specially designed dual index primers were used for indexing. All samples were pooled in equal ratios and a 4 nM pool was sequenced on a MiSeq desktop sequencer with v 3 600 cycles

chemistry with 5 % PhiX according to manufacturer's guidelines. FASTQ files were used for further data analysis after demultiplexing and chimera identification.

Raw sequence data were trimmed with Trim Galore for quality assurance and dereplicated with Vsearch. The final analysis was performed using MetaPhlAn2 and HUMAnN2. For all analysis steps the Medical University Graz private Galaxy instance running on a HPC infrastructure (MedBioNode) was used.

2.1.4. Metabolomics of the AAD10 Supernatant

Liquid chromatography - high resolution mass spectrometry was executed for the metabolomic analysis of cell free supernatant samples. The overnight metabolite extraction was performed with cold methanol. All samples were measured in one run with a Vanquish ultrahigh performance liquid chromatography (UHPLC) system coupled to a mass spectrometer as described before. For quality assurance the samples were measured in duplicates using a stratified randomized sequence (108).

For analysis, raw data was converted into mzXML. PeakScout (109) was consumed to identify substances in the samples applying a reference list with exact mass and retention times of known metabolites.

Quality control of all detected metabolites was performed as described previously (110, 111) using The Information Bus Company (TIBCO) Spotfire. All substances were categorised into two classes suitable for multivariate and univariate analysis (MVA_UVA) and suitable for univariate analysis (UVA). The relative standard deviation (RSD) of the quality control (QC) samples was calculated to assess the technical variability. Using t-Distributed Stochastic Neighbour Embedding (t-SNE) a subsequent unsupervised dimensionality reduction analysis was performed to assess clustering of QCs and sample replicates. Two different correlations were investigated. Firstly the correlation between metabolites and inoculation time was calculated and secondly the correlation between metabolites and relative bacterial abundance was computed.

2.1.5. Susceptibility Testing of the AAD10 Supernatant

All bacteria for susceptibility testing were ordered at Aurosan GmbH. Candida albicans was kindly provided by Angela Horvath. The following germs were selected for antimicrobial activity testing (Table 2):

- *Clostridium (C.) difficile* (ATCC 700057)
- *Enterococcus (E.) faecium* (ATCC 27270)
- *Escherichia (E.) coli* (ATCC 25922)
- *Listeria (L.) monocytogenes* (ATCC 15313)
- *Pseudomonas (P.) aeruginosa* (ATCC 27853)
- *Propionibacterium (Pr.) acnes* (ATCC 6919)
- *Staphylococcus (S.) aureus* (ATCC 29213)
- *Staphylococcus (S.) epidermidis* (ATCC 12228)
- *Streptococcus (Str.) agalactiae* (ATCC 13813)

Species	Phylum	Class	GRAM
Clostridium difficile	Firmicutes	*Clostridia*	+
Listeria monozytogenes	Firmicutes	*Bacilli*	+
Enterococcus faecium	Firmicutes	*Bacilli*	+
Staphylococcus aureus	Firmicutes	*Bacilli*	+
Staphylococcus epidermidis	Firmicutes	*Bacilli*	+
Streptococcus agalactiae	Firmicutes	*Bacilli*	+
Propionibacterium acnes	Actinobacteria	*Actinobacteria*	+
Escherichia coli	Proteobacteria	*Gammaproteobacteria*	-
Pseudomonas aeruginosa	Proteobacteria	*Gammaproteobacteria*	-
Campylobacter jejuni	Proteobacteria	*Epsilonproteobacteria*	-

Table 2: Bacteria used for resistance testing

All bacteria were delivered frozen as sticks. After thawing bacteria were cultured on different agarose plates following the manufacturer's instructions. Next, bacteria were cultured as broth cultures in the fitting broth each and stocks were frozen from the plates. Aerobic bacteria were cultivated for a total of 24 h at 37°C and 120 rpm in sterile 50 ml

plastic tubes and anaerobic bacteria for 48 h at 37°C and 120 rpm in closed sterile glass vials flooded with N_2. These growing times allowed a dense bacterial lawn visible on the plates. 300 µl of each single preculture were spread on 15 cm diameter plates with the fitting agarose for each germ. Tryptic soy agar (30 g/l TSB, 15 g/l Agar-Agar; TSA) was used for *E. Coli, P. aeruginosa* and *S. aureus*. TSA with additional 5 % freshly obtained sheep blood was taken for *C. difficile*, *Pr. acnes* and *Str. agalactiae*. Brain heart infusion agar (52 g/l Brain-Heart-Infusion, 15 g/l Agar-Agar) was spent for *E. faecium* and *L. monocytogenes.* Nutrient agar (1 g/l beef extract powder, 5 g/l peptone, 5 g/ l NaCl and 15 g/l Agar-Agar) fitted for *S. epidermidis*. *C. difficile* and *Pr. acnes* as anaerobic bacteria were cultured on the plates for 48h in airtight boxes with oxid Anaerogen 2.5 l. Following pre-cultivation on broth culture, brain heart infusion agar plates (52 g/l Brain-Heart-Infusion, 15 g/l Agar-Agar) were utilised to cultivate *C. albicans* at 25 °C for 24 hours and an agitation of 120 rpm for both culture steps until a dense fungal lawn was achieved.

Every microorganism was cultured on its specific plate. Cultures were repeated 5 times. Nine disks for resistance testing were positioned with a stencil on each culture plate using a prepared scheme. For susceptibility testing, supernatant was slowly thawed to 4°C in the fridge and then brought to room temperature shortly before application. Each plate was treated exactly the same and the preparation of the different loading substances for the disks was also exactly the same for all replicates. The disks were loaded with 20 µl each according to the following scheme starting at position 1:

- pure supernatant
- 1:2 dilution
- 1:4 dilution
- buffered to pH 7 with 1 n NaOH,
- cooked at 100 °C in a Thermomixer for 30 min without rotation,
- mixed with 1 n HCl (1:1)
- treated with 1 mg/ml Proteinase K
- Pure MRS culture medium (negative control)
- fitting antibiotics for each germ (positive control)

Either Vancomycin (0.03 mg/disk) for *C. difficile*, *E. faecium*, *L. monocytogenes, S. aureus, S. epidermidis* and *Str. agalactiae* or Piperacillin/Tazobactam (0.1 mg/disk) for *E. coli, P. aeruginosa* and *Pr. acnes* or Amphotericin B (0.05 mg/disk) for *C. albicans* were used as positive controls. Plates with aerobic bacteria were incubated at 37 °C for 24 h and thus with anaerobic bacteria for 48 h. The plates containing *C. albicans* were incubated for 24 h at 25

°C. Afterwards, photos of all plates were taken on a light plate and inhibition zones were measured with ImageJ 2.0.0-rc-69/1.52p.

2.1.6. Effect of AAD10 Supernatant on the Fecal Murine Microbiome

Filtrated and centrifuged supernatant or MRS culture medium were used for the *in vivo* application. The whole supernatant was aliquoted after thawing and a dilution with sterile water (1:4) into 1.5ml Eppendorf vials was performed. The diluted supernatant or MRS culture medium was stored at -80 °C until use. 20 BALB/c mice were ordered from the Centre for Biomedical Research of the Medical University of Vienna, Austria with 7 weeks of age as one patch of littermates especially for microbiome examination. Following delivery, two weeks of acclimatization period were kept. After this mice were divided into two equal groups with an equal distribution of the mean body weight. The approval of the animal experiments by the veterinary board has the licence number BMBWF-66.010/0153-V/3b/2019. Mice were single-housed in individually ventilated cages under specific pathogen free (SPF) conditions. The animals had free access to food and water at all times and were kept at a 12 h light-dark cycle. Following acclimatization, the intervention group mice were daily gavage fed with supernatant and the control group mice with MRS medium in the same dilutions as the intervention group. Daily gavage was performed with 1:4 dilutions of supernatant and control medium in the first week to check whether the mice accept it. Because of no apparent complications, mice were gavage fed with the undiluted production step 1 supernatant for 4 more weeks. After the 4 weeks a fecal sample of every animal was taken. Animals were given a gavage feeding break of 4 weeks. Afterwards, mice were gavage fed with the undiluted production step 2 supernatant for 4 more weeks. Euthanasia was performed after 13 weeks with 9 treatment weeks. At euthanasia, organs (lung, spleen, kidneys, white adipose tissue and liver) were harvested and weighed as previously described (107). Again, stool samples were stored at -80°C until 16S based microbiome analysis.

2.1.7. Analysis of the Murine Fecal Microbiome

Fecal samples were isolated with the Magna Pure LC DNA III Isolation Kit according to published protocols for total DNA (112). Abbreviated, each stool pellet was homogenized in 500 µl PBS and 250 µl bacterial lysis buffer. The fecal samples were bead beaten in Magna Lyzer Green bead Tubes in a Magna Lyzer instrument at 6,500 rpm for 30 seconds two times. The enzymatic lysis was performed with 25 µl lysozyme concentrated at 100 ng/ml at 37 °C for 30 minutes and 43,4 µl proteinase K concentrated 20 mg/ml at 65 °C for 60 minutes. Afterwards, all samples were heat inactivated at 95 °C for 10 minutes. Total DNA was purified in a MagnaPure LC instrument according to the manufacturer`s guidelines. The purified total DNA was eluted in 100 µl elution buffer and stored at -20 °C. 2 µl of total DNA was used for 16S PCR in a 25 µl PCR reaction with the FastStart™ High Fidelity PCR-System following the manufacturer's guidelines. The target specific primers 515F and 806R were used in the PCR reaction for 30 cycles. All samples were analysed in triplicates. The results were pooled, indexed, normalized and purified following public available protocols (112). An Illumina MiSeq desktop sequencer at 9 pM and v 3 600 cycles chemistry was used to sequenze the final pool. FastQ raw files were used for further data analysis.

2.479.083 MiSeq paired end FASTQ reads were used in the DADA2 pipeline for correcting and modelling Illumina-sequenced amplicon errors for quality-filtering (113) with standard settings for dereplicating, denoising, merging and checking for chimeras as implemented in QIIME2 2018.4 microbiome bioinformatics platform (114). Non-public QIIME2 was integrated in an own instance of Galaxy (115). Taxonomic assignment of the representative sequences was performed out with a QIIME2 sklearn-based classifier against SILVA rRNA database release 132 at 99 % identity (116). Abundance changes were calculated using absolute counts from OTU table on genus level. 16S rRNA data was transmitted to the Calypso online software to compare and interpret taxonomic information (117). After that, samples were rarefied to a depth of 20,220 reads. Alpha diversity was calculated through Chao1 estimator, inversed Simpson and Shannon index. Beta diversity was analysed through a redundancy analysis (RDA) and coloured principal component analysis plots (PCoA) based on the Bray-Curtis dissimilarity score. All P-values were adapted to multiple testing by FDR. A linear discriminant effect size (LEfSe) analysis was performed for discriminating taxa within and between the groups. For verified differences between groups by ANOVA ($p<0.1$), LEfSe analysis was performed and differentially abundant taxa identified were considered relevant.

2.2. *Supernatant of L. diolivorans Cultures (Reuterin System)*

The headspace of this culture contains substances of the Reuterin system. The production, primary analysis and stability testing were conducted at our partner at the University of Life Sciences Vienna.

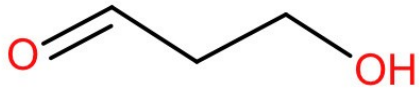


Figure 5: 3-Hydroxypropionaldehyde (Reuterin), created with KingDraw®.

2.2.1. Reuterin System Production

L. diolivorans LMG 19668 was chosen as most efficient 3-HPA production strain following a preliminary screening of different *L. reuteri* and *L. diolivorans* strains. 3-HPA was produced biosynthetically in a 2-step production process with a few modifications according to Lindlbauer et al. (86). Glycerol containing water was used as fermentation medium. A preculture was performed by inoculating 40 ml MRS broth (10 g/l peptone from casein, 5 g/l yeast extract, 10 g/l meat extract, 2 g/l dipotassium hydrogen phosphate, 1 ml/l Tween 80, 20 g/l glucose, 2g/l ammonium citrate tribasic, 5 g/l sodium acetate, 0.2 g/l magnesium sulphate, 0.05 g/l manganese sulfate; pH 5.7 with 1 ml cryostock of the strain *L. diolivorans* LMG 19668 (86). *L. diolivorans* was incubated in the medium above overnight at 150 rpm and 30 °C in an anaerobic atmosphere. Optical density (OD) of 0.1 (absorbance unit) at 600 nm was measured in an aliquot of the preculture in 800 ml MRS broth supplemented with 5 mg/l vitamin B12, 5.17 g/l 1,2-PDO and additional 10 g/l glucose. The above mentioned growth medium was previously added into one reactor of the Eppendorf parallel fermentation system with four parallel bioreactors. 5 ml of a 10% Struktol® SB2121 solution were added to the reactor. The inoculated medium was incubated at 400 rpm and at 30°C. The pH of 5.7 and the anaerobic condition were kept constant by gassing with N_2 (2 sl/min) and automatic addition of NH_3 (12.5%). Growth and biomass production were regularly checked by measuring the OD600 in a photometer 2 after diluting the sample 1:10 in water. Samples were taken every 60 minutes of biomass generation starting at timepoint 0. The cells were harvested by centrifugation at 4,248 g for 8 min at 20 °C towards the end of the exponential

growth phase and washed in 200 ml water. The end of the growth phase was identified by a stop in NH_3 consumption and a decrease in CO_2. The obtained cell pellet was resuspended in 400 ml of a 2 % glycerol solution with a pH of 7 and maintained at that value using NH_3 (12.5 %). The biotransformation of glycerol took place using an Eppendorf parallel fermentation system at 30 °C, 400 rpm agitation and 2 sl/min N_2-gassing. The concentrations of the extracellular metabolites glycerol and 3-HPA were identified by HPLC analysis with an Aminex HPX-87H column (300mm x 7.8mm) operated at 60 °C, 0.6 ml/min flow rate and 0.004 M H_2SO_4 as mobile phase and equipped with a Micro-Guard Cation H Cartridge (30mm x 4.6mm) at defined time points (00:00 h; 00:50 h; 01:45 h; 02:50 h and 03:20 h) as reported before (118). When the amount of 3-HPA stopped increasing the process was finished and the solution was centrifuged at 4,248 g for 8 min at 20 °C and filter-sterilized. The gained substance subsequent referred to as Reuterin system was aliquoted into 50 ml screw cap tubes and stored at -80 °C until provision.

2.2.2. Storage Stability Testing of the Reuterin System

0.9 ml of sterile-filtrated Reuterin system was portioned into 1.5 ml Eppendorf vials and stored at -20°C. At every time point throughout one year two tubes of the frozen supernatant were thawed slowly at 4°C for 1 hour and the concentrations of 3-HPA and glycerol were determined by HPLC analysis as described above.

For stability determination of 3-HPA in the Reuterin system after various freeze-thaw cycles sterile-filtrated supernatant was aliquoted into 50 ml and 15 ml plastic tubes and stored at -20°C. Over a period of 4 weeks both tube sizes were regularly thawed to 4°C and then examined weekly for the presence of 3-HPA and glycerol by HPLC.

2.2.3. Susceptibility Testing of the Reuterin System

These methods have already been described in detail above (see 2.1.5).

2.2.4. Effect of the Reuterin System on the Murine Fecal Microbiome

A supernatant containing 13.4 g/l 3-HPA and 6.9 g/l glycerol was used for *in vivo* experiments and will further be called Reuterin complex supernatant. A biotransformation medium containing the same amount of glycerol as the supernatant was used as control substance. The Reuterin complex supernatant was thawed, aliquoted and 1:4 diluted with sterile water into 1.5 ml Eppendorf vials before storage at -80°C. 20 BALB/c mice with 7 weeks of age were ordered as one patch of littermates especially for microbiome examination. Following delivery, two weeks of acclimatization period were kept. After this mice were divided into two equal groups with an equal distribution of the mean body weight. The approval of the animal experiments by the veterinary board has the licence number BMBWF-66.010/0153-V/3b/2019. Mice were single-housed with free access to food and water in individually ventilated cages under SPF conditions and a 12 h light-dark cycle. Following acclimatization, the intervention group mice were daily gavage fed with Reuterin system at a dilution of 1:41.2 which corresponds to 0.1 mg Reuterin per dose to accustom to the substance in week 1. In the second week of gavage feeding the daily dose was raised up to 0.15 g Reuterin per dose followed by 4 more weeks with 0.2 mg Reuterin per dose. The control group received biotransformation medium containing 6.9 g/l glycerol in the same dilutions as the Reuterin system group. After 6 weeks of treatment mice were euthanized.

The methods for VOC analysis and 16S microbiome analysis have already been described in detail above (see 2.1.2 and 2.1.7).

2.3. (S) - Reutericyclin

(S) - Reutericyclin with the chemical name 4-acetyl-1-[(E)-dec-2-enoyl]-3-hydroxy-2-(2methylpropyl)-2H-pyrrol-5-one was obtained from BioCrick Biotech Co., Ltd. For susceptibility testing (S) - Reutericyclin was resolved at a concentration of 62.5 µg/ml in autoclaved water containing 1‰ H_3PO_4 which served as negative control too. As described in the previous sections the solution was also diluted 1:2 and 1:4, buffered, treated with HCl and proteinase K. Autoclaved water with 1‰ H_3PO_4 served as negative and antibiotics/amphotericin B as positive control. For *in vivo* application (S) - Reutericyclin was administered to a group of 10 mice (Reutericyclin group) as following: week 1:12.5

µg/mouse/day in 400 µl 1 ‰ H_3PO_4; week 2-5:25 µg/mouse/day in 400 µl 1 ‰ H_3PO_4; week 6-7: 50 µg/mouse/day in 400 µl 1 ‰ H_3PO_4. Autoclaved water containing 1 ‰ H_3PO_4 served as control and was also administered to 10 mice (control group). The methods for VOC analysis and 16S microbiome analysis have already been described in detail above (see 2.1.2 and 2.1.7).

