UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Robert ŠKET

PHYSICAL INACTIVITY RELATED CHANGES IN HUMAN FECAL MICROBIOTA

DOCTORAL DISSERTATION

Ljubljana, 2018
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»Maturing as years go by,
slowly, in silence, within,
wherever to go and each way to try,
each time anew he will have to begin.«
(From: A Poem about the Stars)

»Vsak tiho zori
počasi in z leti,
a kamor že greš, vse poti
je treba na novo začeti.«
(Iz: Pesem o zvezdah)

Tone Pavček
(Slovenian poet)
On the basis of the State of University of Ljubljana and by decisions of Senate of the Biotechnical Faculty and the decision of the University Senate dated from 13.09.2016 and from 12.09.2017, the continuation of Interdisciplinary Doctoral Programme in Bioscience, field: Bioinformatics, was approved. Assoc. Prof. Blaž Stres as a supervisor and Assist. Prof. Tadej Debevec as co-supervisor were confirmed.


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Doktorsko delo je bilo opravljeno na Katedri za mikrobiologijo in mikrobno biotehnologijo, Oddelek za zootehniko, Biotehniška fakulteta, Univerza v Ljubljani, ob podpori Javne agencije za raziskovalno dejavnost Republike Slovenije (št. pogodbe 6316-2/2013-750).

Supervisor (mentor): Assoc. Prof. Blaž STRES, PhD
University of Ljubljana, Biotechnical Faculty, Faculty of Civil and Geodetic Engineering, Faculty of Medicine, Slovenia

Co-supervisor (somentor): Assist. Prof. Tadej DEBEVEC, PhD
Jožef Stefan Institute, Slovenia; University of Ljubljana, Faculty of Sport, Slovenia

Committee for the evaluation and the defense (Komisija za oceno in zagovor):

Chair (predsednik): Prof. Andrej BLEJEC, PhD
University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Member (član): Prof. Gregor ANDERLUH, PhD
National Institute of Chemistry, Department for Molecular Biology and Nanobiotechnology, Slovenia

Member (član): Prof. Michael SCHLOTER, PhD
Technische Universität Muenchen – TUM and Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH) – HMGU, Germany

Date of the defense (datum zagovora): Robert ŠKET
We explored the assembly of intestinal microbiota in healthy male participants during the randomized crossover design of run-in (5 day) and experimental phases (21-day normoxic bed rest (NBR), hypoxic bed rest (HBR) and hypoxic ambulation (HAmb) (hypoxic ~4000 m simulated altitude)) in a strictly controlled laboratory environment, with balanced fluid and dietary intakes, controlled circadian rhythm, microbial ambiental burden and 24/7 medical surveillance. Intestinal transit spanning Bristol Stool Scale, defecation rates, zonulin, α1-antitrypsin, eosinophil derived neurotoxin, bile acids, reducing sugars, short chain fatty acids, total soluble organic carbon, water content, diet composition and food intake, intestinal electrical conductivity, sterol and polyphenol content and diversity, indole, aromaticity and spectral characteristics of dissolved organic matter, along with nuclear magnetic resonance metabolomics and trace metal makeup of intestinal environment were assessed. Furthermore (i) abundance, structure and diversity of intestinal butyrate producing microbial community sequencing butyrate pathway genes but and buk, (ii) structure and diversity of microbial community sequencing bacterial and archaeal 16S rRNA and (iii) taxonomic and functional description of bacteria, archaea and fungi using shot-gun metagenomics were investigated. Inactivity negatively affected fecal consistency and in combination with hypoxia aggravated the state of gut inflammation (p < 0.05). On the other hand many of the microbial parameters such as butyrate producing microbial community, the general bacterial and archaeal microbial community were shown to lag behind the changes in human physiology and intestinal environment, since significant changes in bacterial community were delayed until week four in HBR only, where members of the genus Bacteroides and proteins involved in iron acquisition and metabolism, cell wall, capsule, virulence and mucin degradation were enriched. This suggest a time-dependent and complex interplay between the host physiology (including apparent constipation), immunity (inflammation), controlled diet, intestinal environment variables and microbiome physiology during the acute cessation of exercise. Finally, yo-yo dieting and active/inactive lifestyle, along with its effects, seems not to be a human peculiarity but rather a common evolutionary adaptation of mammals to survive food shortage and seasons rotation.
KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK UDK 612.33:579.6:577.121:575.112(043)=163.6
KG človeška fekalna mikrobiota/mikrobn združbe/Bacteroides/hipoksija/fizična neaktivnost/vnetje/zaprtje/čevesni metaboliti
AV ŠKET, Robert, mag. mikr.
SA STRES, Blaž (mentor)/ DEBEVEC, Tadej (somentor)
KZ SI-1000 Ljubljana, Jamnikarjeva 101
ZA Univerza v Ljubljani, Biotehniška fakulteta, Interdisciplinarni doktorski študij
Bioznanosti, področje Bioinformatika
LI 2018
IN SPREMEMBE HUMANE FEKALNE MIKROBIOTE POVEZANE S TELESNO NEAKTIVNOSTJO
DT Doktorska disertacija
OP XII, 102 str., 32 sl., 187 vir.
IJ EN
JI en/sl
AB Preiskovali smo sestavo črevesne mikrobiote pri zdravih moških preiskovanceh v navzkrižno zasnovanem poskusu s 5 dnevnim prilagajanjem na kontrolirano okolje in tremi 21-dnevnimi izvedbami: (i) horizontalnega mirovanja v normoksičnih pogojih (NBR), (ii) horizontalnega mirovanja v hipoksičnih pogojih (HBR) in (iii) gibanja v hipoksičnih pogojih (HAmb) (hipoksično ~ 4000 m simulirane nadmorske višine), v strogo nadzorovanem laboratorijskem okolju, z uravnoteženimi vnosmi tekočine in prehrane, kontroliranimim cirkadianim ritmom, minimaliziranim mikrobnim vnosom in 24-urnim zdravstvenim nadzorom. Preučevali smo parametre črevesnega okolja: zaprtost črevesja, zonulin, α1-antitripsin, nevrotoksin eozinofilcev, žolčne kisline, reducirajoče sladkorje, kratko-verižne maščobne kisline, vsebnost vode, črevesne električne prevodnosti, sterole, polifenole, indol, spektralne lastnosti raztopljenih organskih snovi, celokupne črevesne metabolite in elemente v sledovih. Črevesno mikrobioto smo opredelili glede na: (i) preučevanje številčnosti in raznovrstnosti bakterij, (ii) preučevanje struktur in raznovrstnosti bakterijske in arhejske mikrobiote na osnovi 16S rRNA genov, in (iii) taksonomski in funkcionalni opis bakterij, arhej in gliv na osnovi sekvenciranja celokupne mikrobiote DNA. Telesna neaktivnost je povzročila zaprtje črevesja in v kombinaciji s hipoksijo še poslabšala vnetje v črevesju. Po drugi strani je mikrobiota oz. njen oziv zaostajal za spremembami v človeški fiziologiji in v črevesnem okolju, saj so se bistvene spremembe v bakterijski mikrobioti zgodile v zadnjem tednu poskusa v fiziološko najbolj prizadeti skupini (HBR), kjer je prišlo do obogatitve bakterij rodu Bacteroides in genov vključenih v privzem železa, ter genov za celično steno, kapsulo, virulentnost in razgradnjo mukoznega sloja gostiteljevega črevesja. Slednje kaže na časovno odvisen in zapleten medsebojni vpliv med gostiteljsko fiziologijo (vključno z očitnim zaprtjem), imunskim odgovorom (vnetje črevesja), parametri črevesnega okolja in lastnostmi mikrobiote ob nadzorovanem prehranskem vnosu in med akutnim prenehanjem fizične aktivnosti. Drastične spremembe v prehrani (angl. yo-yo dieting) in nihanje med aktivnim ter neaktivnim življenjskim slogom, ki smo jim priča v vsakdanj življenju, skupaj s pripadajočimi učinki, niso človeška posebnost, temveč skupina evolucijska prilagoditev sesalcev, razvita kot posledica preživetja zaradi pomanjkanja hrane in prilagajanja na spremembe v letnih časih.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEY WORDS DOCUMENTATION</td>
<td>III</td>
</tr>
<tr>
<td>KLJUČNA DOKUMENTACIJSKA INFORMACIJA</td>
<td>IV</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>V</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>VIII</td>
</tr>
<tr>
<td>LIST OF SUPPLEMENTARY MATERIAL</td>
<td>X</td>
</tr>
<tr>
<td>ABBREVIATIONS AND SYMBOLS</td>
<td>XI</td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 PROBLEM DESCRIPTION</td>
<td>1</td>
</tr>
<tr>
<td>1.2 PURPOSE OF THE RESEARCH</td>
<td>2</td>
</tr>
<tr>
<td>1.3 HYPOTHESES</td>
<td>2</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>2.1 INTESTINAL MICROBIOTA</td>
<td>3</td>
</tr>
<tr>
<td>2.2 ASSESSMENT OF INTESTINAL MICROBIOTA</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1 Amplicon sequencing of genes involved in butyrate synthesis pathways</td>
<td>5</td>
</tr>
<tr>
<td>2.2.2 16S rRNA amplicon sequencing</td>
<td>6</td>
</tr>
<tr>
<td>2.2.3 Metagenome sequencing</td>
<td>7</td>
</tr>
<tr>
<td>2.2.4 Metabolome analysis</td>
<td>7</td>
</tr>
<tr>
<td>2.3 HUMAN SYSTEMS BIOLOGY AND HEALTH</td>
<td>8</td>
</tr>
<tr>
<td>2.3.1 Exercise</td>
<td>8</td>
</tr>
<tr>
<td>2.3.2 Diet</td>
<td>9</td>
</tr>
<tr>
<td>2.4 EXERCISE AND INTESTINAL MICROBIOTA</td>
<td>10</td>
</tr>
<tr>
<td>2.5 PLANHAB BED-REST STUDY</td>
<td>12</td>
</tr>
<tr>
<td>2.5.1 Bedrest</td>
<td>12</td>
</tr>
<tr>
<td>2.5.2 Hypoxia</td>
<td>12</td>
</tr>
<tr>
<td>2.5.3 PlanHab project</td>
<td>13</td>
</tr>
<tr>
<td>3 MATERIALS AND METHODS</td>
<td>15</td>
</tr>
<tr>
<td>3.1 PLANHAB BED-REST STUDY</td>
<td>15</td>
</tr>
<tr>
<td>3.1.1 Study design and setting</td>
<td>15</td>
</tr>
<tr>
<td>3.1.2 Test participants</td>
<td>17</td>
</tr>
</tbody>
</table>
3.1.3 Bed rest and environmental protocol ......................................................... 17
3.1.4 Diet .............................................................................................................. 18
3.1.5 Sampling ..................................................................................................... 18
3.2 BUTYRATE PRODUCING MICROBIAL COMMUNITY .................................. 18
3.2.1 Characterization of fecal samples: BSS, metabolites, pH, MWI ................. 20
3.2.2 Gut barrier integrity, permeability and inflammation .............................. 21
3.2.3 DNA extraction ........................................................................................ 21
3.2.4 Amplicon sequencing of butyrate producing bacterial communities .......... 22
3.3 MICROBIAL 16S RRNA AMPLICON SEQUENCING .................................. 23
3.3.1 Establishment of the PlanHab database ................................................. 23
3.3.2 Intestinal electrical conductivity as measure of fecal ionic strength ......... 25
3.3.3 Fecal polyphenols and sterols as determined by HPLC ......................... 25
3.3.4 Deconvolution of dissolved organic matter spectral derivatives of biological importance .......................................................... 25
3.3.5 Amplicon sequencing of microbial 16S rRNA ......................................... 26
3.3.6 Bioinformatic and statistical analysis of 16S rRNA sequencing .............. 27
3.4 MICROBIAL METAGENOMES AND INTESTINAL METABOLOMES .......... 29
3.4.1 Microbial metagenome sequencing ......................................................... 30
3.4.2 Bioinformatic and statistical analysis of microbial metagenome ............. 31
3.4.3 Intestinal metabolome analysis using proton nuclear magnetic resonance ... 31
3.4.4 X-ray fluorescence spectrometry of intestinal metal content .................. 32
3.4.5 Bayesian network modelling ..................................................................... 33
4 RESULTS ........................................................................................................... 34
4.1 BUTYRATE PRODUCING MICROBIAL COMMUNITY ............................... 34
4.1.1 Inactivity affects fecal consistency ......................................................... 34
4.1.2 Inactivity and hypoxia aggravate the state of gut inflammation but not permeability ................................................................. 34
4.1.3 Inactivity and hypoxia did not affect gut metabolic markers .................... 36
4.1.4 Diversity and abundance of butyrate producing microbial community was not influenced by bedrest and hypoxia ......................... 38
4.1.5 Correlative analysis .................................................................................. 42
4.2 MICROBIAL 16S RRNA AMPLICON SEQUENCING .................................. 42
General microbial diversity and composition are largely unaffected by 21-day inactivity and hypoxia ................................................................. 42

Intestinal environmental parameters are congruent with human physiology markers .................................................................................. 47

Identification of the key structuring parameters ........................................... 51

MICROBIAL METAGENOMES AND INTESTINAL METABOLOMES ...... 52

Taxonomic annotation of bacteria, archaea and fungi .................................. 52

Functional annotation of metagenomes ....................................................... 55

Microbial metabolome in the intestinal environment .................................. 58

Electrical conductivity governs intestinal metal availability ....................... 59

Bayesian network as a model of interactions at systems level .................... 61

DISCUSSION............................................................................................... 63

CHANGES IN HUMAN PHYSIOLOGY WITHIN THE PLANHAB PROJECT .... 63

INTESTINAL ENVIRONMENT .................................................................. 63

INTESTINAL MICROBIOTA ..................................................................... 65

REVERSIBILITY OF PHYSIOLOGICAL SYMPTOMS AS A PART OF INBUILT MAMMALIAN PHYSIOLOGY ............................................ 70

HOST AND MICROBIOTA DIALOGUE .................................................. 71

SYNTHESIS AND STUDY SIGNIFICANCE ............................................. 72

CONCLUSIONS......................................................................................... 74

SUMMARY (POVZETEK)........................................................................... 75

SUMMARY ............................................................................................... 75

POVZETEK ................................................................................................. 77

REFERENCES .......................................................................................... 88

ACKNOWLEDGEMENTS

SUPPLEMENTS
LIST OF FIGURES

Figure 1. Microbiota assessment from seven human body sites according to: (a) taxonomy (16S rRNA amplicon sequencing) and (b) function (whole metagenome shotgun sequencing) (The Human Microbiome Project Consortium, 2012). .............................................. 4

Figure 2. Four pathways for butyrate synthesis and corresponding genes (protein names) (Vital et al., 2014). ............................................................................................................. 6

Figure 3. 16S rRNA highly conserved domains (white), punctuated by V1 to V9 highly variable regions (orange). ............................................................................................................. 6

Figure 4. Physiological responses to acute endurance exercise (Mach and Fuster-Botella, 2016). .................................................................................................................................................. 9

Figure 5. A schematic presentation of interactive and dose dependent responses to physical inactivity and overeating over the course of human evolution (Ske et al., 2017b). .............................................................................................................. 10

Figure 6. Human systems biology exploration space mapping exercise with oxygen saturation levels and human population diversity (Clarke et al., 2014; Debevec et al., 2014; Liao et al., 2016; Ske et al., 2017a)......................................................................................... 11

Figure 7. The difference between accumulation and short-term real-time experiments. The inset shows a tentative scheme of dose dependent benefits derived from various levels of exercise (Bermon et al., 2015; Cronin et al., 2016; Egan and Zierath, 2013; Ringseis et al., 2015; Ske et al., 2017a, 2017b, 2018). ........................................................................................ 11

Figure 8. PlanHab study experimental setup (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Ske et al., 2017a, 2017b, 2018; Stavrou et al., 2016; Strewe et al., 2017). ................................................................................................................. 14

Figure 9. CONSORT flowchart of participants recruitment and the cross-over designed PlanHab study trial flow (Ske et al., 2017b). ............................................................................. 16

Figure 10. Schematic outline of the PlanHab experiments and analysis workflow described in first part of our study (Ske et al., 2017a). ................................................................................................. 19

Figure 11. Schematic outline of the PlanHab experiments and analysis workflow described in second part of our study (Ske et al., 2017b). ................................................................. 24

Figure 12. Schematic outline of the PlanHab experiments and analysis workflow described in third part of our study (Ske et al., 2018). ................................................................................................. 30

Figure 13. Changes in Bristol stool scale values (BSS) (A) and retention time (as time between particular defecations) (B) during run-in (week 1) and subsequent 3-week experimental phase (Ske et al., 2017a). ................................................................................................. 34

Figure 14. Changes in immunological markers present in fecal samples: zonulin (A), α1-antitrypsin (B), eosinophil-derived neurotoxin (EDN) (C) and bile acids (D) during run-in (week 1) and subsequent 3-week experimental phase (Ske et al., 2017a). ........................................................................................................................... 35