2.4. Statistics

Data was administrated with Microsoft Excel 2016® spreadsheets. Because of the small sample size normal distribution was ruled out. Statistical analysis was performed with SPSS 26.0®. GraphPad Prism 9® was used for all graphical workup. The heatmap analysis of the supernatant results: relevant results for gene families were drawn with the heatmap function of gplots package version 3.1.1 for Rstudio® version 1.4.1106 after norming the results to the maximum for each bacterium. A Spearman-Rho analysis was performed to detect significant marker correlations obtained from different analyses. A $p < 0.05$ was considered statistically significant. Metabolite and inoculation time correlation graphs, as well as t-SNE plots were generated using the Orange Data Mining Toolbox v3.31.0.

3. Results

3.1. OMNi BiOTiC® AAD10 Supernatant

3.1.1. Shotgun Analysis of AAD10 Supernatant

Shotgun analysis was not able to discriminate between the two *L. acidophilus* strains and between *B. lactis* and *B. longum.* Therefore, these were combined for further analysis. The abundances of *B. longum/lactis, E. faecium, L. salivarius* and *L. paracasei* decreased time-dependently (Figure 6). During culture *L. plantarum* increased in contrast to this. *L. rhamnosus* also increased with a peak at 96 h and then decreased again. The two *L. acidophilus* strains remained relatively constant (Figure 6).

E. faecium showed the highest gene expression changes over the culture time in the analysis of the concerning pathways. Table 3 shows that this was followed by *L. paracasei*, *B. longum/lactis*, *L. salivarius*, *L. rhamnosus*, *L. plantarum*, *L. acidophilus* and *B. bifidum.* The distribution of the gene expression differed between the bacteria of the co-culture. While *L. plantarum* had the highest expression at 196 h (Figure 7), *E. faecium* and *L. paracasei* had the highest gene expression at the start of the culture (Figure 8). *L. rhamnosus* and *L. salivarius* exhibited the top level at 96 h of culture time (Figure 9). The combination of *B. longum/lactis* had a peak in gene activity right at the beginning of the culture followed by a plateau and a second peak after 48 h of culture time ending with a quick decrease. *B. bifidum* and *L. acidophilus* showed no clear pattern in view of culture time. Table 3 presents the gene activities of all different bacteria according to MetaCyc.

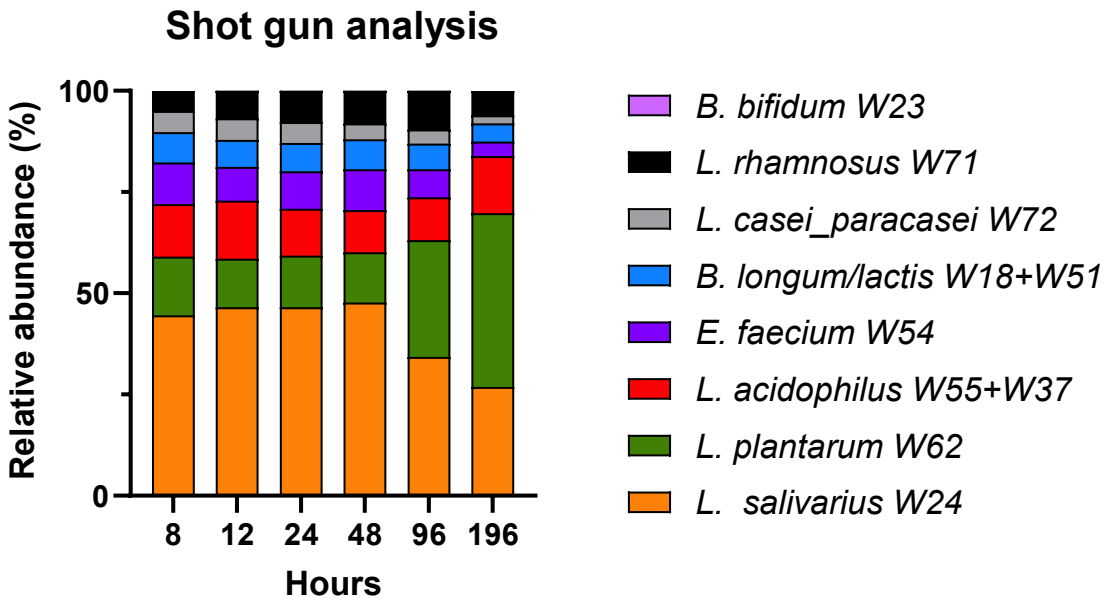


Figure 6: Shotgun analysis (retrieved from Figure 3 of the original publication (104); recoloured; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

Species	W24	W62	W54	W18+51	W72	W71
Total genes changed (n)	40	32	48	44	44	41
Amino acid biosynth.	↑↓13%	↑23%	↓2%	↓45%	↓11%	↑↓12%
Nucleoside/nucleotide biosynth.	↑↓38%	↑26%	↓52%	↓16%	↓39%	↑↓39%
Carbohydrate biosynth.	↑↓5%	↑6%	↓6%	↓9%	↓4%	↑↓5%
Fermentation	↑↓3%	↑3%	↓4%	↓9%	↓2%	↑↓2%
Carbohydrate degradation	↑↓13%	↑9%	↓8%	-	↓9%	↑↓12%
Cellwall component biosynth.	↑↓8%	↑6%	-	↓7%	↓7%	↑↓7%
Cofactor/vitamin/carrier biosynth.	↑↓8%	↑9%	↓6%	↓7%	↓9%	↑↓7%
Fatty acid and lipid biosynth.	-	↑9%	↓4%	↓5%	-	-
Glycolysis	↑↓8%	↑3%	↓6%	↓2%	↓7%	↑↓7%
Other	4%	6%	12%	-	12%	9%

Table 3: Genes with changed expression over culture time for bacteria varying in their relative abundance. ↑…increased activity, ↓…decreased activity, ↑↓…increase followed by decrease. %...per cent of total number of genes with changed expression.

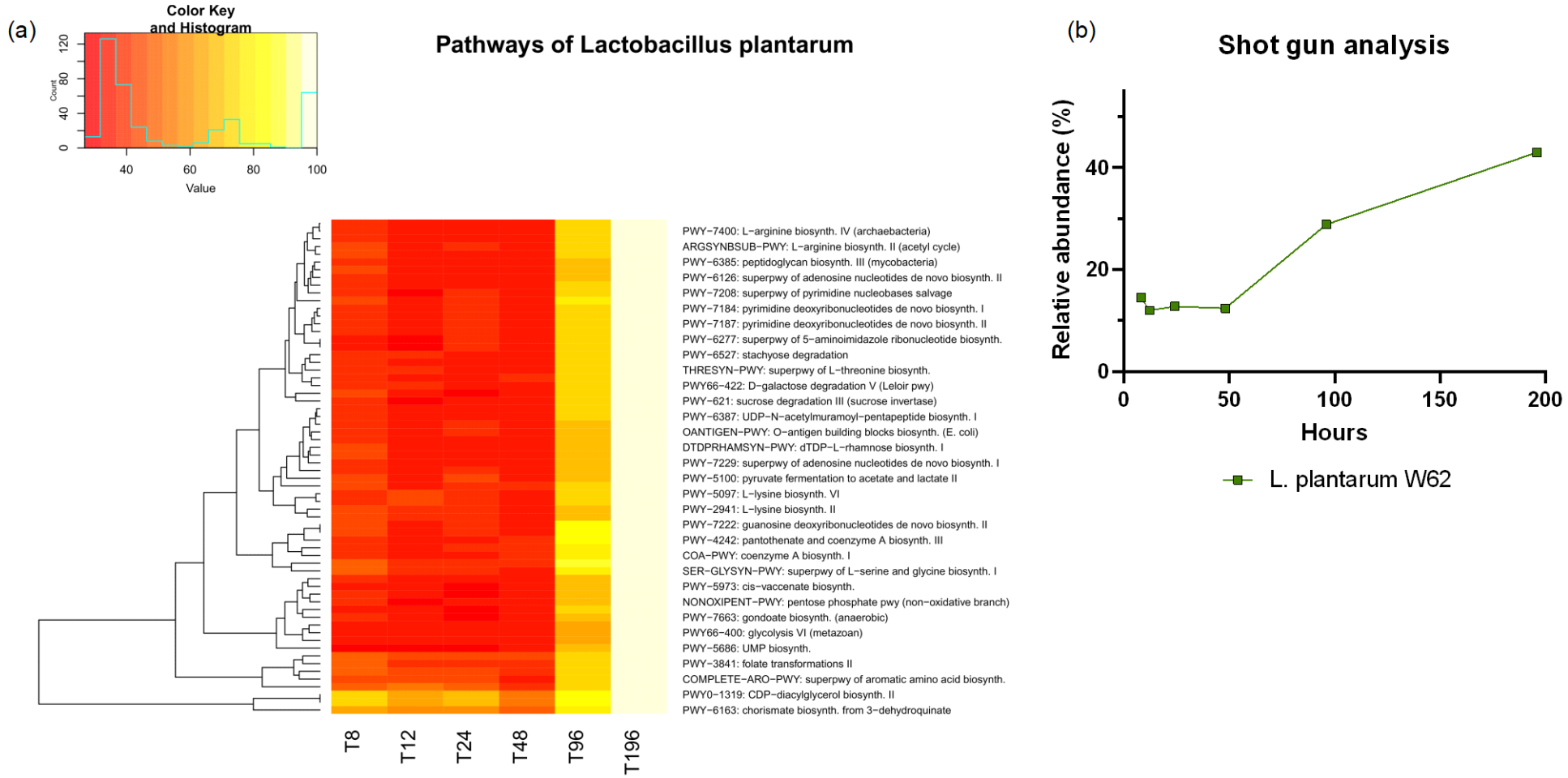


Figure 7: Heatmap (a) and relative abundance (b) of *L. plantarum*. 0% of maximum coloured in red and 100% in yellow (retrieved from Figure 3 of the original publication (104); added relative abundance of *L. plantarum* over culture time additionally to the right; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

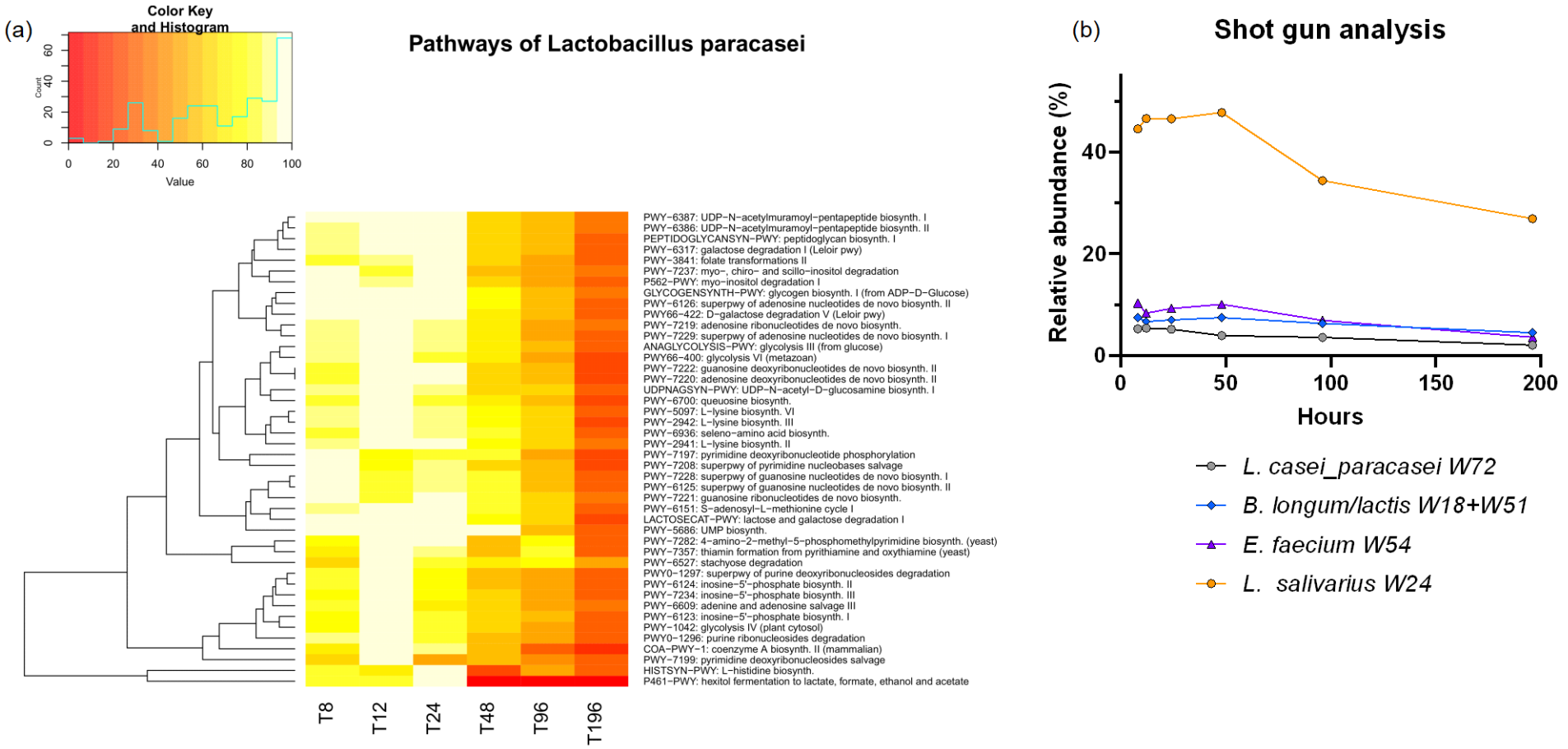


Figure 8: Heatmap (a) and relative abundance (b) of *L. paracasei*. 0% of maximum coloured in red and 100% in yellow (retrieved from Figure 3 of the original publication (104); added relative abundances of decreasing bacteria over time additionally to the right; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