Figure 15. Variation in water content (A), pH (B), and total soluble organic carbon (TSOC) in fecal samples (C) during run-in (week 1) and subsequent 3-week experimental phase (Ske et al., 2017a). ........................................................................................................................... 36
Figure 16. Changes in concentration of C1–C6 short chain fatty acids (SCFA) present in fecal samples during run-in (week 1) and subsequent 3-week experimental phase (Sket et al., 2017a). ................................................................. 37

Figure 17. Changes in molecular weight indices (MWI) of fecal samples during run-in (week 1) and subsequent 3-week experimental phase (Sket et al., 2017a). ................................................................. 38

Figure 18. A schematic representation of closest matches of buk (A) and but (B) gene sequences describing butyrate producing communities in PlanHab samples (Sket et al., 2017a). ................................................................. 39

Figure 19. NM-MDS ordination showing a host-specific grouping of butyrate communities based on combined but and buk gene datasets for all participants in experimental variants (Sket et al., 2017a). ................................................................. 40

Figure 20. Abundance of butyrate producing community members as estimated by two approaches, qPCR (A, B), and band intensities (C, D) during run-in (week 1) and subsequent 3-week experimental phase (Sket et al., 2017a). ................................................................. 41

Figure 21. Schematic overview of the detected changes in microbial communities. The congruency of four statistical tests was used to detect significant differences in the structure of microbial community (Sket et al., 2017b). ................................................................. 43

Figure 22. Heatmap plot of the genus Bacteroides sequences (Sket et al., 2017b). ................................................................. 44

Figure 23. The overview of core microbiomes at the start-up and endpoint of the PlanHab experiment (Sket et al., 2017b). ................................................................. 46

Figure 24. Weekly defecation rates as a direct measure of constipation due to increased retention times of organic matter in intestinal system throughout the PlanHab experiment (Sket et al., 2017b). ................................................................. 47

Figure 25. Fluctuations in the intestinal parameters over the course of PlanHab experiment (Sket et al., 2017b). ................................................................. 48

Figure 26. Heatmap plot showing the relationship between parameters describing intestinal environment that differed significantly by week four at the end of PlanHab experiments (Sket et al., 2017b). ................................................................. 50

Figure 27. Heatmap plot showing the relationship between parameters describing human physiology that differed significantly by week four at the end of PlanHab experiments (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a, 2017b, 2018; Stavrou et al., 2016; Strewe et al., 2017). ................................................................. 51

Figure 28. Schematic representation of the significant changes in the taxonomic annotation of metagenomes (Sket et al., 2018). ................................................................. 53

Figure 29. Schematic representation of the significant changes in the functional annotation of microbiomes (Sket et al., 2018). ................................................................. 56

Figure 30. Schematic representation of the relationships between microbial fecal 1H-NMR metabolomes (Sket et al., 2018). ................................................................. 59

Figure 31. Schematic representation of the relationships between trace metal compositions based on X-ray fluorescence (XRF) spectrometry (Sket et al., 2018). ................................................................. 60

Figure 32. Bayesian network analysis (Sket et al., 2018). ................................................................. 61
LIST OF SUPPLEMENTS

SUPPLEMENT A: Publications produced within the duration of PhD project.
SUPPLEMENT B: Journals permissions to reproduce published articles.
SUPPLEMENT C: Limitations of the PlanHab study.
SUPPLEMENT D: Ethical aspects of the PlanHab study.
SUPPLEMENT E: Additional figures of our sub-study conducted within the PlanHab project (Sket et al., 2017a, 2017b, 2018).
SUPPLEMENT F: Additional tables of our sub-study conducted within the PlanHab project (Sket et al., 2017a, 2017b, 2018).
### ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H-NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>a</td>
<td>Napierian absorption coefficient</td>
</tr>
<tr>
<td>A1AT</td>
<td>$\alpha_1$-antitrypsin</td>
</tr>
<tr>
<td>AMOVA</td>
<td>Analyses of molecular variance</td>
</tr>
<tr>
<td>AWKS</td>
<td>Abundance-Weighted Kolmogorov-Smirnov statistics</td>
</tr>
<tr>
<td>BA</td>
<td>Bile acids</td>
</tr>
<tr>
<td>BDC</td>
<td>Baseline data collection</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BSS</td>
<td>Bristol stool scale</td>
</tr>
<tr>
<td>buk</td>
<td>Butyrate kinase</td>
</tr>
<tr>
<td>but</td>
<td>Butyryl-CoA:acetate CoA-transferase</td>
</tr>
<tr>
<td>Corbata</td>
<td>CORe MicroBiome Analysis Tools</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
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<tr>
<td>EDN</td>
<td>Eosinophil-derived neurotoxin</td>
</tr>
<tr>
<td>ESA</td>
<td>European Space Agency</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>F_iO_2</td>
<td>Fraction of inspired O_2</td>
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<td>GIT</td>
<td>Human gastrointestinal tract</td>
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<td>HAmb</td>
<td>Normobaric hypoxic ambulatory confinement</td>
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<tr>
<td>HBR</td>
<td>Normobaric hypoxic bed rest</td>
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<tr>
<td>HIA</td>
<td>Health Impact Assessment</td>
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<td>HMP</td>
<td>Human Microbiome Project</td>
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<tr>
<td>HOMOVA</td>
<td>Homogeneity of molecular variance</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence spectrum</td>
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<tr>
<td>IEC</td>
<td>Intestinal electrical conductivity</td>
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<tr>
<td>MG-RAST</td>
<td>Metagenomics Rapid Annotation using Subsystem Technology server</td>
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<tr>
<td>miRNA</td>
<td>micro RNAs</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>MMC</td>
<td>Migrating motor complexes</td>
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<td>MWI</td>
<td>Molecular weight indices</td>
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<tr>
<td>NASA</td>
<td>National Aeronautics and Space Administration</td>
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<tr>
<td>NBR</td>
<td>Normobaric normoxic bed rest</td>
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<tr>
<td>NM-MDS</td>
<td>Non-metric multidimensional scaling</td>
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<tr>
<td>NP</td>
<td>Non-parametric</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer membrane vesicles</td>
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<td>OTUs</td>
<td>Operational taxonomic units</td>
</tr>
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<td>PAHBAH</td>
<td>4-hydroxybenzoic acid hydrazide</td>
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<td>P&lt;sub&gt;O2&lt;/sub&gt;</td>
<td>Partial pressure of inspired O&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RDP</td>
<td>Ribosomal Database Project</td>
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<tr>
<td>SCFA</td>
<td>C1-C6 short chain fatty acids</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
</tr>
<tr>
<td>SpO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Capillary oxyhemoglobin saturation</td>
</tr>
<tr>
<td>Sr</td>
<td>Ratio slopes between 275 nm - 295 nm slope and 350 nm - 400 nm slope</td>
</tr>
<tr>
<td>SUVA&lt;sub&gt;254&lt;/sub&gt;</td>
<td>Specific ultraviolet absorbance</td>
</tr>
<tr>
<td>SViA&lt;sub&gt;420&lt;/sub&gt;</td>
<td>Specific visible absorbance</td>
</tr>
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<td>TDLP&lt;sub&gt;9&lt;/sub&gt;</td>
<td>Nine p-hydroxy, vanillyl, and syringyl lignin phenols</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total correlated spectrum</td>
</tr>
<tr>
<td>TSOC</td>
<td>Total dissolved organic matter concentration</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole metagenome shotgun sequencing</td>
</tr>
<tr>
<td>XRF</td>
<td>X-ray fluorescence spectrometry</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 PROBLEM DESCRIPTION

Modern life in the western societies is characterized as a hectic and stressful. The increasingly rapid pace, continuous stress, westernized (high-fat/high-sugar) nutrition and lack of regular exercise represent everyday life issues that might result in increased occurrence of cardiovascular disease, obesity, metabolic syndrome, pulmonary disorders associated respiratory insufficiency and many other ailments. "What is the role of gut microbiota in the development of the above-mentioned alterations to human health?" So far there has been no uniform answer to this question. The reasons for this are multivariate: (i) the complexity of the microbiota present in human gastrointestinal tract; (ii) variability in host metabolism and microbiota metabolism; (iii) the large number of internal and external factors that influence the composition of the intestinal microbiota; (iv) interaction between space and time; (v) the resulting large variability in parameters between different individuals.

To determine the role of microorganisms in a specific disease/condition it is first necessary to define the composition of the microbiota in the gut of healthy humans, as is the objective of human microbiome project (HMP) and MetaHIT project (Metagenomics of the Human Intestinal Tract) (Lloyd-Price et al., 2017; Qin et al., 2013; The Human Microbiome Project Consortium, 2012).