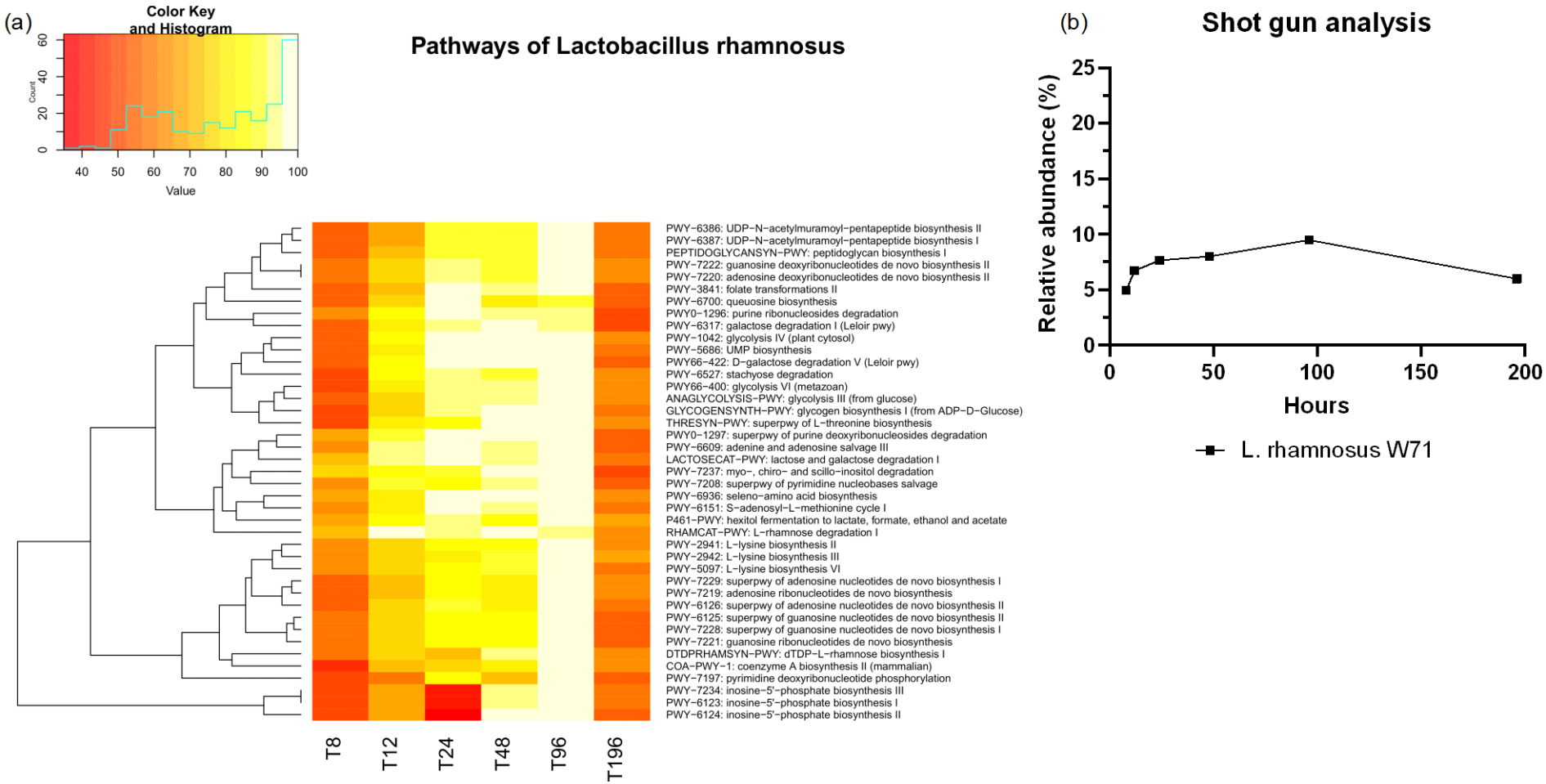
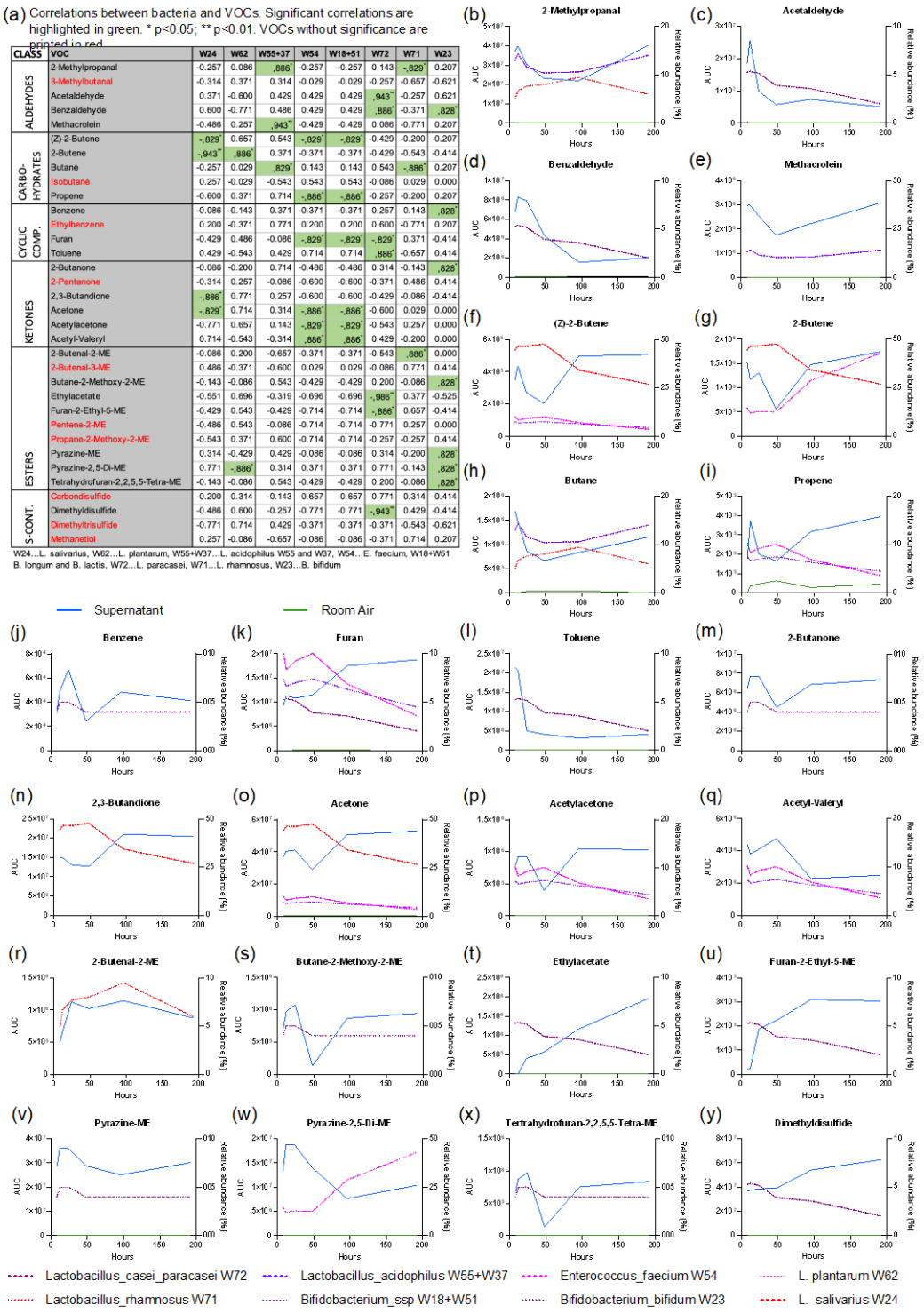


Figure 9: Heatmap (a) and relative abundance (b) of *L. rhamnosus*. 0% of maximum coloured in red and 100% in yellow (retrieved from Figure 3 of the original publication (104); added relative abundance of *L. rhamnosus* over time additionally to the right; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.1.1. Supernatant VOC Analysis



CLASS	VOC	W24	W62	W55+37	W54	W18+51	W72	W71	W23
ALDEHYDES	2-Methylpropanal	-0.257	0.086	,886*	-0.257	-0.257	0.143	-,829*	0.207
	3-Methylbutanal	-0.314	0.371	0.314	-0.029	-0.029	-0.257	-0.657	-0.621
	Acetaldehyde	0.371	-0.600	0.429	0.429	0.429	,943**	-0.257	0.621
	Benzaldehyde	0.600	-0.771	0.486	0.429	0.429	,886*	-0.371	,828*
	Methacrolein	-0.486	0.257	,943**	-0.429	-0.429	0.086	-0.771	0.207
CARBO-HYDRATES	(Z)-2-Butene	-,829*	0.657	0.543	-,829*	-,829*	-0.429	-0.200	-0.207
	2-Butene	-,943**	,886*	0.371	-0.371	-0.371	-0.429	-0.543	-0.414
	Butane	-0.257	0.029	,829*	0.143	0.143	0.543	-,886*	0.207
	Isobutane	0.257	-0.029	-0.543	0.543	0.543	-0.086	0.029	0.000
	Propene	-0.600	0.371	0.714	-,886*	-,886*	-0.257	-0.200	0.207
CYCLIC COMP.	Benzene	-0.086	-0.143	0.371	-0.371	-0.371	0.257	0.143	,828*
	Ethylbenzene	0.200	-0.371	0.771	0.200	0.200	0.600	-0.771	0.207
	Furan	-0.429	0.486	-0.086	-,829*	-,829*	-,829*	0.371	-0.414
	Toluene	0.429	-0.543	0.429	0.714	0.714	,886*	-0.657	0.414
KETONES	2-Butanone	-0.086	-0.200	0.714	-0.486	-0.486	0.314	-0.143	,828*
	2-Pentanone	-0.314	0.257	-0.086	-0.600	-0.600	-0.371	0.486	0.414
	2,3-Butandione	-,886*	0.771	0.257	-0.600	-0.600	-0.429	-0.086	-0.414
	Acetone	-,829*	0.714	0.314	-,886*	-,886*	-0.600	0.029	0.000
	Acetylacetone	-0.771	0.657	0.143	-,829*	-,829*	-0.543	0.257	0.000
	Acetyl-Valeryl	0.714	-0.543	-0.314	,886*	,886*	0.429	-0.200	0.000
ESTERS	2-Butenal-2-ME	-0.086	0.200	-0.657	-0.371	-0.371	-0.543	,886*	0.000
	2-Butenal-3-ME	0.486	-0.371	-0.600	0.029	0.029	-0.086	0.771	0.414
	Butane-2-Methoxy-2-ME	-0.143	-0.086	0.543	-0.429	-0.429	0.200	-0.086	,828*
	Ethylacetate	-0.551	0.696	-0.319	-0.696	-0.696	-,986**	0.377	-0.525
	Furan-2-Ethyl-5-ME	-0.429	0.543	-0.429	-0.714	-0.714	-,886*	0.657	-0.414
	Pentene-2-ME	-0.486	0.543	-0.086	-0.714	-0.714	-0.771	0.257	0.000
	Propane-2-Methoxy-2-ME	-0.543	0.371	0.600	-0.714	-0.714	-0.257	-0.257	0.414
	Pyrazine-ME	0.314	-0.429	0.429	-0.086	-0.086	0.314	-0.200	,828*
	Pyrazine-2,5-Di-ME	0.771	-,886*	0.314	0.371	0.371	0.771	-0.143	,828*
	Tetrahydrofuran-2,2,5,5-Tetra-ME	-0.143	-0.086	0.543	-0.429	-0.429	0.200	-0.086	,828*
S-CONT.	Carbondisulfide	-0.200	0.314	-0.143	-0.657	-0.657	-0.771	0.314	-0.414
	Dimethyldisulfide	-0.486	0.600	-0.257	-0.771	-0.771	-,943**	0.429	-0.414
	Dimethyltrisulfide	-0.771	0.714	0.429	-0.371	-0.371	-0.371	-0.543	-0.621
	Methanetiol	0.257	-0.086	-0.657	-0.086	-0.086	-0.371	0.714	0.207

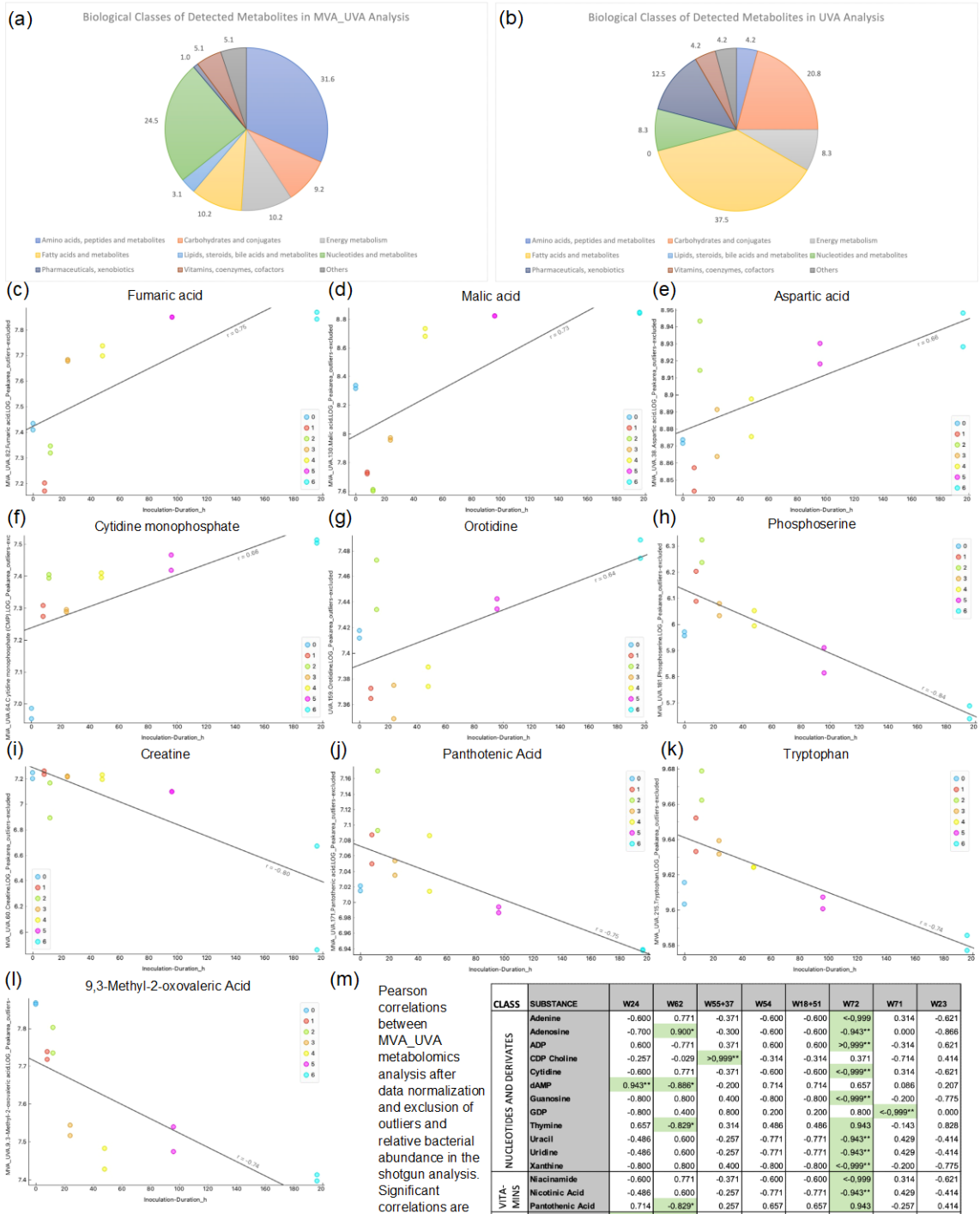
Figure 10: VOC analysis. The table (a) gives an overview of all VOCs and their correlation with the relative abundance of probiotic bacteria. The graphs (b-y) show the profile of the VOC (blue solid line), the room air (green solid line) and the relative abundance of the correlating probiotic bacterium (dashed lines) (retrieved from Figure 4 of the original publication (104); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

GC-MS identified 36 substances in VOC analysis. Propanal and pentane were traced back to room air contamination and were therefore excluded from further analysis. 24 substances significantly correlated with the relative abundance of one or even more of the probiotic bacteria at the different time points. The relevant substances and their correlations to the relative abundances of the bacteria are represented in Figure 10.

3.1.2. Metabolomics Analysis

122 metabolites were identified after quality control. Multivariate analysis and univariate analysis (MVA_UVA) substances (n=98) allocated most of the found substances to the groups amino acids/metabolites/peptides and nucleotides/metabolites. Univariate analysis (UVA) substances (n=24) mostly represent the group's fatty acids and their metabolites, carbohydrates and conjugates as well as pharmaceuticals and xenobiotics (Figure 11). The technical variability with a median RSD of 4.4% in the QC samples was almost perfect for the 98 MVA_UVA metabolites and visually confirmed using t-SNE, which showed a compact clustering of the QCs, and overlapping replicates in most cases.

Fumaric acid was found in the correlation analysis with an R of 0.75, malic acid with an R of 0.73, aspartic acid with a R of 0.66, cytidine monophosphate with an R of 0.66 and orotidine with an R of 0.64 increasing with culture time. The decrease of phosphoserine with an R of -0.84, creatine with an R of -0.80, pantothenic acid with an R of -0.75, tryptophan with an R of -0.74, and 9,3-methyl−2-oxovaleric acid with an R of -0.74 was also observed. The 98 metabolites of MVA_UVA and the relative abundance of bacteria over the culture time revealed significant correlations in 25 cases (Figure 11). The highest correlation was found between *L. paracasei* and the metabolites of all investigated bacteria, while *B. bifidum* showed no significant correlations.



(m)

Pearson correlations between MVA_UVA metabolomics analysis after data normalization and exclusion of outliers and relative bacterial abundance in the shotgun analysis. Significant correlations are highlighted in green. * p<0.05; ** p<0.01.

CLASS	SUBSTANCE	W24	W62	W55+37	W54	W18+51	W72	W71	W23
NUCLEOTIDES AND DERIVATES	Adenine	-0.600	0.771	-0.371	-0.600	-0.600	<-0,999	0.314	-0.621
	Adenosine	-0.700	0.900*	-0.300	-0.600	-0.600	-0.943**	0.000	-0.866
	ADP	0.600	-0.771	0.371	0.600	0.600	>0,999**	-0.314	0.621
	CDP Choline	-0.257	-0.029	>0,999**	-0.314	-0.314	0.371	-0.714	0.414
	Cytidine	-0.600	0.771	-0.371	-0.600	-0.600	<-0,999**	0.314	-0.621
	dAMP	0.943**	-0.886*	-0.200	0.714	0.714	0.657	0.086	0.207
	Guanosine	-0.800	0.800	0.400	-0.800	-0.800	<-0,999**	-0.200	-0.775
	GDP	-0.800	0.400	0.800	0.200	0.200	0.800	<-0,999**	0.000
	Thymine	0.657	-0.829*	0.314	0.486	0.486	0.943	-0.143	0.828
	Uracil	-0.486	0.600	-0.257	-0.771	-0.771	-0.943**	0.429	-0.414
	Uridine	-0.486	0.600	-0.257	-0.771	-0.771	-0.943**	0.429	-0.414
	Xanthine	-0.800	0.800	0.400	-0.800	-0.800	<-0,999**	-0.200	-0.775
VITAMINS	Niacinamide	-0.600	0.771	-0.371	-0.600	-0.600	<-0,999	0.314	-0.621
	Nicotinic Acid	-0.486	0.600	-0.257	-0.771	-0.771	-0.943**	0.429	-0.414
	Pantothenic Acid	0.714	-0.829*	0.257	0.657	0.657	0.943	-0.257	0.414
AMINO ACIDS & METABOLITES	Alanine	-0.886*	0.943**	0.086	-0.257	-0.257	-0.600	-0.429	-0.621
	Aspartic Acid	-0.486	0.600	-0.257	-0.771	-0.771	-0.943**	0.429	-0.414
	Indoxyl Sulphate	0.371	-0.600	0.429	0.429	0.429	0.942**	-0.257	0.621
	N-Acetylputrescine	-0.143	-0.143	0.943**	-0.029	-0.029	0.600	-0.771	0.414
	Phosphoserine	0.600	-0.771	0.371	0.600	0.600	>0,999	-0.314	0.621
	Taurine	0.657	-0.600	-0.143	0.943**	0.943**	0.714	-0.257	0.000
ORGANIC ACIDS	Citric Acid	0.371	-0.600	0.429	0.429	0.429	0.943**	-0.257	0.621
	Glyceric Acid	-0.600	0.771	-0.371	-0.600	-0.600	<-0,999**	0.314	-0.621
	Malic Acid	-0.600	0.771	-0.371	-0.600	-0.600	<-0,999**	0.314	-0.621
	Pyroglutamic Acid	-0.600	0.486	0.314	<-0,999**	<-0,999**	-0.600	0.200	0.000

W24...L. salivarius W62...L. plantarum, W55+37...L. acidophilus W55 and W37, W54...E. faecium, W18+51...B. longum and B. lactis, W72...L. paracasei, W71...L. rhamnosus, W23...B. bifidum

Figure 11: Multivariate metabolomics analysis and correlation of metabolites with the relative abundance of probiotic bacteria (only metabolites with significant correlations are displayed) (retrieved from Figure 5 of the original publication (104); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.1.3. Antimicrobial Activity of AAD10 Supernatant

Antimicrobial activity was tested for cell free AAD10 supernatant harvested after 48 hours (production step 1) or 196 hours (production step 2) of fermentation. The activity differed between the two time points (Figures 12 and 13).

At 48 h the antimicrobial activity was more distinctive than after 196 h. The activity was independent of cooking, buffering or proteinase K treatment for *S. epidermidis* and *Str. agalactiae*. For *L. monocytogenes*, *E. faecium* and *P. aeruginosae* buffering led to a loss of antimicrobial activity. Furthermore, HCl treatment was associated with a loss of antimicrobial activity in *P. aeruginosae* and *E. faecium*.

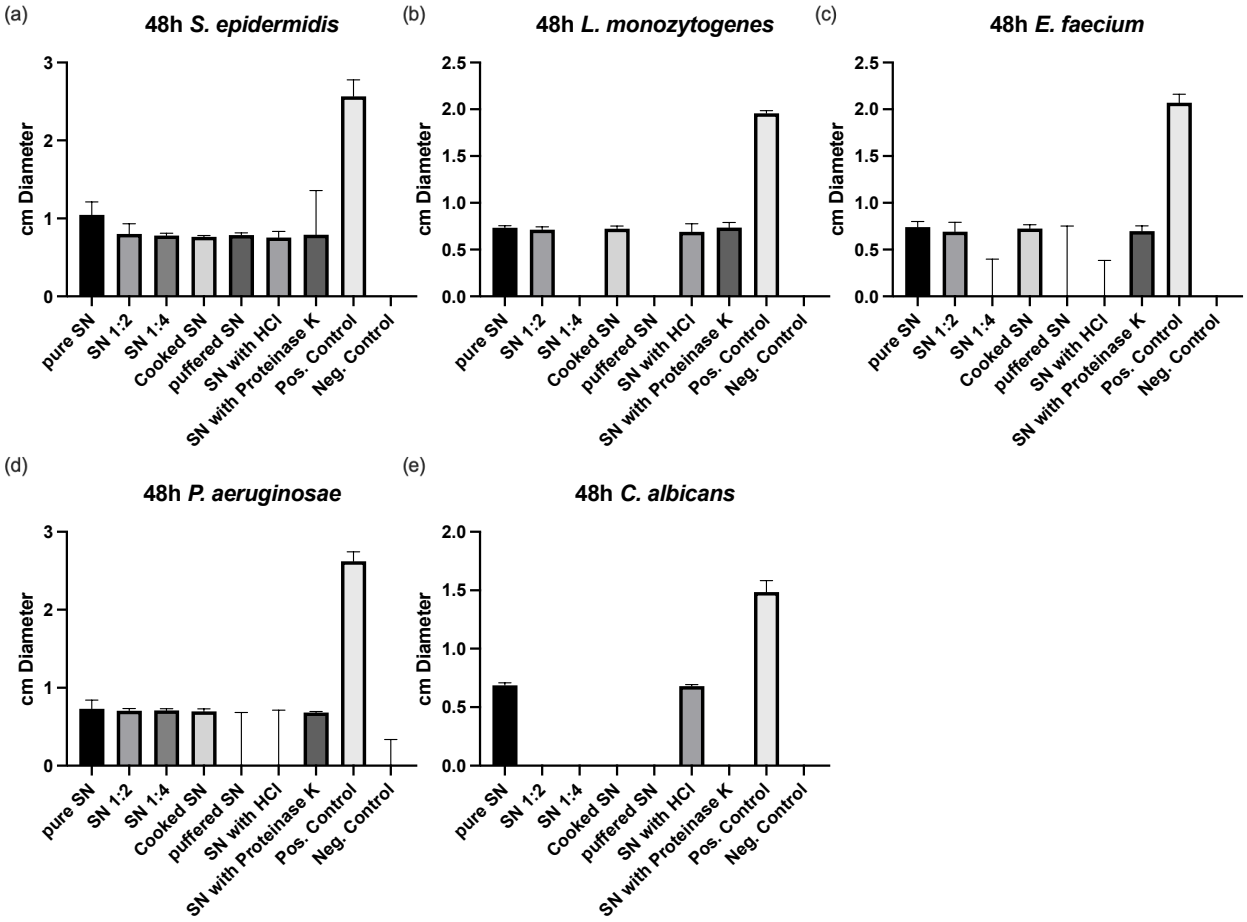


Figure 12: Antimicrobial activity of AAD10® supernatant after 48h (Panels (a)-(e) retrieved from Figure 2 of the original publication from (104); rearranged order / only 48h results; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

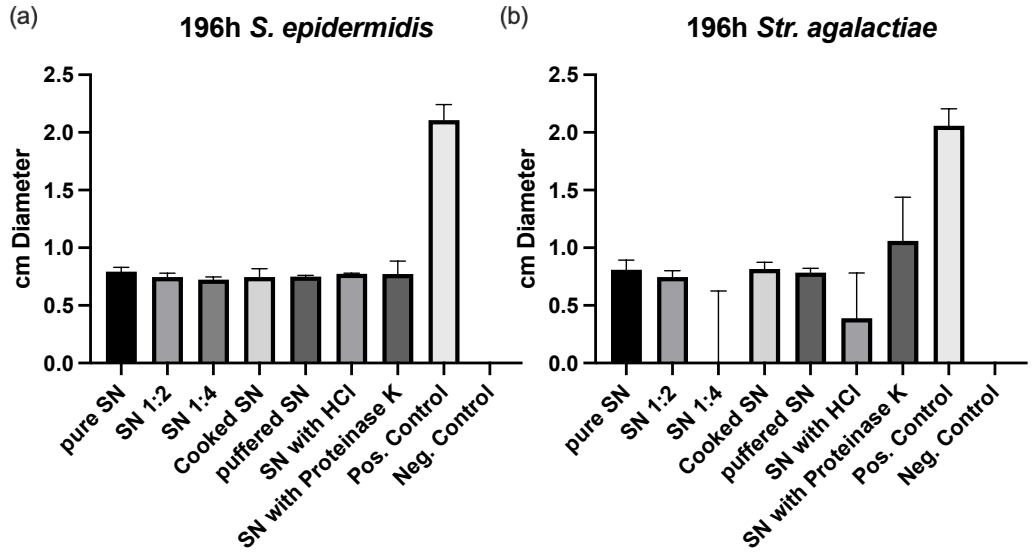


Figure 13: Antimicrobial activity of AAD10® supernatant after 196 h (Panel (a)-(b) retrieved from Figure 2 of the original publication (104); rearranged order / only 196 h results; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.1.4. Effect of AAD10 Supernatant on the Murine Fecal Microbiome

At euthanasia no significant differences were found for body weight and organ weights between animals receiving AAD10 supernatant and those mice receiving control medium. Neither alpha- nor beta-diversity markers were significantly different in the fecal microbiome analysis between animals which received the postbiotic supernatant of AAD10 of one and the other time point (48 h and 196 h) and those mice which were gavage fed with culture medium (Figure 14).

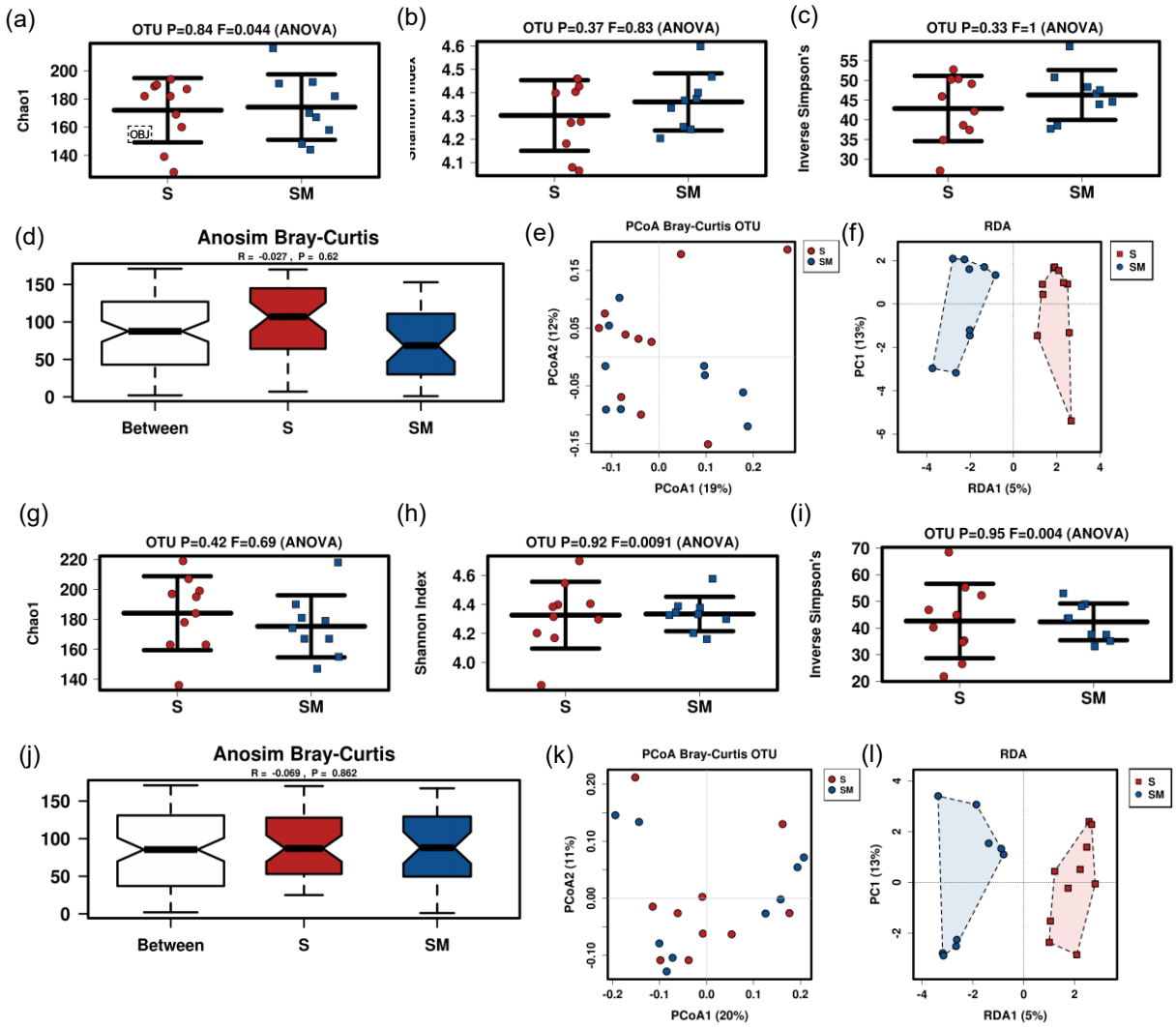


Figure 14: Alpha and beta diversity markers of supernatant after 48 h ((a)-(f); rarefaction 14420 reads) and 196 h ((g)-(l); rarefaction 14111 reads) culture time versus growth medium. RDA for 48 h supernatant: p=0.893; F 0.85; Var 18.34. RDA for 196 h supernatant: p=0.699; F 0.92; Var 19.3. S…supernatant; SM…medium group (retrieved from Figure 6 of the original publication (104); rearranged order of the panels; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

Neither the family nor the phylum levels results showed significant differences. Mice gavage fed with the 48 h postbiotic supernatant showed lower abundances of *Lachnospiraceae_FCS020_group* in LEfSe and ANCOM analysis compared to the control group. LEfSe additionally demonstrated a lower abundance of *Rikenellaceae_RC9_group* and a higher abundance of *Anaeroplasma_uncultured* in the group receiving the postbiotic supernatant (Figure 15). The 196 h postbiotic supernatant displayed a higher number of altered taxa in the murine stool microbiome. LEfSe analysis and ANCOM showed increased

Ruminoclostridium_5_uncultured_Clostridium_bacterium. Moreover, LEfSe also indicated a lower relative abundance of *Lachnoclostridium_Dorea_sp_52* and *Ruminoclostridium_6_uncultured* as well as an increase of *Anaeroplasma_uncultured*, *Odoribacter_uncultured*, *Tyzerella_uncultured* and *Fecalibacterium prausnizii* related with the postbiotic supernatant.

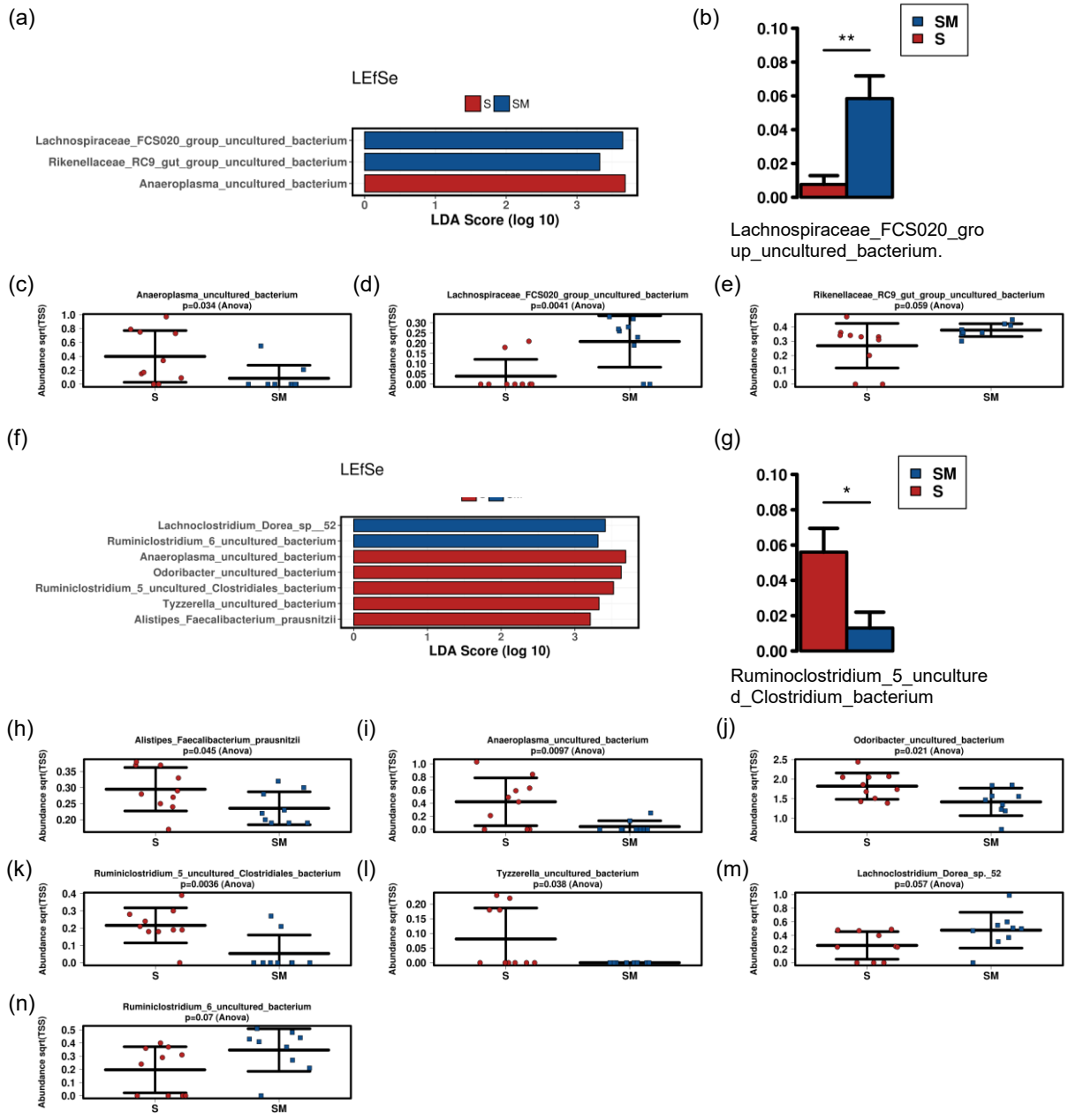


Figure 15: LEfSe and ANCOM analysis with bar charts of the 48h and the 196h postbiotic supernatant versus culture medium. S... supernatant; SM... medium control (retrieved from Figure 7 of the original publication (104); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.2. *Lentilactobacillus Diolivorans Supernatant – Reuterin System*

The production of *L. diolivorans* supernatant was conducted at our partner at the University of Life Sciences in Vienna.

3.2.1. Fermenter Culture of *L. diolivorans (Reuterin System)*

A Reuterin System supernatant with a maximum of 13.4 g/l 3-HPA and 6.9 g/l glycerol was produced at our partner as described in the materials and methods section above (Table 4).