Many studies investigating host–microbiota interactions have demonstrated that numerous parameters drive the structural and functional changes of the human gut microbiome and that microbiome varies between different individuals due to various factors. Its critical role in maintaining gut homeostasis and host health has been confirmed in a number of studies comparing various end-point groups with significantly different symptoms (Barton et al., 2018; Clarke et al., 2014; Schloss et al., 2014) and also in investigations employing longitudinal approach over time (David et al., 2014a, 2014b; Thaiss et al., 2014; Vital et al., 2013). Factors such as genotype, age, diet and a variety of health conditions, e.g. functional bowel disorders, inflammatory bowel disease, cancer and obesity have been shown to influence structural and functional milieu of gut microbiome. Obesity-related disorders are often associated with improper diet and lack of exercise, physical activity and/or sedentary lifestyle. Findings from recent studies by Clarke et al and Barton et al, where the intestinal microbiota of professional athletes has been studied, suggest that the microbiota of highly active individuals differ from the microbiota of the average population, and that increased physical activity has a positive impact on the increase of microbiota biodiversity (Barton et al., 2018; Clarke et al., 2014). “What happens with the composition of the intestinal microbiota in the event of reduced physical activity, or even a longer standstill due to various medical conditions or other reasons?” Most studies so far considered random samples from general population leading to uncertain answers to the question above. In order to solve that problem, the multifaceted relationship between the decreased physical exercise (i.e.
inactivity), human physiology, intestinal microbiota, accompanying intestinal metabolites and diet needs to be explored during acute cessation of exercise in healthy individuals.

The PlanHab project (Planetary Habitat Simulation project EU FP7-space; http://cordis.europa.eu/project/rcn/104127_en.html; PI: Igor Mekjavić, IJS: Jožef Stefan Institute, Ljubljana) provided a unique opportunity for studying the dynamics of human intestinal microbiota and the associated parameters in healthy individuals during prolonged physical inactivity. The basic aim of the PlanHab project was to investigate the separate and joint effects of physical inactivity, a reduction in gravity due to the horizontal immobilization (hydrostatic pressure) and the partial reduction of oxygen (hypoxia) on the human physiological systems in healthy people who were bedridden, under medical supervision, under controlled conditions for 21 days (5 days of baseline data collection (BDC), 21 intervention days, and 5 days of medical follow-up). The above-mentioned factors are known to significantly affect the nutritional status, the regulation of body composition and overall health status (Debevec et al., 2014). Such factors can therefore trigger host responses at different levels, which can lead to changes in the gastrointestinal micro-environment, interaction with the host and, consequently, affects the intestinal microbiota (Sket et al., 2017a, 2017b, 2018).

1.2 PURPOSE OF THE RESEARCH

The aim of our research was to determine the response of (i) the structure of the intestinal microbiota, (ii) the intestinal metabolites, and (iii) the immune status of the subjects during the physical inactivity and exposure to hypoxia (n = 28 days "before" (-7 days); " after "(+21 days)), (iv) to reformat and coordinate information on changes in human physiology from the database of PlanHab partners (IJS) in an appropriate form; and (v) to explore the quadripartite relationship between the responses of the host (human physiology), immune status, metabolites and intestinal microbiota during the experiments on healthy subjects.

1.3 HYPOTHESES

- Ho: Among the groups of subjects there are no differences.
- H1.1: The differences in metabolites, immune status and composition of the microbiota due to interpersonal variability between subjects within the same experimental group, will be smaller than the differences in the composition of the microbiota due to the influence of either bedrest or hypoxia or combined.
- H1.2: Physical inactivity leads to significant changes in metabolites, immune status and composition of the microbiota.
- H1.3: Hypoxia leads to significant (characteristic) changes in metabolites, immune status and composition of the microbiota.
- H1.4: Responses to the four levels (human physiology, metabolites, immune status and microbiota) are interconnected. Changes in human physiology are larger than the changes in the microbiota, metabolic and immune status.
2 LITERATURE REVIEW

2.1 INTESTINAL MICROBIOTA

Intestinal microbiota plays an important role in the human gastrointestinal tract. It enables the dynamic relationship with the host, helps maintain homeostasis of epithelial cells, plays a fundamental role in the induction and function of the host immune system, affects the acquisition of nutrients and regulation of energy with promotion of digestion and food absorption, physically prevents the colonization of the intestine surface by pathogenic microorganisms and it is involved in the production of vitamins along with short chain fatty acids (Belkaid and Hand, 2014; Lloyd-Price et al., 2017; The Human Microbiome Project Consortium, 2012).

Main reason for vast exploration of human gastrointestinal tract (GIT) microbiome is, beside its role in human health and disease, the fact that it contains the vast majority of microbial biomass present within or on the surface of the human body and can be sampled relatively easily by the collection of fecal material (Eckburg et al., 2005; Greenhalgh et al., 2016). Consequently the microbiota compositions of other body habitats remains much less explored, in comparison to the intestinal microbiome. To overcome that problem Human Microbiome Project (HMP) and shortly after MetaHIT (Metagenomics of the Human Intestinal Tract) projects were introduced. In addition to the collection of clinical samples from up to 18 body sites from healthy subjects, both projects strived to introduce and clarify all aspects of human metagenomics, from protocol design, data analysis and visualization, to correlation with various diseases (Highlander, 2012; The Human Microbiome Project Consortium, 2012).

Human intestinal microbiota, in addition to the fungi, archaea, and viruses, consists of 3.8 x 10^{13} bacteria, which are classified into at least a thousand different bacterial species (Sender et al., 2016; The Human Microbiome Project Consortium, 2012). Intestinal microbiota in healthy adult human consists of 9 bacterial phyla of wich Firmicutes, Bacteroidetes and Actinobacteria are dominant (Greenhalgh et al., 2016; Ley et al., 2006). The most attention so far was given to Firmicutes and Bacteroidetes, which constitute over 90 % of the known phylogenetic categories and as such dominate the intestinal microbiota on the level of biomass. Firmicutes are interesting because of their vast capabilities for energy harvesting from food and Bacteroidetes are responsible for many conditions associated either to health and disease, and are vital in complex sugar degradation and metabolizing of proteins into short chain fatty acids (SCFA) (Greenhalgh et al., 2016; Ley et al., 2006; The Human Microbiome Project Consortium, 2012).

Human intestinal microbiome differentiates not only between different individuals, but also significantly over human lifespan. In that regard considering age-specific differences may be key to understanding the effects of microbiota on health. Although the variability of microbial species between unrelated individuals, or comparing different body sites, was
large, the microbial functional potential in gastrointestinal tract seemed to be comparable between individuals and with functional potential of microbes from different body sites (Figure 1) (Greenhalgh et al., 2016; Lloyd-Price et al., 2017; Marchesi et al., 2015; The Human Microbiome Project Consortium, 2012).

Figure 1. Microbiota assessment from seven human body sites according to: (a) taxonomy (16S rRNA amplicon sequencing) and (b) function (whole metagenome shotgun sequencing) (The Human Microbiome Project Consortium, 2012).

Sequencing enables us to determine the nucleotide sequence of DNA molecules and represents the gold standard of microbial characterization, revealing the composition and identity of archaeal, bacterial and viral communities at many sites, in and on the human body (Highlander, 2012). There are different types of sequencing: (i) sequencing of specific microbial section (amplicon sequencing), (ii) sequencing of whole microbial gene pool (metagenome sequencing), or (iii) sequencing of total microbial RNA (metatranscriptome sequencing). Among the above listed methods, amplicon sequencing has been the most commonly used method for base determination of microbial segment, which represents a phylogenetic marker for taxonomic classification of organisms. Examples of such markers are genes of bacterial or archaeal ribosomal RNA (16S rRNA) or functional genes, such as...
bacterial butyrate synthesis pathways genes (butyryl-CoA: acetate CoA-transferase \((but)\) and butyrate kinase \((buk)\) genes) or fungal 18S rRNA and internal transcribed spacer (ITS). Using amplicon sequencing methods alpha diversity measures on how many different members exist within a community or beta diversity measure on the differences (and similarities) between communities can be determined (Highlander, 2012).

On the other hand studies encompassing metagenomics and functional omics, as shotgun metagenome sequencing of total microbial DNA, enable us to get the information not only who there is, but also what they are doing and help us identify complements of microbiota associated with health or disease (Greenhalgh et al., 2016).

### 2.2.1 Amplicon sequencing of genes involved in butyrate synthesis pathways

Butyrate-producing bacteria are important for healthy colon. Short chain fatty acid butyrate as their final fermentation product of mainly dietary fibre, helps maintaining gut homeostasis and epithelial integrity and serves as the main energy source for colonocytes. On the other hand changed butyrate producing community can be involved in diseases such as ulcerative colitis and type II diabetes (Vital et al., 2013, 2014, 2015). There are four known main pathways for butyrate production among which acetyl-CoA pathway is present in the majority of butyrate producers. Other pathways are: glutarate, 4-aminobutyrate and lysine pathway (Figure 2). The main obstacle in identification of butyrate producers is their polyphyletic personality which prevents accurate detection of 16S rRNA structure. To overcome that problem, main butyrate-producing pathway genes can be targeted to assess abundance, structure, and diversity of butyrate producing microbial community. Examples of those genes are butyryl-CoA:acetate CoA-transferase \((but)\) and butyrate kinase \((buk)\), that are involved in final catalyzation from butyryl-CoA to butyrate (Figure 2). The bulk of butyrate producers consist of Firmicutes. Other phyla also identified as potential butyrate producers are: Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae (Vital et al., 2014).
Figure 2. Four pathways for butyrate synthesis and corresponding genes (protein names) (Vital et al., 2014).

Slika 2. Shema štirih poti sinteze maslene kisline in pripadajoči geni (imena proteinov) (Vital in sod., 2014)

2.2.2 16S rRNA amplicon sequencing

Up to date studies based on 16S rRNA gene sequencing still helps exhibit certain broad features of archaeal and bacterial communities, associated with investigated factors (Sket et al., 2017b; Tringe and Rubin, 2005). The 16S rRNA molecule is highly conserved and has functionally constrained tertiary structure, composed of highly conserved domains, punctuated by V1 to V9 highly variable regions (Figure 3) (Gutell, 1994).

Figure 3. 16S rRNA highly conserved domains (white), punctuated by V1 to V9 highly variable regions (orange).

Slika 3. 16S rRNA visoko ohranjene regije (bela), prekinjene z V1-V9 visoko variabilnimi regijami (oranžna).
Approximately 1500 base pairs long 16S rRNA genes are present in all bacteria and archaea on one hand and possess enough interspecies variability that can be used as a molecular tool for microbial identification on the other, since the 3% difference across the 16S rRNA genes usually discriminates between species, phylotypes or operational taxonomic units (OTUs) (Highlander, 2012). Usually the most abundant microbes are the most represented in results. On the other side also unidentified microbes and the ones in smaller quantities, which would be otherwise unnoticed, using standard identification methods, are detected to some extent (Tringe and Rubin, 2005). Although we can survey specific distributions of microbial phyla, diversity and relative stability over time (Greenhalgh et al., 2016; Kolbert and Persing, 1999), main restriction of 16S rRNA gene amplicon sequencing is limited resolution, usually to the genus or at best to the species level of bacteria or archaea. On the other hand identification of microbial structure based on 16S rRNA genes can presents starting point for further investigations of microbiome (Figure 1) (Barton et al., 2018; Clarke et al., 2014; Sket et al., 2017b, 2018).

2.2.3 Metagenome sequencing

Advances in bioinformatics, refinements of DNA amplification and computational power have risen to the task, enabling us analysis of DNA sequences recovered from environmental samples and as such allow the adaptation of genome shotgun sequencing to metagenomic samples. In whole metagenome shotgun sequencing (WGS) we are targeting random genome fragments from a heterogeneous mix of species, strains and subpopulations out of the total microbial gene-pool in community. WGS enables sequencing a large amount of different microbes and is not restricted to issues associated with 16S rRNA gene phylogenies (Highlander, 2012). The acquired results contain information on which microbes are present in the community and more important, what the catalogue of their genes present in community is (Figure 1), suggesting an introductory idea on the function of these microbes in specific environment (Turnbaugh and Gordon, 2008). In addition to metagenomics also single-cell genomic, transcriptomic and epigenomic sequencing (Wang and Song, 2017) expanded our view of microbial diversity on functional and phylogenetical scales and enable us to add new branches (bacterial and archaeal phyla with no cultivated representatives) to overall topology of the tree of life (Woyke et al., 2017).

2.2.4 Metabolome analysis

Work in metagenomic field enable us to functionally assess the intestinal microbiota, but clear understanding of central molecular mechanisms that are the key to bacteria-host interactions in metabolic diseases, is still missing (Clavel et al., 2014; Turnbaugh and Gordon, 2008). The attempt to overcome this barrier is the use of metabolomics i.e. quantification of small molecules, metabolites in tissue, cell, biofluid or better in whole organism. Metabolome analysis enable a better understanding of the microbiome dynamics and its functional contributions to the various body habitats, also with functional capacities.
not coded in human genome, such as digest of plant glycans and synthesis of vitamins (Turnbaugh and Gordon, 2008). Most commonly used methods for quantifying a large number of metabolites are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) in use are also high-pressure liquid chromatography, thin layer chromatography (TLC) and capillary electrophoresis (CE).

2.3 HUMAN SYSTEMS BIOLOGY AND HEALTH

Many positive and negative factors can affect human health. To identify them, method of Health Impact Assessment (HIA) has been proposed. Consequently factors affecting human health were divided into categories: (i) biological factors e.g. age, sex, genetics, (ii) physical environment e.g. clean air, water, housing, healthy workspace etc., (iii) social and economic status e.g. education, employment etc., (iv) individuals characteristics and behavior e.g. diet, exercise etc. (Lock, 2000). Usually it is hard or even impossible to take into the account all the effects of so many determinants of health, but some of them are more flexible to manipulate and record then the others.

2.3.1 Exercise

Inactive life-style can cause changes in the physiology of the human body, such as skeletal muscles atrophy (Agostini et al., 2010; Biolo et al., 2008; Iovino et al., 2013; Pišot et al., 2008) bone demineralization (Berg et al., 2007; Frings-Meuthen et al., 2013; Rittweger et al., 2009), heart failure, hypotension (Eiken et al., 2008), systemic inflammation, insulin resistance (Hamburg et al., 2007; Mazzucco et al., 2010), constipation (Iovino et al., 2013). On the other hand physical activity or exercise, defined as cardiovascular exercise performed for an extended period of time (running, skiing, cycling, aerobic exercise, swimming, etc.), provokes both muscle and systemic responses. Muscle tissue adaptations to exercise are: (i) improvement of mechanical, metabolic, neuromuscular and contractile functions, (ii) rebalance of electrolytes, (iii) decrease in glycogen storage, (iv) an increase in mitochondrial biogenesis, (v) decrease in muscle damage and (vi) high consumption of oxygen and nutrients. Systemically speaking exercise increase: (i) oxidative stress, (ii) intestinal permeability, (iii) systemic inflammation responses with immune responses and (iv) ventilation and heart pumping function (Figure 4) (Mach and Fuster-Botella, 2016).
2.3.2 Diet

It has been shown that many chronic health problems, such as obesity and inflammatory bowel disease that were first noted in Western countries, are now expanding worldwide. Those diseases can be related to diet, usually the high-fat/high-sugar ‘Western’ diet. Problem usually occurs when nutrition, either carbohydrates, fats or proteins, is excessive and the body is not capable of quantitative control of the nutrients absorption and storage. Over-nutrition on one hand or lack of physical activity, under same level of nutrition on the other, can lead to alterations of human health such as: diabetes, cardiovascular diseases, obesity, hypertension, and hyperlipidemia (Figure 5) (Shridhar et al., 2015). Different diet mainly induced changes to gut-associated microbial communities and those then contribute to growing epidemics of chronic illnesses (David et al., 2014b). It was shown that short-term consumption of entirely animal products and consequently high protein diet or consumption of plant products with high carbohydrates levels, alters GIT microbiota and overcome inter-individual differences in GIT microbiota structure and function (David et al., 2014b).
Considering that, normalized and tailored diet is of most importance in order to successfully link the effects of investigated factors to changes in GIT microbiota (Clarke et al., 2014; David et al., 2014b).

![Figure 5](image)

**Figure 5.** A schematic presentation of interactive and dose dependent responses to physical inactivity and overeating over the course of human evolution (Sket et al., 2017b).

**Slika 5.** Schematic presentation of interactive and dose dependent responses to physical inactivity and overeating over the course of human evolution (Sket et al., 2017b).