Bioreactor 1/2	Time (hh:mm)				
	00:00	00:50	01:45	02:50	03:20
3-HPA (g/l)	1.1/0.7	7.8/6.6	10.4/9.6	10.3/9.4	13.4/11.9*
Glycerol (g/l)	23.2/23.48	15.5/15.63	9.3/10.9	9.0/10.6	6.9/8.3

Table 4: Concentration of 3-HPA and glycerol over time; *The supernatant with the higher 3-HPA value was used for the further experiments.

3.2.2. Storage Stability of the Reuterin System

Every thawing and freezing cycle of the Reuterin system supernatant slowly decreased the amount of 3-HPA. This decline was more pronounced in the 50 ml screw cap vials compared to the 15 ml screw cap vials used for storage (Table 5).

Day	3-HPA (g/l)		Glycerol (g/L)	
	15 ml	50 ml	15 ml	50 ml
0	100%	100%	100%	100%
7	97%	89%	102%	93%
14	95%	86%	101%	93%
21	94%	85%	101%	93%
28	93%	85%	102%	94%

Table 5: Concentration changes depending on storage vial size

The 3-HPA concentration dropped within the first seven days by 6%. Starting on day 7, a plateau was reached for 28 days with a stabile 3-HPA content -20°C. This plateau was

followed by a decrease and an increase until the 3_HPA content levelled off around 66 % in the following measurements (Table 6).

Day	3-HPA (g/l)	Glycerol (g/l)
0	100%	100%
7	94%	101%
9	94%	101%
12	94%	101%
14	94%	101%
35	94%	101%
63	68%	101%
211	72%	123%
244	70%	123%
302	69%	124%
342	66%	123%
369	66%	122%

Table 6: Storage concentration (3-HPA; Glycerol) over time

3.2.3. Antimicrobial Activity of the Reuterin System

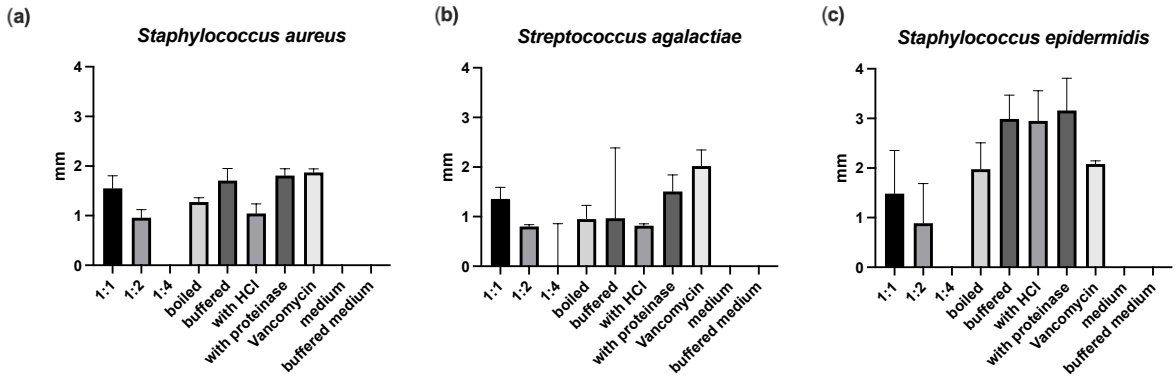


Figure 16: Antimicrobial activity of the Reuterin system (retrieved from Figure 1 of the original publication (119); removed ATCC numbers; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

The Reuterin system supernatant showed antimicrobial activity only against *S. aureus*, *S. epidermidis* and *Str. agalactia*, however there was no activity found against the remaining bacteria tested (Figure 16). The antimicrobial activity declined with increasing dilution of the Reuterin system supernatant. No relevant effects could be found with buffering or boiling the Reuterin system supernatant on the antimicrobial activity. Also Proteinase K

treatment or the treatment with HCl did not relevantly influence the antimicrobial activity of the Reuterin system supernatant. HCl was chosen to simulate the exposure to the acidic gastric environment.

3.2.4. Effect of the Reuterin System on the Fecal Murine Microbiome

At euthanasia, no significant differences for body and organ weights were found between the groups. In the 16S analysis Chao1, Inversed Simpson and Shannon were calculated as alpha diversity indices, but none of them significantly different between the two groups. Referring to beta-diversity, neither Anosim Bray-Curtis, PCoA Bray Curtis nor Redundancy Analysis (RDA) revealed significant differences between the two groups (Figure 17).

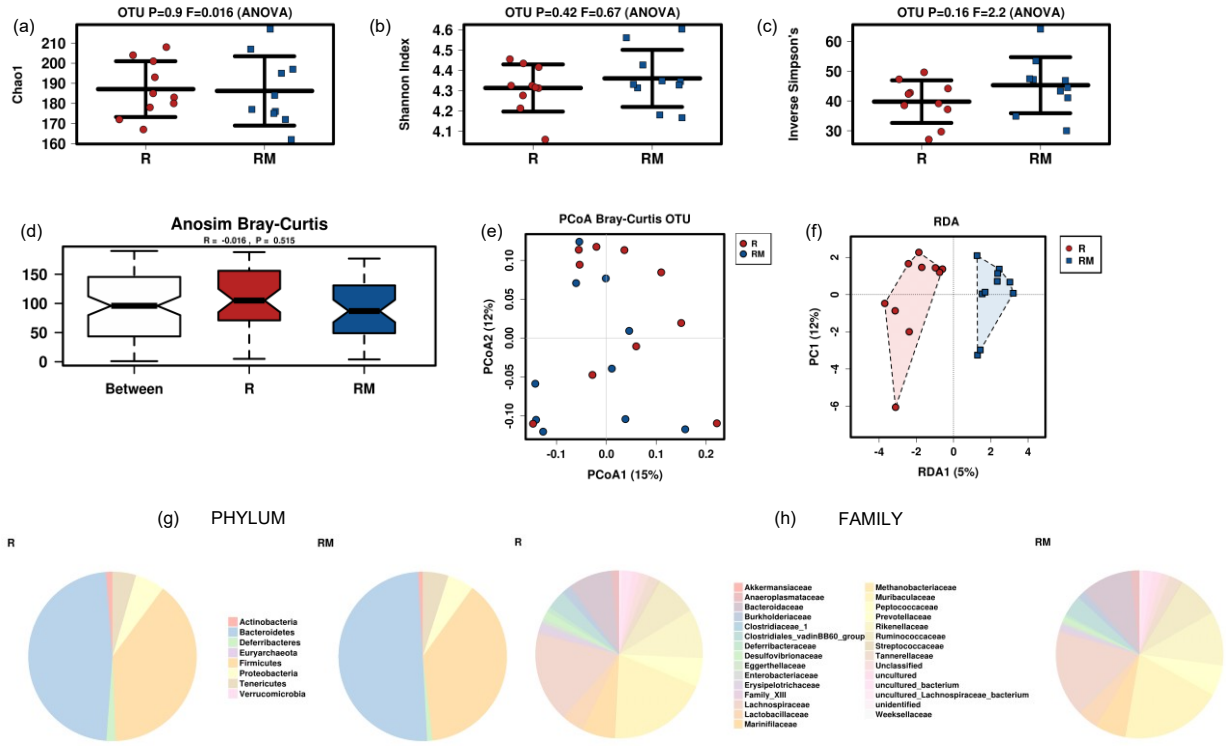


Figure 17: Alpha and beta diversity and relative abundances in the two groups. R...Reuterin group; RM...Reuterin medium group (retrieved from Figure 2 of the original publication (119); relabelled letters; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

The LEfSe analysis and ANOVA group comparison exposed a decrease of *Ruminoclostridium_5_uncultured_Clostridiales_bacterium* and *Lachnospiraceae_bacterium_COE1* in the Reuterin group. *Candidatus Arthromitus*, *Desulfovibrio_uncultured_bacterium, Eubacterium_xylanophilum_group* and *Ruminococcae_NK4A214_group* were risen in the group treated with the Reuterin system supernatant (Figure 18).

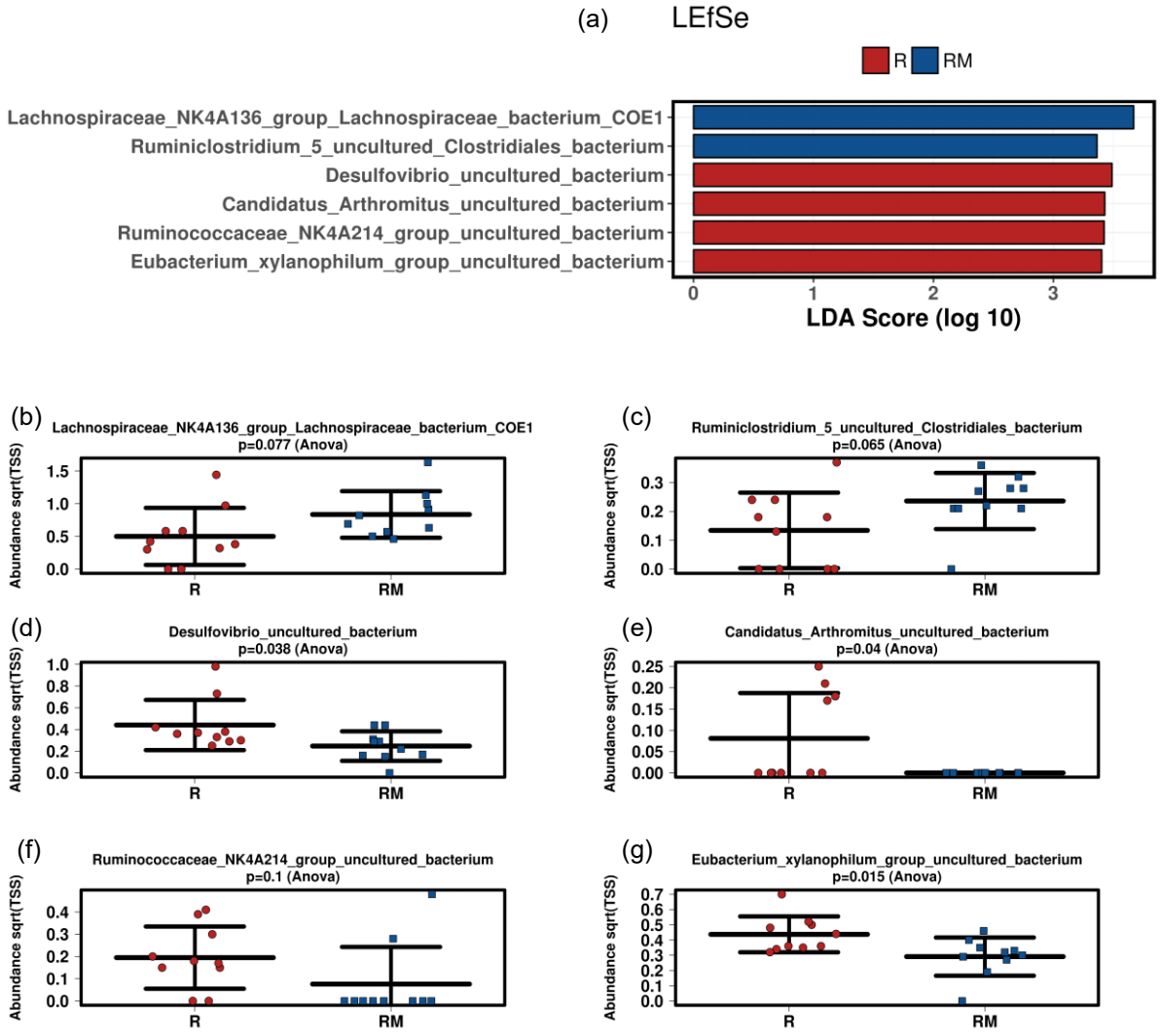


Figure 18: LEfSE Analysis of the two groups. R...Reuterin group; RM...Reuterin medium group (retrieved from Figure 3 of the original publication (119); relabelled letters; 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

Biomarker analysis found the same bacteria for the group discrimination as LEfSE Analysis (Figure 19).

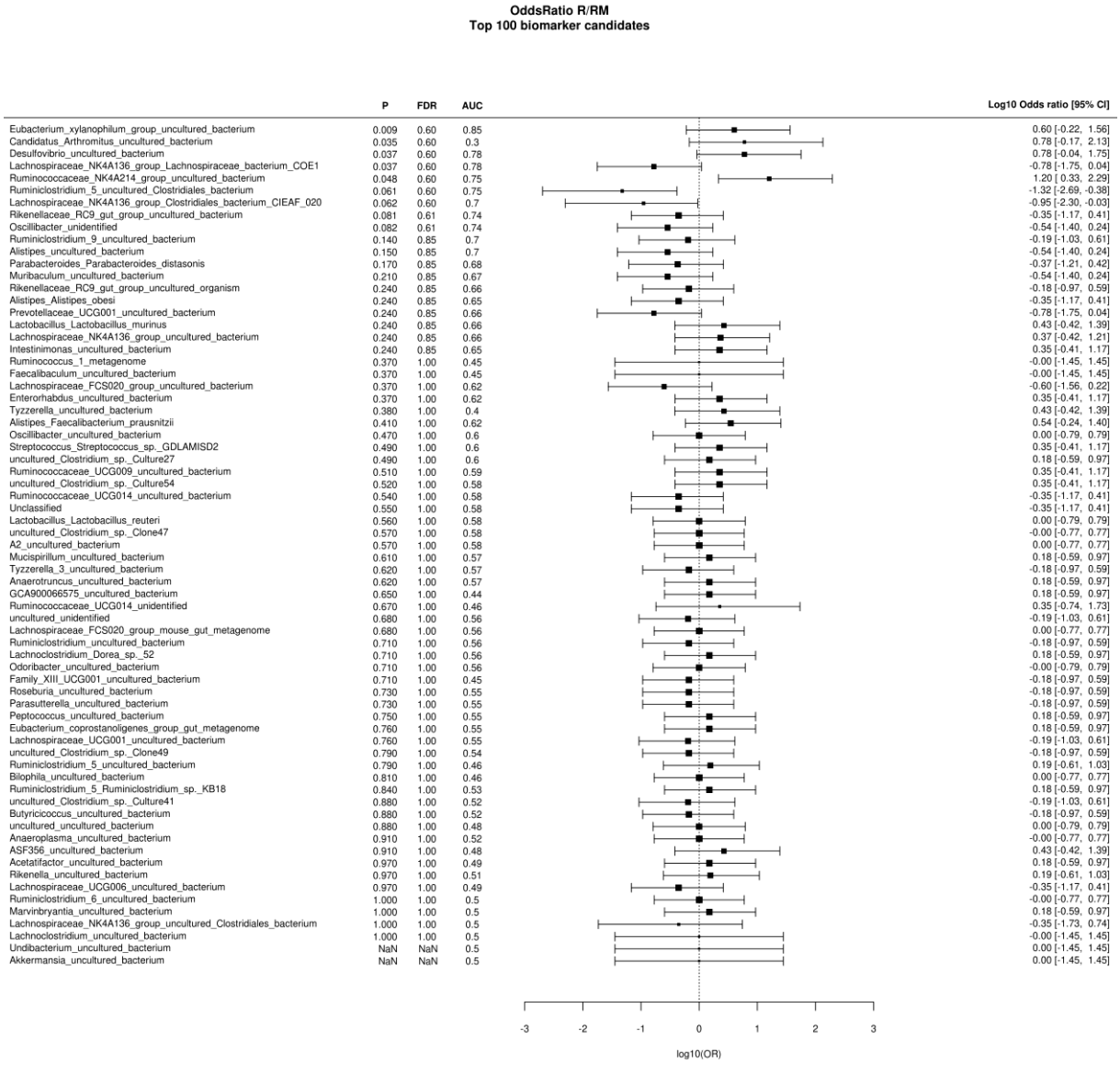


Figure 19: Biomarker search (retrieved from Figure 4 of the original publication (119); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.2.5. Fecal VOC Analysis after Reuterin Administration

GC-MS identified 42 substances in VOC analysis. Propene, isopropyl alcohol, isoflurane and propanol were attributed to room air contamination and thus were excluded from further analysis leaving 38 substances for group comparison. Only two substances, 3-methylbutanal and heptane, significantly differed between the two tested groups (Figure 20). While heptane significantly decreased following treatment with the Reuterin system supernatant, 3-methylbutanal significantly increased. These two substances, however, did not correlate significantly. The relative abundance of fecal bacteria showed significant group differences in the LEfSe analysis.