### 2.4 EXERCISE AND INTESTINAL MICROBIOTA

Despite the clear impact of inactivity on human physiology, the exact relationship between the gut microbiota and inactive life-style is still unclear (Berton et al., 2016; Clarke et al., 2014). Although the role of gut microbiome and its effect on individual’s exercise performance remains unknown, the effect on alterations of gut microbiome, because of the exercise, is well established (Barton et al., 2018; Clarke et al., 2014; Mach and Fuster-Botella, 2016). In that regard the role of exercise as an effector on the gut microbiome has received attention as a possibility of reducing the risk for several metabolic, inflammatory and neoplastic diseases for inactive individuals (Cronin et al., 2016; van Dijk et al., 2012; Egan and Zierath, 2013; Pham et al., 2012). Various levels of physical exercise were recently linked to modifications of the microbiota in test-participants (Berton et al., 2016; Clarke et al., 2014), increased vagal-nerve tone at rest (Cronin et al., 2016), gene expression of transport proteins (Jayewardene et al., 2016) and exercise related immunological responses (Bermon et al., 2015; Ringséis et al., 2015). Furthermore, Clarke et al. (2014) clearly outlined the close link between the triad of diet, metabolism and exercise as increased exercise and dietary extremes strongly influenced the microbial diversity in professional athletes.

However, most studies so far have focused on the magnitude of symptoms as a result of prolonged inactivity and the effects of the restoration of physical activities, in an unfit population (Figure 6, Figure 7).
Figure 6. Human systems biology exploration space mapping exercise with oxygen saturation levels and human population diversity (Clarke et al., 2014; Debevec et al., 2014; Liao et al., 2016; Sket et al., 2017a).


Figure 7. The difference between accumulation and short-term real-time experiments. The inset shows a tentative scheme of dose dependent benefits derived from various levels of exercise (Bermon et al., 2015; Cronin et al., 2016; Egan and Zierath, 2013; Ringseis et al., 2015; Sket et al., 2017a, 2017b, 2018). Abbreviations: body mass index (BMI), hypoxic bedrest (HBR), normoxic bedrest (NBR), hypoxic ambulation confinement (HAmb), National Aeronautics and Space Administration (NASA), European Space Agency (ESA), German Aerospace Center (DLR).

From a physiological perspective, inactivity was shown to result in tissue hypoxia through reduced capillary oxyhemoglobin saturation, changes in muscle composition, modified expression of genes and cellular metabolism and release of pro-inflammatory cytokines (Bermon et al., 2015; Ringseis et al., 2015). On the other hand, adaptation to hypoxia results in coordinated cascade-like down-regulation of metabolic demands and supply to prevent a mismatch in ATP utilization and production in cell tissues (Wheaton and Chandel, 2011). Hypoxia and inflammation therefore appear to be interdependently related as many publications implicated inflammation during hypoxic conditions to be related to a wide range of human diseases (Bartels et al., 2013). For instance, life-long inactivity and obesity-related disorders were clearly associated to differences in human intestinal microbiome between healthy and affected groups (Cronin et al., 2016; Turnbaugh, 2009).

2.5 PLANHAB BED-REST STUDY

2.5.1 Bedrest

Bed rest is characterized as immobilization, inactivity, confinement and elimination of gravitational stimuli, such as posture change and direction, which affect body sensors and responses (Pavy-Le Traon et al., 2007). In nineteenth century bed rest was first introduced as a medical treatment, but later it was recognized as not so good for individual’s health. Its effects are: (i) cardiovascular changes due to lower plasma levels, (ii) increased calcium excretion, (iii) reduced body weight, (iv) reduced muscle mass, strength and resistance of muscle to insulin, (v) altered bone stiffness in lower limbs and spinal cord, (vi) shifts in circadian rhythms. Bed rest can be used also for academic purposes to study its physiological effects and clinical implications in healthy individuals, and the importance of activity to health (Debevec et al., 2014; Pavy-Le Traon et al., 2007).

2.5.2 Hypoxia

Reduced systemic oxygen availability, also termed systemic hypoxia is a common physiological phenomenon, expressed under various cellular, environmental and clinical conditions such as: (i) high altitude, (ii) physical exercise, (iii) pregnancy, (iv) aging, (v) inflammation, (vi) cardiovascular and respiratory failures, (vii) wounds and (viii) late-stage cancer. Studies exploring that phenomenon are defining the field of high altitude medicine. Alterations in human health due to systemic and tissue hypoxia were shown to result in correlated implications such as chronic heart failure, obstructive pulmonary disease and obesity related syndromes (Liao et al., 2016).
2.5.3 PlanHab project

To improve our understanding regarding the pathophysiological consequences of inactivity and hypoxia on the dynamics of intestinal microbiota, controlled studies incorporating hypoxia exposure (e.g. normobaric hypoxia simulation) and inactivity (usually simulated by continuous bed rest) are needed. In such studies healthy, preselected subjects are subjected to complete whole body inactivity and confinement in either normoxic or hypoxic conditions for a designated time period. In order to link the influence of inactivity to changes in microbiota structure it is necessary to minimize the impact of various external factors. Hence the following approaches for bed rest study standard operational procedures of European Space Agency (ESA) and NASA core bedrest data collection SOP (Standardization of bed rest study conditions 1.5, August 2009) are utilized: prescreening the population of test subjects, balanced died, identical general environment of experiments, conserved interaction with research / technical staff, 24/7 medical care and thereby reduced risk of differential bacterial, viral or other infections that could lead to drastic changes in the composition of the intestinal microbiota or dysbiosis. In order to draw proper conclusions such a study should be conducted in a controlled environment under the permanent supervision of qualified persons, and should be a priori approved by the ethics committee. As noted above the PlanHab project was performed according to the above.

The experimental setup of the PlanHab project was designed to investigate in controlled manner the combined effects of prolonged (21 day) inactivity/unloading and hypoxia on healthy-volunteers (Debevec et al., 2014; Simpson et al., 2016). The challenge of the project was in the complexity of the experimental interventions where healthy humans were confined to a hypoxic environment during prolonged bedrest. A series of studies were conducted at the Olympic Centre Planica, Slovenia hypoxia facility capable of housing 20 subjects at any simulated altitude (Debevec et al., 2014). Subjects remained in horizontal position (bedrest) or were ambulatory, but confined to the facility (ambulation) for 21 days/trial. Each subject participated in three trials: hypoxic bedrest (HBR) (simulated altitude 4000m), normoxic bedrest (NBR), and hypoxic ambulation (HAmb) (Figure 8).

The effects concerning metabolic, cardiorespiratory, musculoskeletal, haematological, immunological and thermoregulatory functions with addition of intestinal microbiome functions were investigated (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a, 2017b, 2018; Stavrou et al., 2016; Strewe et al., 2017). Study was explorative in the sense that it collected a broad spectrum of basic data corresponding to 21-day bedrest experiments conducted by ESA/NASA (bedrest core data) and is as such readily comparable with the core data obtained in previous bedrest studies using the same methodology (Debevec et al., 2014).
PlanHab Study Concept:

to investigate the combined effects of hypoxia and sustained bedrest on human physiological systems.

Bed rest study (NASA/ESA SOP) -> immobilization, inactivity and confinement for 21 days, minimum impact of various external factors

Standardization of bed rest study conditions

Prescreening results in harmonious test population of 9 healthy male volunteers

One week before START - Acclimation of the subjects to controlled environment

-5 -1 3 10 18 21

Run in period

Day

Experiment duration = 21 days

Stool, urine sample collection

Legend:
Normoxic: PO₂ (Partial Pressure of Oxygen) ~ 400m HA
Hypoxic: PO₂ ~ 4000m HA

Ambulatory: subjects were allowed to move around the facility and performed low-intensity exercise sessions to mimic their habitual levels of activity.

Figure 8. PlanHab study experimental setup (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a, 2017b, 2018; Stavrou et al., 2016; Strewe et al., 2017).

3 MATERIALS AND METHODS

3.1 PLANHAB BED-REST STUDY

In the framework of the present sub-study conducted within the PlanHab project (registration number NCT02637921 at http://cordis.europa.eu/project/rcn/104127_en.html) the dynamics and diversity of the gut microbiome was studied in response to reduced physical activity and hypoxia using stool samples as proxy. The study had received approval by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia. The detailed outline of the PlanHab project (Debevec et al., 2014; Simpson et al., 2016; Sket et al., 2017a) is summarized below.