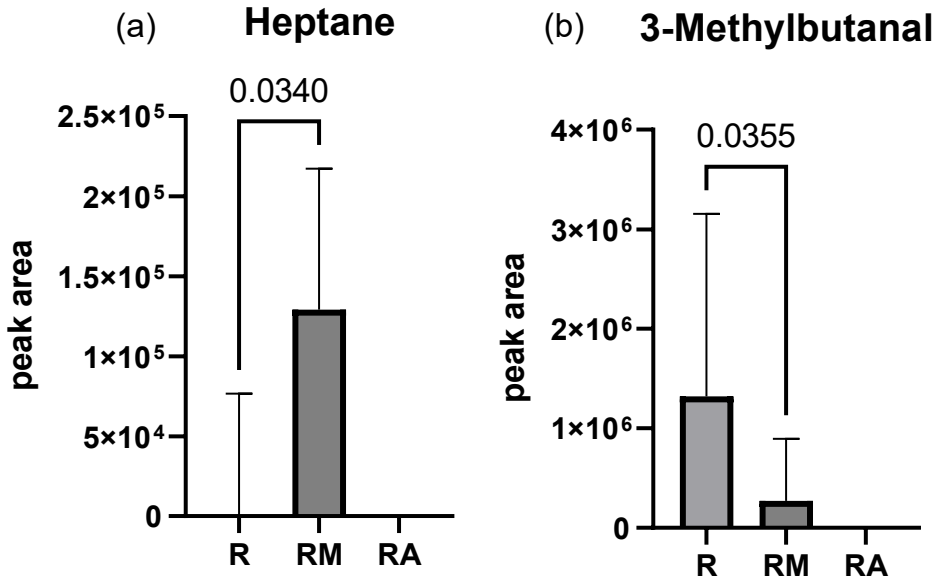


Figure 20: Significant results of the VOC analysis. R...Reuterin group; RM...Reuterin medium group; RA...room air (retrieved from Figure 5 of the original publication (119); relabelled letters, 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.3. *(S) - Reutericyclin*

3.3.1. Antimicrobial Activity Testing of (S) - Reutericyclin

Antimicrobial activity testing of (S)-Reutericyclin revealed activity against *S. epidermidis* only. This activity was not affected by buffering, by treatment with HCl or by proteinase K treatment (Figure 21).

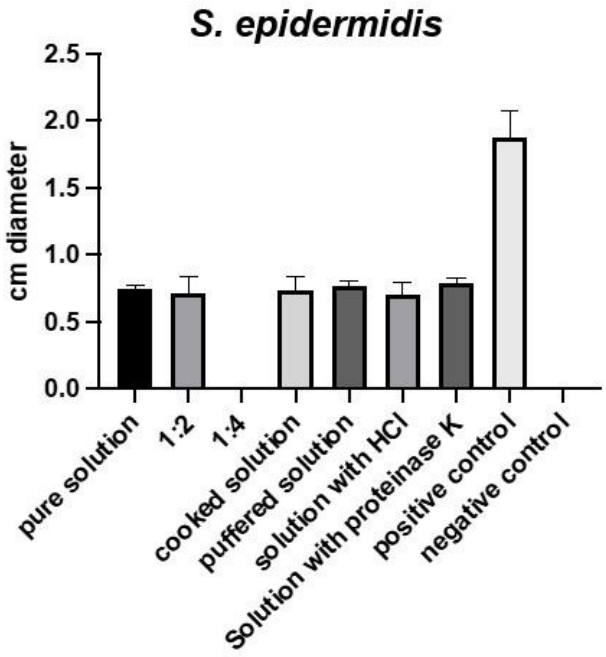


Figure 21: Antimicrobial activity of (S) - Reutericyclin (retrieved from Figure 1 of the original publication (120); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.3.2. Effect of (S) – Reutericyclin on the Fecal Murine Microbiome

Similar to the other postbiotics there were no significant group differences regarding body and organ weights at euthanasia. The analysis of the microbiome samples originated a trend towards a reduced alpha diversity with Chao1, Shannon and Inversed Simpson calculated. Beta diversity was significantly reduced in animals treated with (S) – Reutericyclin (Figure 22).

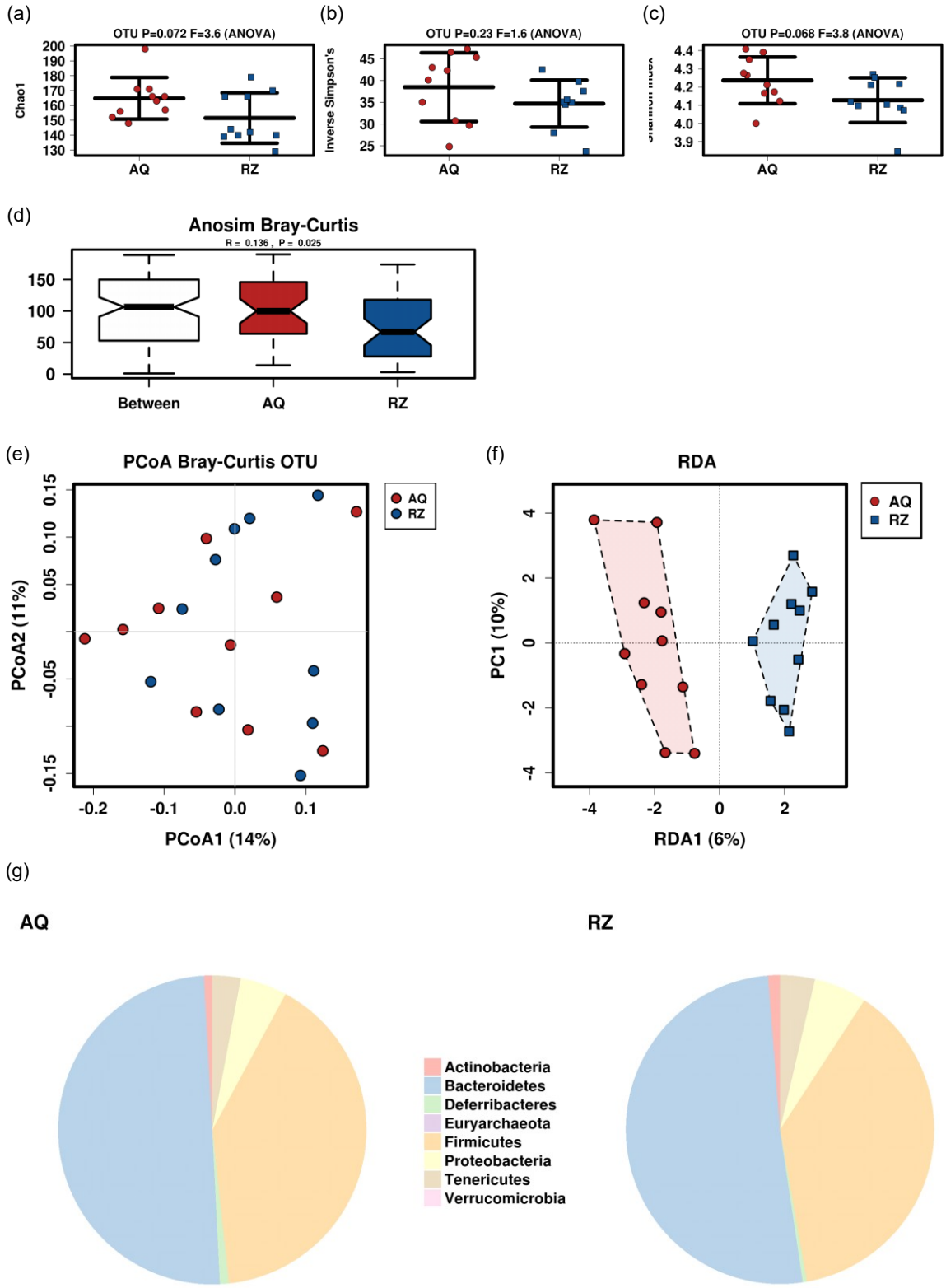


Figure 22: Alpha and beta diversity markers and relative abundance at the phylum level. The p-value of the RDA analysis is 0.07. AQ...Aqua group; RZ...(S) - Reutericyclin group (retrieved from Figure 2 of the original publication (120); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

LEfSE analysis showed significant changes in animals under (S) -Reutericyclin treatment (Figure 23).

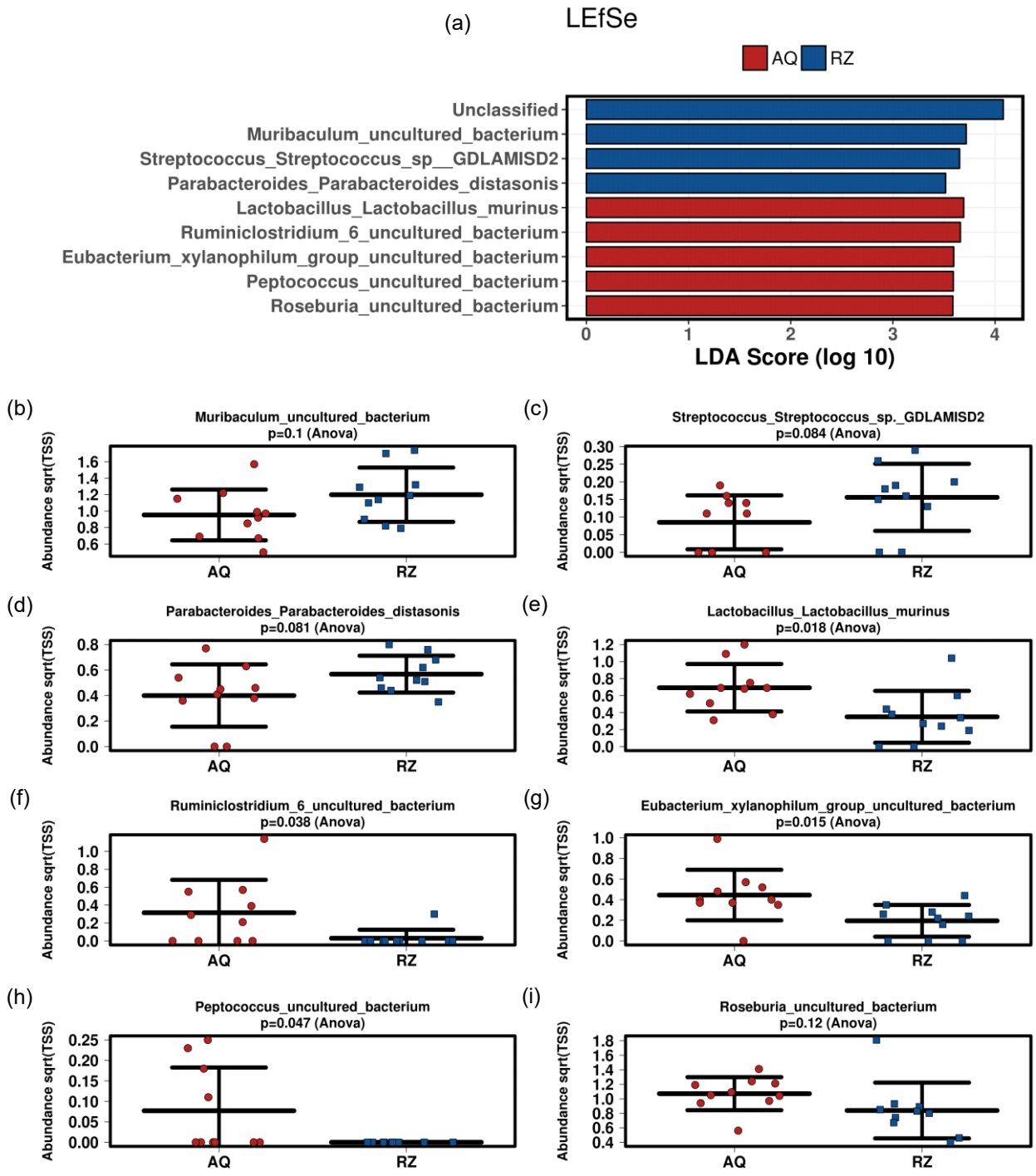


Figure 23: LEfSe analysis and bar charts of the two groups. AQ…Aqua group. RZ...Reutericyclin group (retrieved from Figure 3 of the original publication (120); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

3.3.3. Fecal VOC Analysis after (S) – Reutericyclin Administration

GC-MS identified 42 substances in VOC analysis. Isoflurane, isopropyl alcohol, o-xylene and propene were ascribed to contamination of room air and were consequently excluded from further analysis. Furthermore 3-methylpentane, 2-methylpentane, acetic acid, butanoic acid butyl ester and propanol were found in one single sample only and so these were no further analyzed. So, 33 substances were left for group comparison. In mice of the (S) - Reutericyclin group a significant increase of heptane and pentane and a significant decrease of 2-heptanone and 2,3-butanedione was found. Also three trends could be identified. Butanoic acid propyl ester tended to an increase and acetoacetate methyl ester and (z)-2-butene tilted to a decrease in mice treated with (S) - Reutericyclin.

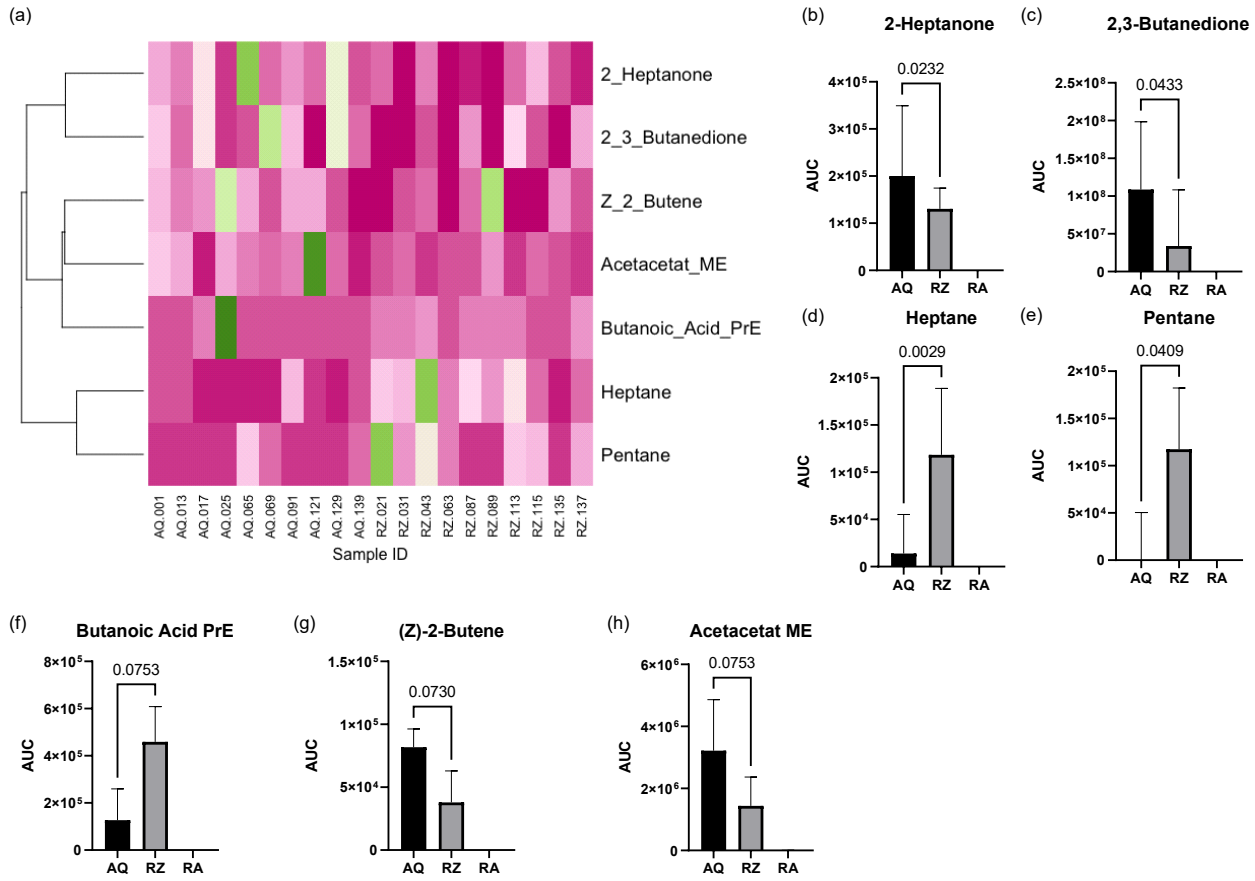


Figure 24Figure 24: Results of the VOC analysis. (a) heatmap with dendrogram; substances with significant changes (b)-(e) and with a trend between (f)-(h) the groups. AQ…aqua control group; RZ…(S) - Reutericyclin group; RA…room air samples; PrE…propyl ester; ME…methyl ester (retrieved from Figure 4 of the original publication (120); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

The dendrogram analysis of the results showed clustering within esters, ketones and short-chained carbohydrates (Figure 24). A Spearman-Rho correlation analysis was carried out. The correlating results with a group difference of p<0.1 between bacteria altered VOCs and in LEfSe analysis are presented in Figure 25.