3.1.1 Study design and setting

The PlanHab study experiments were performed between June 2012, when recruitment started and January 2014, when the last follow-up periods ended (Figure 9). The study was performed within the premises of the normobaric hypoxic facility of the Olympic Sport Center Planica in Rateče, Slovenia, located at 940 m of altitude and was conducted according to the European Space Agency's standardization plan for bed rest studies (ESA, 2009) (Debevec et al., 2014, 2016; ESA; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a; Stavrou et al., 2016; Strewe et al., 2017), including sample size calculation. For this study, each participant underwent 5 days of baseline data collection (BDC) during which participants were ambulant, 21 intervention days (during which the participants underwent wither normoxic bed rest, hypoxic bed rest, or hypoxic ambulation), and 5 days of medical follow-up. For BDC, operational days are indicated by ‘BDC-n’, meaning n days before the onset of bed rest. In particular, the participants underwent the following three protocols in a randomized and counterbalanced manner: (1) normobaric normoxic bed rest [NBR; fraction of inspired O\(_2\) (F\(_{\text{O}_2}\)) = 0.209; partial pressure of inspired O\(_2\) (P\(_{\text{O}_2}\)) = 133.1 ± 0.3 mmHg]; (2) normobaric hypoxic ambulatory confinement [HAmb; F\(_{\text{O}_2}\) = 0.141 ± 0.004; P\(_{\text{O}_2}\) = 90.0 ± 0.4 mmHg; ~4,000 m simulated altitude]; and (3) normobaric hypoxic bed rest [HBR; F\(_{\text{O}_2}\) = 0.141 ± 0.004; P\(_{\text{O}_2}\) = 90.0 ± 0.4 mmHg; ~4,000 m simulated altitude] (Figure 9, Figure S1A). In addition to the present sub-study, other published sub-studies or in preparation focused upon cardiorespiratory, muscular (Debevec et al., 2014), immunological (Keramidas et al., 2016), psycho-neuroendocrine responses (Keramidas et al., 2016; Strewe et al., 2017) next to appetite-regulation (Debevec et al., 2016), skeletal muscle miRNA expression (Rullman et al., 2016) and mineral metabolism (Rittweger et al., 2016) within the same participants.
Figure 9. CONSORT flowchart of participants recruitment and the cross-over designed PlanHab study trial flow (Sket et al., 2017b). Interventions: normoxic bed rest (NBR), hypoxic bed rest (HBR) and hypoxic ambulation (HAmb).

3.1.2 Test participants

Inclusion and exclusion criteria for the study were based on the European Space Agency's standardization plan for bed rest studies (ESA, 2009; Debevec et al., 2014, 2016) and aimed at selecting participants who could safely undergo bed rest (e.g. exclusion of people with osteoporosis, with blood clotting disorders, history of deep vein embolism, lower back pain, and respiratory disorders). In addition, participants assessed were also excluded from this study if they had been exposed to altitudes ≥ 2000 m, two months prior to study commencement. Altogether 11 healthy men underwent all 3 campaigns in randomized crossover design of PlanHab project (Figure 9). The participants were all physically active male engaged in recreational sports activities for 2-4 hours per week. Their habitual daily physical activity was assessed via SOP questionnaire prior to the inclusion in the study (Debevec et al., 2014). Sample size was determined based on previous reports of bed rest studies to obtain sufficient predictive power ≥ 0.80 (Debevec et al., 2014, 2016; Simpson et al., 2016). Subjects were enrolled by project manager and randomly allocated between campaigns using latin square design method (Figure 9) (Debevec et al., 2014, 2016; Keramidas et al., 2016). Due to the lack of overlapping defecation time-points the number of participants was decreased to 9 with a mean age (± SD) of 27.4 ± 5.6 years, a height of 180.2 ± 5.0 cm, a mass of 75.1 ± 10.3 kg and a body mass index of 23.1 ± 2.7 kg/m² (Table S1, Figure 9) (Sket et al., 2017b). Participants were given concise explanation regarding the experimental procedures and potential risks before giving their written informed consent. Exclusion criteria included history of any cardiorespiratory, musculoskeletal, neuro-logical or vascular disease. Except for transient headaches and backaches, all 9 participants concluded the PlanHab experiment without any significant injurious health-related issues.

3.1.3 Bed rest and environmental protocol

Environmental conditions were controlled (ambient temperature: 24.4 ± 0.7 °C, relative humidity: 53.5 ± 5.4 %) or assessed (ambient pressure: 91.2 ± 5.3 kPa). The light:dark cycle was set to 16:8 h, with bed time between 23:00 and 7:00. SpO₂ was measured daily at 7:00 AM with a finger oximetry device 3100 WristOx device (Nonin Medicals, Minnesota, USA) and also as part of a sleep polysomnographic study. To the latter purpose, full ambulatory polysomnography (Nicolet One, Viasys, Healthcare, Neurocare, Madison, WI, USA) was performed using standard setups (Debevec et al., 2016).

During the bed rest phase of the NBR and HBR campaigns, the participants were confined to bed in the horizontal position for 24 h/day (no negative tilt), and all activities of daily life took place in bed. One pillow was allowed for head support. Showers were taken on a specific gurney, and hospital equipment was used for bowel movements and urine collection. Compliance with the bed rest protocol was ascertained by supervision through members of staff and through closed-circuit television. No physical activity was allowed during NBR and HBR campaigns, except for changing position between supine, prone and lateral. During
HAmb, participants were confined to the hypoxic area, but remained ambulatory and out of bed during the day. In order to replicate their habitual bone loading during the confinement periods, participants performed low-level physical activity in two 30-minute bouts per day. The exercise was rotated using stepping, cycling and dancing. Telemetric heart rate monitoring was used to achieve the targeted heart rate (123 ± 4 beats/min) during the exercises, which was set to the heart rate observed at 50 % of hypoxia specific peak power output assessed at the onset of the intervention (for more details see Debevec et al., 2014).

During HBR and HAmb campaigns, normobaric hypoxia was generated within the hypoxic area by a vacuum pressure swing adsorption system (b-Cat, Tiel, the Netherlands). Regulation of O2 concentration was actuated within each room at 15-minute intervals. For safety reasons, participants carried portable O2 sensors (Rae PGM-1100, California, USA) at all times (Debevec et al., 2014, 2016).

3.1.4 Diet

The participants were provided with an individually tailored, standardized and controlled diet throughout the intervention. Energy requirements were assessed with the Harris-Benedict method, and correction factors of 1.4 and 1.2 were used to account for activity levels in the ambulatory phases and the bed rest phases, respectively. In addition to a controlled intake of fat (30 %) and protein (1.2 g per kg body mass), sodium intake was set to 3500 mg per day. Participants were supplemented with 1000 IU vitamin D3 per day. Fluid intake was ad libitum, but participants were encouraged to drink at least 28.5 mL per kg per day. Importantly, menu plans were cycled in the same way for each participant across the three experimental conditions, adjusting the quantity according to activity factors above.

3.1.5 Sampling

Fecal samples were collected at the time of defecation in aseptic containers to prevent cross-contamination. Longitudinal sampling was performed for 9 participants with sampling at days -5 and -1 before the onset of experiments and days 3, 10, 18 and 21 of treatment. Altogether 54 samples were collected and immediately frozen at -20 °C. Samples were subsequently aliquoted under frozen conditions (-20 °C walk-in room) for analyses of metabolites, immunological markers and DNA extraction preceding subsequent molecular analyses.

3.2 BUTYRATE PRODUCING MICROBIAL COMMUNITY

In the frame of the first publication (Sket et al., 2017a) the dynamics and diversity of fecal butyrate producing bacterial communities were studied as in response of reduced activity and hypoxia and linked to fecal metabolites (SCFA, reducing sugars) as well as immunological markers of the gut barrier integrity (zonulin, α1-antitrypsin (A1AT), eosinophil-derived neurotoxin (EDN) and bile acids (BA)) (Figure 10).
**Butyrate producing microbial community (Sket et al., 2017a)**

- DNA of human intestinal microbiota
- Amplicon sequencing of butyryl-CoA: acetate CoA-transferase (but) and butyrate kinase (buk)
  - α-diversity and β-diversity
  - NM-MDS of butyrate community

**Characterization of fecal samples:**
- Bristol stool scale, pH
- TSOH, SCFA
- Reducing sugars content (PAHBH)
- Molecular weight indices (MWI)

**Gut barrier integrity, permeability and inflammation:**
- Zonulin,
- α2-antitrypsin (A1AT),
- Eosinophil-derived neurotoxin (EDN),
- Bile acids (BA)

**Variation partitioning (n = 167; Table S2)**

**ENVIRONMENTAL DATA MATRIX**
- Experimental design
- Inactivity, hypoxia, participant...

**SPECIES DATA MATRIX**
Butyrate producing microbial community:
- butyryl-CoA: acetate CoA-transferase (but) and butyrate kinase (buk) genes. No significant changes.