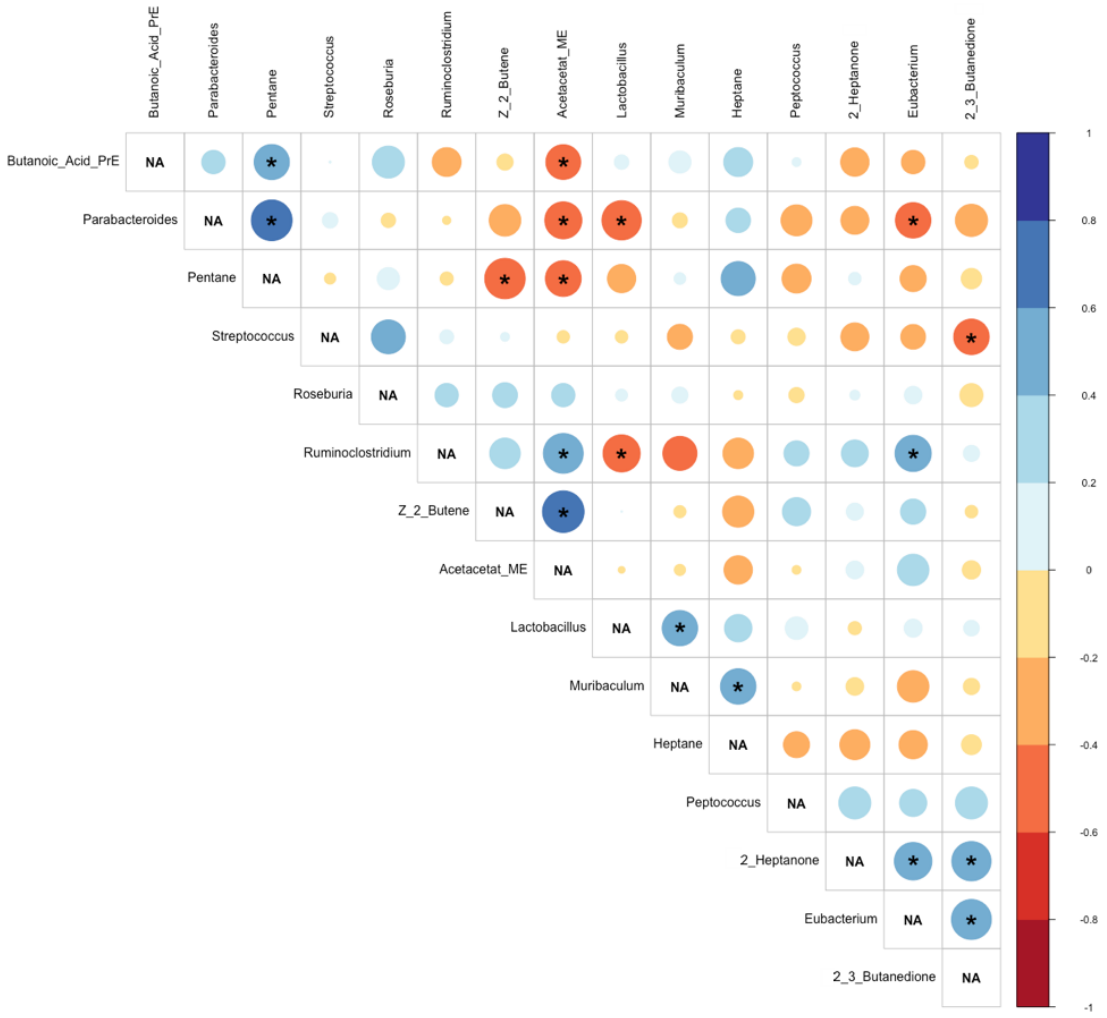


Figure 25: Correlation analysis (Spearman-Rho). Significant correlations (p<0.05) are marked with * (retrieved from Figure 5 of the original publication (120); 2022 with permission of the authors; license: https://creativecommons.org/licenses/by/4.0).

4. Discussion

In these studies, we investigated the antimicrobial activity of different bacteriocins / antimicrobial peptides either as pure substance or in form of postbiotic culture supernatants. The postbiotic candidate substances investigated in this thesis were chosen according to a database search (bactibase.hammamilab.org). The main focus was put on possible inhibitory effects on *Clostridia*, *Enterococcus*, *Listeria*, *Staphylococcus* and *Pseudomonas* and a promotion of the growth of *Lactobacillus*. As such the antimicrobial substance Reuterin and the bacteriocin Reutericyclin were identified by our partners at the University of Life Sciences in Vienna. Despite various efforts Reutericyclin could not be produced in suitable amounts in fermenter cultures by our partner. As such we chose to buy and test (S) – Reutericyclin as pure substance. The supernatant of OMNi BiOTiC® AAD10 was chosen in accordance to the interests of our industrial partner in this probiotic mixture.

4.1. General Considerations Regarding Postbiotics

Recently, the acceptance of probiotic therapy has substantially increased. However, a certain risk for adverse side effects in vulnerable patients including elderly, neonates or immunocompromised patients is still left, although probiotic therapy is considered safe in general (65). Potential problems that may occur include probiotic bacteremia, fungemia, increase of antimicrobial resistance and altered long-term immune responses (65-70). Therefore, alternative possibilities to positively influence intestinal dysbiosis are warranted (71). Bacteriocins, antimicrobial bacterial metabolites or postbiotics may be potential alternatives to live microorganisms positively influencing dysbiosis (121). Concerning probiotics, it still remains unclear whether the probiotic effect is caused by the bacteria *per se* or by bacterial metabolites or components. These may include short-chained fatty acids (SCFAs), secondary bile acids or microbe associated molecular patterns such as lipopolysaccharide or peptidoglycan for instance (121).

A preparation of inanimate microorganisms and/or their components conferring a direct or indirect health benefit are called postbiotics (72) with any substance released or produced through the metabolic activity of microorganisms included (73, 74). These substances may include bacterial lysates, cell free supernatants (CFS), cell wall fragments, enzymes, exopolysaccharides or short-chained fatty acids (SCFAs) (74). CFS are known to

contain amino acids, carbohydrates, electrolytes, lactate, bacterial toxins like bacteriocins and antimicrobial peptides and other bacterial metabolites. In contrast to probiotics, postbiotics have some advantages including a relative stability in industrial processes and storage. Intellectual property protection because no alive microorganisms can be acquired from the postbiotic and a better safety profile are two additional advantages (72).

4.2. Culture Supernatant of OMNi BiOTiC® AAD10

OMNi BiOTiC® AAD10 is a mixture of 10 different probiotic strains with beneficial effects (described in (65)) to the host as reported before. These effects include decrease of the severity of antibiotic-associated diarrhea, travelers' diarrhea, prevention of constipation, prevention and treatment of necrotizing enterocolitis, reduction of radiation induced diarrhea or reduction of the risk of alimentary allergies (122-127). In comparison to products containing only one probiotic strain the combination of more than one strain may be beneficial. A previous study with the multi-species probiotic VSL#3 could show benefits compared to single strain probiotics. VSL#3 contains *B. breve*, *B. infantis*, *B. longum, E. faecium*, *L. acidophilus*, *L. casei, L. delbrueckii, L. plantarum* and *S. thermophilus* which is close to the composition of OMNi BiOTiC® AAD10(128). OMNi BiOTiC® AAD10 composition focused on therapy and prevention of antibiotic-associated diarrhea (AAD). Several clinical reports have proven the therapeutic potential of AAD and even the potential in prevention of AAD (129-131). Only scarce information is available concerning the antimicrobial activity and the effect on the fecal microbiome of probiotics containing single species. Even less information about growth, metabolic activity and gene expression of a combination of different probiotic bacteria can be found when combined in a co-culture.

To gain an insight into the starting phase of the co-culture the time point of production step 1 was chosen at 48h supported by the findings of other studies which have shown the highest activity then (132). The culture was not stopped here. Only a part was harvested and the rest was continued to 196 h of cultivation time to obtain an impression of the interaction between the different strains under competitive prerequisites in the long-term culture. To win more knowledge on the course of the culture shotgun, metabolomics and VOC analysis was performed at certain time points throughout the culture period.

In this study, all of the 10 probiotic strains named above as members of the OMNi BiOTiC® AAD10 composition were co-cultured in only one flask resembling one niche. This

is the first time the alterations of the relative abundances of the probiotic strains in such a co-culture have been described. Consequently, there is no literature available allowing a more thorough discussion of these findings. The cause of the distinct growth behaviour in the culture bottle may be accredited to shifts in every niche's conditions as modified chemical composition as a consequence of metabolism with its pathways or the production of bioactive substances or consumptions of nutrient resources and space (133).

An untargeted metabolome analysis was performed. Comparative analysis between metabolites and the time of culture revealed several correlations. Malic and fumaric acid increased over culture time, maybe impacting differing growth patterns. The addition of organic acids may stunt the growth of several cultured bacteria. As described previously, malic acid was found to inhibit growth of *Escherichia coli*, *Salmonella typhimurium, Listeria monocytogenes* (134) and *Shigella flexneri* (135) effectively. Likewise, fumaric acid acted clearly antimicrobially against *Campylobacter jejuni* (136). In this experiment the antimicrobial activity of the production step 1 cell culture supernatant (48h) was higher compared to the later supernatant (196h). In detail fumaric acid increased towards the end of the culture time but only the early (48h) and not the late (196h) supernatant exhibited activity against *L. monocytogenes*. Furthermore, malic acid also increased throughout the culture period, but neither supernatant took influence on *Campylobacter jejuni*. Consequently, the abundance of these metabolites is an unlikely occasion for the antimicrobial activity observed for the cell culture supernatant. The underlying mechanisms as well as the effect on the growth behaviour of single metabolic products of several probiotic strains remains unclear.

The bacteria are supposed to directly interact besides environmental changes. The interaction between diverse strains of bacteria and the underlying mechanisms are very complex; quorum sensing seems to play a major role. Quorum sensing is a bacterial cell-to-cell communication process (reviewed in (137)) potentially inducing bacteria to produce bacteriocins or/and other antimicrobial molecules to help them to defend their niche. Against this background, the co-culture of diverse bacteria causes advantageous impact in triggering the induction of non-ribosomally and ribosomally generated secondary metabolites (137). For example, the nisin production raised by 85% in a co-culture of *Saccharomyces cerevisiae (known to produce nisin)* with *Lactococcus lactis* (138). Nevertheless, none of the substances which are known to modulate quorum sensing (small peptides, acetylated homoserine lactones or autoinducer-2) (137) could be identified in our untargeted metabolomic analysis.

The shotgun analysis uncovered the underlying pathways of the bacterial gene expression. Most of them showed up at nucleoside/nucleotide biosynthesis, protein/amino acid synthesis and energy production as fermentation or glycolysis. *L. Salivarius* showed that the activity peaked at 96 h although the relative abundance already decreased. In other bacteria the gene expression was similar with their relative abundance. Therefore, the results of the untargeted metabolome analysis exposed amino acids/peptides, nucleotides and their metabolites and energy metabolites as the most predominant classes. The measured distinct substances correlated with the abundance of certain bacteria. *L. paracasei* correlated highest of the probiotic bacteria used here while *B. bifidum* did not correlate. A possible explanation for this finding could be a great metabolic activity of *L. paracasei.* In combination with both a low activity as well as relative abundance of *B. bifidum* the results of this co-culture might be explained. The bacterial emitted substances measured in the VOC measurement were attributed to ketones, esters and aldehydes. It is not possible to identify which substance was produced by which bacteria influencing other bacteria because of the co-culture. There is no possibility to conclude on the bacterial activity directly.

In vitro antimicrobial activity of the supernatant produced for this study was found against the bacteria *E. faecium, L. monozytogenes, P. aeruginosae, S. epidermidis, Str. agalactiae* and the fungus *C. albicans.* The activity of the supernatant of production step 1 was more pronounced than the supernatant of production step 2. We chose the time point for harvesting of production step 1 after 48 h of culture time and the time point for harvesting of production step 2 after 196 h. The first harvesting point has been chosen because of prior investigations of monocultures which postulated the highest antimicrobial activity of monoculture supernatants at this time point (132). However, the antimicrobial activity of longer culture periods have not been investigated so far. Thus, we were also inquisitive in results from later time points. The supernatant´s antimicrobial activity of production step 2 differed from production step 1. The activity against *C. albicans, E. Faecium, L. monocytogenes* and *P. aeruginosae* could not be confirmed with the supernatant of production step 2 but the growth of *Str. agalactiae* was inhibited as a new finding.

There are various reports examining the antimicrobial effects of CFS of either monocultures or merged CFS of different cultured bacteria (139). Danilova et al. exhibited the antimicrobial potential of *L. plantarum* culture supernatant against *S. pyogenes, E. coli, P. aeruginosa* and *S. aureus* (132). In comparison to the data of Danilova et al., we have examined an antimicrobial activity against *P. aeruginosae* but not against *S. aureus* and *E. coli*. However, we were not able to attribute the antimicrobial effects to either low- or high-

molecular weight fractions since we did not distinguish between those two. Fredua-Agyeman et al. have reported inhibitory effects of a combination of culture supernatants of *B. lactis, L. acidophilus* and *B. bifidum* against *P. aeruginosa* (139). Different cultured strains affect each other competing for the same niche. Several bacteriocins can be produced by culturing more than one strain at the same time in one flask. With merging supernatants of different strains of monocultures these substances might be lost and so the merged supernatant will differ from the supernatant produced in a co-culture. Antifungal processes comparable to our results were also shown for *Lactobacillus* strains (140) and cell-free supernatant of *Pediococcus acidilactici HW01* (141).

Neither subjection to proteinase K nor cooking influenced the antimicrobial activity of postbiotic supernatants confirming previous findings (132). However, the antifungal activity was lost thereby suggesting that the impact on *C. albicans*, but not the antibacterial activity may depend on a peptide/protein. Since the untargeted metabolomic approach revealed no apparent bacteriocin, the cause for the antimicrobial activity of the postbiotic supernatant used in our study has still to be elucidated.

Sometimes the administration of probiotics could be critical, for example in immunocompromised patients (71). Bacteriocins or postbiotic supernatant could serve as an alternative when live bacteria are not administrable (74). Postbiotics from *Lactobacilli* have proven antimicrobial, immunomodulatory, anti-tumour and barrier-preserving effects (142). Prior studies found an effect of postbiotics on the bacterial composition of the intestinal microbiota in a variety of different models. A study examining a postbiotic derived from *Lactobacillus* caused significant increase of richness (Chao1) and diversity (Shannon) in rainbow trouts. The same study also revealed decreases of *Fusobacteria* and increases of *Bacteroides, Tenericutes* and *Spirochaetes* in fish fed with postbiotics (143). In suckling rats, postbiotic supplementation caused an increase of richness and decreases of *Chitinophagaceae* (144). Heat treated postbiotics from *L. fermentum* and *L. delbrueckii* led to reduced abundances of *Turicibacter*, *Clostridium sensu strictu* and *Dorea* in a study with mice (145). The human gut in a fecal fermentation model reacted to application of the same probiotic with an enzyme- and heat-stable bifidogenic effect (71). We could not find changed alpha or beta diversity in our supernatant administered mice. The supernatant from OMNi BiOTiC® AAD10 is here contradictory to other studies (143, 144). We detected a differential impact of cell-free supernatant of production step 1 and 2 at genus level. The production step 1 supernatant harvested after 48 h caused increases of *Anaeroplasma* and decreases of *Rikenellaceae_RC9_group* and *Lachnospiraceae_FCS020_group* with potential beneficial

effects (146). The growth of potentially health promoting *Aneroplasma* stayed present when gavage feeding postbiotic supernatant after 196 h of culture. In addition, *Ruminoclostridium_5*, *Tyzzerella*, *Odoribacter* and *Fecalibacterium (F.) prausnitzii* increased while *Dorea* and *Ruminoclostridium_6 decreased*. Particularly the rise of *F. prausnitzii* has been linked to favourable aspects like SCFA production or anti-inflammatory effects (7, 147). *Dorea* has been connected with non-alcoholic fatty liver disease (148, 149). As a result, reducing *Dorea* could also be helpful for the host. Several studies in different models including mice, fish, rats or even fermenter cultures showed several effects of postbiotic CFS and also different classes of postbiotics such as CFS, cell wall fragments, exopolysaccharides, enzymes, bacterial lysates, SCFAs or bacteriocins (74).

Limitations of this study concerning AAD10 supernatant include the setting characterisation of the cell-free supernatant culture. The culture was performed only one time. The results of the shotgun, VOC and metabolomic examinations for the supernatant rely on only two samples per time point. Hence, we did not perform statistical comparisons. Even though the data presented here have to be verified in future studies, we are able to present a first glimpse into the effects of a probiotic co-culture. The efforts will be much higher in the search of bacteriocins with targeted approaches. The nomenclature of the probiotic bacteria used as applicable before the changes made in 2020 (150), because these names are still used by the manufacturer of OMNi BiOTiC® AAD10.

4.3. Reuterin System

The relative storage stability of the synthesised 3-HPA in this study was 35 days. 3-HPA exhibited *in vitro* antimicrobial activity against three aerobic Gram-positive cocci. Furthermore, in our *in vivo* experiment the treatment with the reuterin system caused changes of the fecal microbiota and volatile organic compounds.

Additional to an adequate bacterial strain, its age and concentration, the fermentation for bioconversion should also include a suitable medium (151, 152). Other studies have demonstrated that the production of 3-HPA depends on the concentration of glycerol as well as other compounds of the medium to a great extent (152). Utilising growth media like MRS extended with glycerol, great quantities of 3-HPA were secreted by *L. reuteri* (83). Nonetheless, the specimen has still contained accidental secondary products following purification (83). Hence, a 2-step process was selected for the production of 3-HPA in this

experiment. The gold of step 1 was the generation of an active biomass in a suitable growth medium under ideal conditions, followed by the second step, a quick bioconversion in glycerol-containing water as biotransformation medium (86). This process has been described previously to reach an excellent outcome in terms of oppression of by-product formation and high productivity (86). Another upside is that finally 3-HPA was dissolved in an aqueous solution and not in a complex medium (153). In this arrangement, we were able to achieve a final 3-HPA concentration of 13.4 g/l with *L. diolivorans.* Our outcome confirms other research groups with trials with *L. reuteri* ATCC 53608 mentioned in the literature (83, 152, 154).

Confirming previous reports, 3-HPA was stable for at least six months in aqueous solution at 4°C (83, 152). Additionally, the composition of the HPA-system did not change over a period of 150 days as examined (83). As the firmness of 3-HPA exhibits a decrease with rising temperature (152), a similar reaction could be assumed for declining temperatures. Consequently, we have examined the stability of 3-HPA in our Reuterin system at -20°C and could detect a minor depletion of the 3-HPA concentration at the beginning followed by 35 days of stability with a slow decline throughout the following months. The differently applied methods for 3-HPA quantification are a possible explanation for the discrepancy between our results and previous results reported in the literature. Vollenweider et al. and Lüthi-Peng et al. used other methods than HPLC for the further quantification of 3-HPA in contrast to this study (83, 152). Temperature, free amino groups and sugar have an effect on the stability of 3-HPA. The highly reactive hydroxyl and/or aldehyde groups of 3-HPA react with carboxyl, sulfhydryl, hydroxyl and amino groups of biological molecules (83, 84, 152, 155, 156). Therefore, 3-HPA has been shown to be more stable in water than other media (152). When the storage periods were longer, the pH significantly affected the stability of the HPA-system´s main compounds. Correspondingly, one day or seven days under strong alkaline or acid conditions destroyed the HPA-system (83).

Besides the storage period, we have also investigated the influence of repetitive freeze-thaw cycles. A decrease in the 3-HPA concentration with every freeze-thaw cycle was found. The size of the storage vial exhibited different storage outcome. The 3-HPA concentration declined faster in larger compared to smaller vials. Oxygen or the contact surface might be the influencing factors on the stability of 3-HPA. Because of these findings, the Reuterin system was stored at -20°C and pH 7 for the remaining *in vitro* and *in vivo* examinations avoiding freeze-thaw cycles wherever possible.

The inhibitory effect of Reuterin against Gram-negative and Gram-positive bacteria, fungi, yeasts and protozoa has been reported. In addition, the antimicrobial potential of the Reuterin system against a variety of food spoilers and pathogens, lactic acid bacteria used in food fermentations and microorganisms of the gastrointestinal tract of mammals have been shown (85). Regarding the complexity of the Reuterin system, it is still unclear which molecule (3-HPA, HPA-hydrate or HPA-dimer) serves as the active antimicrobial substance (84). Although inhibition of ribonucleotide reductase (and thus DNA synthesis) (83, 84) and thioredoxin (cytoprotective against oxidative stress) (83, 84, 155) or a toxic effect of acrolein (85) have been discussed previously, the exact mechanism of the antimicrobial activity of the Reuterin system still remains unclear (155).

Cleusix et al. have described a representative number of bacteria residing in the human gastrointestinal tract, which are sensitive to Reuterin (155). This research group tested 19 non-*Lactobacillus* species. 15 species out of those have shown minimal inhibitory concentration values lower than the ones of *E. coli* K12, including *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bifidobacterium (B.) catenulatum*, *B.adolescentis*, *B.bifidum*, *B.longum*, *B.longum* var *infantis*, *Colinsella auerofaciens*, *Clostridium difficile*, *Enterococcus faecium*, *Eubacterium biforme*, *Eubacterium eligens*, *Streptococcus salivarius* and *Listeria ivanovii* (155).

In our investigation, the Reuterin system had antimicrobial effects on the Gram-positive aerobic cocci *S. agalactiae, S. aureus* and *S. epidermidis* but not on *C. difficile*, *E. Faecium, L. monocytogenes*, *P. acnes* and *P. aeruginosa*. Some of these bacteria cannot be found in the literature in relation to the influence of the Reuterin system. The results for *E. faecium* and *C. difficile* in this study are contradictious to reports from other publications (155). Nonetheless, data from various studies are unfrugal to compare because of the different methods used in the studies. In addition, different studies have examined different bacteria and used non-comparable compositions of the Reuterin system. Limitations also occur when extrapolating *in vitro* Reuterin data to *in vivo* gastrointestinal conditions (155). The inhibitory effects of the Reuterin system may be influenced by factors like nutrient competition and synergistic or antagonistic relationships between the intestinal bacteria (155). However, low intestinal concentrations of Reuterin can already alter bacterial growth (155).

The application of Reuterin system in our *in vivo* mouse experiments showed significant changes of the fecal microbiota. The *in vivo* effects of the Reuterin system on the murine fecal microbiome has not been investigated yet. Previously to the *in vivo* application

of the Reuterin system, we have tested the influence of proteinase K and HCl on the Reuterin system. No distinct depletion of its antimicrobial activity was found. Consequently, neither intestinal proteinases nor the acidic gastric milieu should influence the Reuterin activity in the gastrointestinal tract. No difference in alpha or beta diversity was found. We could demonstrate a depletion of *Ruminoclostridium_5_uncultured_Clostridiales_bacterium* and *Lachnospiraceae_bacterium_COE1* and increases of *Desulfovibrio_uncultured_bacterium*, *Candidatus Arthromitus*, *Ruminococcae_NK4A214_group* and *Eubacterium_xylanophilum_group*. The potential of these effects and possible impacts on diseases need to be investigated in future studies.

Volatile organic compounds (VOCs) in the headspace of stool samples are influenced by the intestinal epithelium, the microbiome and diet. VOCs are generated during metabolic processes within the intestinal tract (157). VOCs are accountable for the “smell” of different microbiota (158). As a consequence, cultures of distinct bacteria like *E. coli* and *Mycobacterium spp*. or fungi like *Fusarium spp.* and *Aspergillus* have certain VOC profiles with the permission to identify the species by their “smell” (159-162). In mammals, esters typically dominate the stool VOC profile confirming results of our study.

Changes of heptane have, amongst others, been examined in cultures of adipocytes derived from patients with Simpson Golabi Behmel Syndrome (163), in the supernatant of bacterial cultures associated with infectious bacterial activity (164), in breath samples of patients with, for example, bronchiolitis obliterans syndrome (165) or sleep apnoea (166). 3-methylbutanal has been detected in the VOC profile of stool of patients with a *C. difficile* infection (167) and in cultures of *Pseudomonas aeruginosa* and *S. aureus* (168). Moreover, cultures of mesophilic *Lactobacilli* as *L. paracasei, L. casei*, *L. plantarum* and *L. helveticus 209* were shown to produce 3-methylbutanal (169). Because of numerous intestinal bacteria and their plethora of metabolic processes, specific VOCs cannot be linked to single bacteria. We were not able to prove a significant correlation between bacteria with significant group differences in the LEfSe analysis and fecal VOCs. The microbiome is known to change throughout the whole gut. Therefore, it might be possible, that the VOCs detected were produced and emitted upstream and then excreted with the stool. As we did not harvest intestinal contents from every single level of the gastrointestinal tract, we are not able to address this question at the moment.

Limitations of this study concerning the Reuterin System include that the data for storage stability rely on only on culture supernatant and one repetition. Before further application in the future these tests have to be repeated to gain more reliable data -

especially before commercial application. Like the other postbiotics investigated here, the antimicrobial testing was based on agar diffusion only. It cannot be ruled out, that liquid cultures may have yielded different results – especially for strictly anaerobic bacteria. Furthermore, it is questionable (as for all other studies involving the murine microbiome) to which extent the murine results can be transferred to humans.

4.4. Reutericyclin

As various fermenter culture approaches failed to produce representable amounts of Reutericyclin we chose commercially available (S) - Reutericyclin (N2833) (https://pubchem.ncbi.nlm.nih.gov/compound/54696024) for this project.

The inhibitory bactericidal effect of Reutericyclin on Gram-positive bacteria has been ascribed to its action as a proton ionophore (100) which selectively disperses the bacterial transmembrane potential (102, 103). Fungi, yeasts and Gram-negative bacteria, however, have been shown to act resistant to Reutericyclin (100).

Reutericyclin producing strains of *L. reuteri* are used in food fermentations (97). These bacteria are a stable constituent of the human and animal intestinal microflora (96). Consequently, the Reutericyclin application seems to be a feasible approach.

Antimicrobial effects of (S) - Reutericyclin against *S. epidermidis* were found in the *in vitro* susceptibility testing. No effect on Gram-negative bacteria (*E. coli, P. aeruginosae* and *C. jejuni*) was expected as published before. The effect on the chosen Gram-positive bacteria should have been examinable (100). Nevertheless, we found no distinct *in vitro* antimicrobial activity against other investigated pathogens (see 3.3.1). Hurdle et al. found Reutericyclin to be very effective against the lethal non-growing stage of *C. difficile* which was contrary to our findings (102). In their study, they describe a mean minimal inhibitory concentration (MIC) of 0.09 - 0.5 mg/l, a concentration that may also be attained in the intestinal tract, depending on the strain (102). We applied Reutericyclin at a concentration of 62.5 µg/ml resulting in 1.25 µg/platelet for the 1:1 concentration. Nevertheless, we could not reveal inhibitory effects. The different culture method could be the reason for this outcome. We used tryptic soy broth and sheep blood in contrast to TY broth which was used by Hurdle et al. Reutericyclin disseminated into the culture medium as we found an effect against *S. epidermidis* but it might be possible that the final concentration was too low to inhibit *C. difficile*.

As Hurdle et al. also used different *Clostridium* strains (C. difficile 9689 (toxinotype 0), BAA-1803 (toxinotype III, NAP1) and BAA-1875 (toxinotype V, NAP7)), the difference in the results could be explained as we used non-toxin producing C. difficile ATCC 700057 in our study (102).

The type of Reutericyclin used may be third potential reason. Numerous diverse versions of Reutericyclin are available at the moment. The different types have been described in the literature before (101, 102). Hurdle et al., for instance, have examined Reutericyclin and its modifications 867 and 1138 (102) (Figure 26).

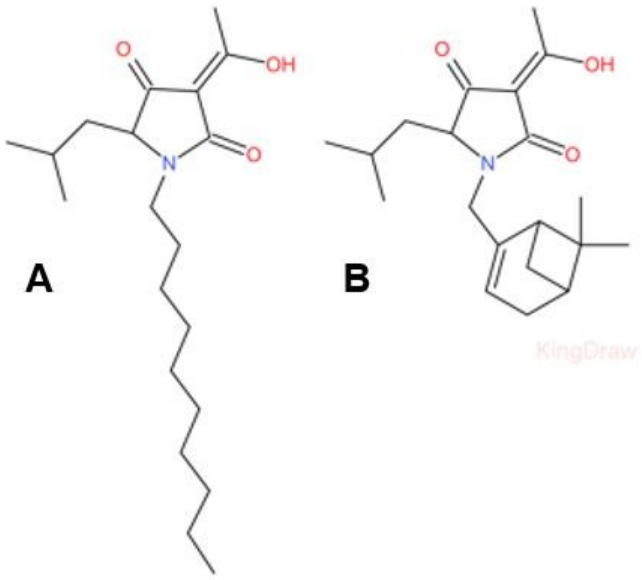


Figure 26: A: Modification 867; B: Modification 1138. Designed with KingDraw®.

They found changed reactivities against different *C. difficile* strains (BAA 9689, 1803 and 1875) for the different substances. Likewise, Cherian et al. noted different MIC against Gram-positive bacteria (*Enterococcus faecalis* ATCC 33186, *Streptococcus pneumonia, Bacillus anthracis sterne*, *Bacillus subtilis* ATCC 23857, *Streptococcus pyogenes* ATCC 700294, *Clostridium difficile* BAA 1803 and methicillin-susceptible *Staphylococcus aureus* N315) according on various modifications of the Reutericyclin side chains (101). It might be possible that the different type of isoform ((R) or (S)) affect the antimicrobial activity of Reutericyclin. Moreover, the S-isoform of Reutericyclin used in this experiment perhaps reveals other antimicrobial activities than the S/R racemate, the R-isoform or other modified versions (101, 102) of Reutericyclin.

Treatment with HCl or proteinase K, buffering or heat did not affect the activity of Reutericyclin. This is in accordance with other reports, where inhibitory substances produced by *Lactobacilli* were isolated from sourdough (99).

In vivo experiments examining the effects of Reutericyclin as a pure substance in mice are not available at the moment. Although a potential weakening of the antimicrobial activity of Reutericyclin by caecal contents can be found, efficient concentrations should be reachable in the colon (102, 170). Only a few reports have described the effect of Reutericyclin producing probiotic *L. reuteri* in piglets (171-173). While these studies showed no distinct effect of Reutericyclin on bacterial diversity, we found significant differences between our treatment groups. We determined a significant depletion of beta diversity markers and a trend towards decreased alpha diversity in the Reutericyclin group.

Piglets administrated with Reutericyclin producing *L. reuteri* TMW1.656 reacted with temporary decreases of the *L. reuteri* group in stool samples. The stool samples also showed reduced proportions of lactobacilli compared to the Reutericyclin-negative mutant (173). The authors interpret these results as Reutericyclin being a modulator of the *Lactobacillus* community in pigs (173). In this respect, we could show a tendency towards a reduced abundance of *L. murinus* in the Reutericyclin treated group. Opposing this, another study found food fermentation with *L. reuteri* interfered with the abundance of other bacteria. The authors of this study also demonstrated that the abundance of *Enterobacteriaceae* was particularly reduced when compared to unfermented controls (172). Another study revealed Reutericyclin producing *L. reuteri* to increase the abundance of *Mitsuokella spp.* and *Dialister spp.*, but Reutericyclin did not influence the abundance of clostridial toxins in the faeces. Lastly, Wang et al report a limited impact of Reutericyclin on intestinal microbiota in piglets (171).

Since we encountered alterations of other additional bacteria, the effect of Reutericyclin seems to be species dependant. While in swine Reutericyclin seems to take a slight effect, our mice reacted to the gavage of Reutericyclin with reduced levels of the potentially beneficial butyrate producing strains *Eubacterium, Roseburia* and *Ruminococcus* (174). Increasing *Streptococcus* has also been found in studies performed in humans – for example under therapy with proton pump inhibitors (175). Among others, a proliferation of *Streptococcus* found in the human stool microbiome has been linked to an increasing risk of *Clostridium difficile* infection (176).

The literature with findings in the strain *Muribaculum* is limited to a SHIP deficient murine model proving that decreased abundances of *Muribaculum* develop inflammatory

bowel disease (177). Therefore, *Muribaculum,* amongst others, may be linked to inflammation within the intestinal wall. Overall, the administration of Reutericyclin decreased potentially beneficial and increased potentially harmful bacteria in our murine model.

Regarding the VOC results, the short carbohydrates heptane and pentane are substances usually found in polluted air. These were increased under (S) - Reutericyclin treatment (178, 179). 2,3-butanedione decreased in mice gavage-fed with (S) - Reutericyclin. This is a plant-growth promoting compound (180) emitted from *Bacillus subtilis* (181). Previous reports showed 2,3-butanedione in the headspace of *Pseudomonas pseudoalcaligenes* (182) and *Bacillus mojavensis* (180) cultures but has not been described in fecal samples of mice yet. 2-heptanone has been found to be increased in fecal and tissue samples of dairy cattle and goats infected with *Mycobacterium avium subspecies paratuberculosis* (183). Amongst many other VOCs, 2-heptanone could also be detected in the urine of healthy human adults (184). Moreover, 2-heptanone was found to decrease in humans exposed to altitude-induced hypoxia (185). We found correlations between VOC measurements and fecal bacterial abundances. Nevertheless, the gut microbiome considerably varies along the gastrointestinal tract (186). Moreover, volatile substances are not exclusively produced by certain species, which makes it almost impossible to relate certain VOC substances directly to specific bacteria *in vivo*.

We tested only one form of Reutericyclin - the (S)-Reutericyclin form which is a possible limitation of our Reutericyclin experiment. However, testing of this isoform can be justified by reports examining (R)-Reutericyclin and all of its modifications. Additionally, we did not examine the susceptibility against other *Lactobacillus* species. In future studies we plan to investigate the effect of diverse isoforms of Reutericyclin on the antimicrobial activity against bacteria also including *Lactobacillus* species.

In a prior report, we could demonstrate the variability of the intestinal microbiome throughout the whole intestinal tract with differences even between the luminal and mucosal microbiome (186). It could therefore be possible, that animals had relevant alterations located at other intestinal levels, which could not be found in stool samples included in our analyisis.

Limitations of the Reutericyclin part of this study include our inability to ferment suitable amounts of Reutericyclin in fermenter cultures. In this regard, future approaches with different bacterial strains will have to be conducted. Furthermore, we relied on the data provided by the manufacturer and did not test the true Reutericyclin contents of the product. Finally, it remains unclear at present, which form of Reutericyclin (or which mixture of

isoforms) is produced by bacteria and which of the isoforms is responsible for the broad antimicrobial effect reported in the literature. As already mentioned above, it also remains unclear to which extent murine data is representative for later human applications.

5. Conclusion

In these studies, co-culture of 10 probiotic bacterial strains composited in the food supplement OMNi BiOTiC® AAD10 were examined. We found a time dependent change of the abundances and the gene expression levels of the tested probiotic bacteria. Gene expressions as well as metabolomic findings could be prevalently assigned to the several pathways of nucleotide and amino acid biosynthesis. The postbiotic supernatant of OMNi BiOTiC® AAD10 produced in co-culture had antifungal and antibacterial effects *in vitro* and improved the growth of beneficial bacteria in our murine model. Further studies will have to infer its possible beneficial use in health and disease of human medicine in the future.

The Reuterin system produced in our study offered acceptable amounts of 3-HPA remaining stable for at least 35 days at -20°C. We found an antimicrobial effect against *S. agalactiae, S. aureus* and *S. epidermidis.* In our *in vivo* experiments, the Reuterin system caused changes in the fecal mouse microbiota. It also increased 3-methylbutanal and decreased heptane in the fecal VOC analysis. Both of this indicates the high potential of the Reuterin system that needs to be further elucidated in future projects.

The (S)-Reutericyclin used in our study had an antimicrobial effect on *S. epidermidis* only, but a different antimicrobial activity than described for the (R)-isoform in previous studies. In addition, its application in a mouse model caused microbial alterations towards dysbiosis.

6. References

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