**COVARIABLE DATA MATRIX**
- Controlled laboratory environment; balanced fluid and dietary intakes (protein, fat, iron, amino acids, carbohydrates, cholesterol, vitamins...), controlled circadian rhythm, 24/7 medical surveillance

**HUMAN PHYSIOLOGY CHANGES**

**SPATIAL DATA MATRIX**
Metabolites and immunological parameters (n = 167; Table S2)

**Figure 10. Schematic outline of the PlanHab experiments and analysis workflow described in first part of our study (Sket et al., 2017a), where dynamics and diversity of fecal butyrate producing bacterial communities were studied and linked to fecal metabolites as well as immunological markers of the gut barrier integrity.**

**Slika 10. Shematična predstavitev poskusov in metodoloških pristopov opisanih v prvem delu naše študije (Sket in sod., 2017a), kjer smo preučevali dinamiko in raznolikost črevesne bakterijske združbe, ki proizvaja masleno kislino in pridobili znanje umestili s podatki o črevesnih metabolitih in imunoloških kazalcih integritete črevesja.**
3.2.1 Characterization of fecal samples: BSS, metabolites, pH, MWI

Stool samples were characterized for a number of parameters (Table S2) as following: Bristol stool scale (BSS) (Degen and Phillips, 1996; Heaton et al., 1992), water content, pH (Stres et al., 2013), TSOC, SCFA (Kolbl et al., 2014), reducing sugars content (4-hydroxybenzoic acid hydrazide (PAHBAH)) (Lever, 1977), molecular weight (MW) and complexity of dissolved organic carbon (DOM) using molecular weight indices (MWI) (Helms et al., 2008; Twardowski et al., 2004).

BSS was used to map the stool consistency into seven consistency categories. The highest scores in general correspond to loose stools and fast intestinal transit, whereas lowest scores correspond to constipation, hard stools and longer colon transit times. In addition, BSS as stool consistency measure was recently implicated as an important environmental variable influencing intestinal microbiota composition (Gilbert and Alverdy, 2016; Tighelaar et al., 2015; Vandeputte et al., 2016). Frequency of defecation was calculated for each week in experiment using all available data points.

TSOC as a measure of the total content of organic carbon concentration in the fecal sample was measured in a miniaturized 96-well format assay using Agilent GC 2.5 mL vials and teflon septa caps. For calculations of TSOC calibration curves prepared from either 1 g/L of 60 °C dried glucose or humic acid (Sigma-Aldrich, Germany) were used to assess the extent of carbon complexity. Dual wavelength spectrophotometry (BIOTEK ELx808, Bio Tek Instruments, USA) was used to acquire two sets of data through monitoring reagent consumption (420 nm) and product formation (595 nm).

The extraction of C1-C6 SCFA (Acetic, Propionic, iso-Butyric, n-Butyric, iso-Valeric, n-Valeric, n-Capric acid) was conducted as described before (Kolbl et al., 2014, 2016) using a gas chromatograph (Agilent 6890, Agilent, Germany) equipped with a capillary column (Agilent J & W GC columns DB-FFAP, 30 m x 0.530 mm x 1 μm layer of stationary phase). The injector and detector temperature (FIS-flame ionizing) were 200 °C and 300 °C, respectively. Initial oven temperature was 70 °C with residence time of 1 min, which was ramped for 20 °C/min to 120 °C and further 10 °C/min to a final oven temperature of 200 °C with a residence time of 3 min. Carrier gas (mobile phase) was Helium with flow rates of 5 mL/min and nitrogen with flow rates of 25 mL/min; the detector gas was hydrogen with flow 30 mL/min and synthetic air with flow 400 mL/min. Among SCFA, butyrate is considered as one of the most important metabolites as it serves as the major energy source for colonocytes, has anti-inflammatory properties, and regulates gene expression, differentiation and apoptosis in host cells (Vital et al., 2014).

Reducing sugar content was determined in microtiter plate format (Stres et al., 2013). Reagent was prepared immediately before analyses (Lever, 1977). After 10 min incubation at 95 °C 200 μL aliquots were transferred to microtiter plates and readings collected at 490 nm. A calibration curve was prepared from serially diluted 1 g/L glucose (Sigma).
The relationship between the DOM MW was characterized using spectrophotometric measurements according to Helms et al. (2008) and Twardowski et al. (2004) generating a number of MWI. Fecal samples were centrifuged at 13,000 g and supernatants were diluted 1:50. 200 µL of each supernatant was transferred in a well of a 96 well transparent microtiter plate (Greiner UV-Star®, Greiner, Germany); spectra from 250 nm to 800 nm with 5 nm step were measured using a photometer (Multiscan® Spectrum # 1500; Thermo Fisher Scientific Inc., USA). Absorbance measurements were transformed to Napierian absorption coefficient (a) using equation: $a = 2.303 \times \frac{A}{l}$, where l represents the length of the beam = 2/3 cm. From the absorption spectra various parameters were calculated according to Helms et al. (2008) to characterize chromogenic dissolved organic matter. These parameters included the linear regression of natural logarithm of the transformed absorption coefficient from 275 nm to 295 nm and from 350 nm to 400 nm, respectively, as well as the ratio of these slopes (SR; 275 nm – 295 nm slope : 350 nm – 400 nm slope). In order to map the DOM MW the following polymers were used: salicylic acid (one aromatic ring), methyl red (two aromatic rings), bromocresol green and resazurin (three aromatic rings), brilliant blue (five aromatic rings), congo red and trypan blue (six aromatic rings).

3.2.2 Gut barrier integrity, permeability and inflammation

In addition to gut metabolites and physicochemical parameters, four immunological markers (zonulin, α1-antitrypsin (A1AT), eosinophil-derived neurotoxin (EDN), bile acids (BA)) measuring colonic permeability, mucus integrity, on-site inflammation and lipid absorption/biocidal effects, respectively, were determined in fecal samples using four distinct ELISA tests according to manufacturer’s instructions (Immundiagnostik AG, Bensheim, Germany) (Table S3).

Measured parameters and host physiology characteristics, generally presented in the form of heatmaps and graphs (mean ± SD), were analyzed for significant differences between two or more groups using non-parametric MANOVA and multiple Benjamini-Hochberg false discovery rate (FDR) multiple test correction (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001).

3.2.3 DNA extraction

Total genomic DNA (gDNA) was extracted from aliquoted fecal samples using MOBIO Power Fecal DNA extraction Kit (MOBIO; California, USA) according to the manufacturer’s instructions. In essence, triplicate homogenized subsamples of 0.25 g fresh weight were adopted for extraction. Concentration and purity (A230, A260 and A280) were determined spectrophotometrically using either NanoVue (Germany) or NanoDrop (USA). Additional external DNA standards were used for verification of quantification. The resulting DNA extracts were stored in 25 µL aliquots at -20 °C until their inclusion in molecular analyses.
3.2.4 Amplicon sequencing of butyrate producing bacterial communities

Abundance and diversity of intestinal butyrate-producing bacterial community was analyzed targeting the two primary bacterial butyrate synthesis pathways, butyryl-CoA: acetate CoA-transferase (*but*) and butyrate kinase (*buk*) as described before (Vital et al., 2013, 2015). On average (± SD) 50,816 ± 9,855 and 73,183 ± 10,169 sequences per sample were retrieved for *but* and *buk*, respectively. Raw reads were merged (Cole et al., 2014) and subjected to FrameBot (Wang et al., 2013), where a manually curated database based on the Functional Gene Database/Repository (Fish et al., 2013) served as reference source as described before (Vital et al., 2015). All amplicons displaying at least 80 % protein homology to a true *but*/*buk* reference were aligned to respective Hidden Markov Models (Fish et al., 2013) and subjected to complete-linkage clustering (95 % protein identity; Cole et al., 2014).

Abundance estimations based on band intensity after PCR amplification was performed according to Vital et al. (2015). Abundances were estimated by categorizing band intensities of PCR products into six distinct brightness groups (0–5) that were related to standard curves established with reference genomes. qPCR on selected taxa (*Roseburia/E. rectale* and *F. prausnitzii*) was performed according to Vital et al. (2013).

A number of ecological indices were used to assess α-diversity of samples: Taxa_S, Individuals, Dominance_D, Simpson_1-D, Shannon_H, Evenness_e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher_a, Berger-Parker, Chao-1 as implemented in mothur (Schloss et al., 2009). Acquired sequencing reads were deposited and are freely accessible on Metagenomics RAST (MG-RAST) database server (Meyer et al., 2008) under project accession number mgp79441 (http://metagenomics.anl.gov/linkin.cgi?project = mgp79441).

Multiple-group comparisons were performed using Benjamini-Hochberg false discovery rate (FDR) multiple test correction (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). Based on presence/absence pattern and abundance of operational taxonomic units (OTUs) in each sample distance matrices were calculated using Bray-Curtis similarity and graphically illustrating each community sample as a point on a two-dimensional graph using non-metric multidimensional scaling (NM-MDS) as implemented in the R package vegan version 2.2-0 (Oksanen et al., 2014).

Variation partitioning of variables (n = 167; Table S2) distributed into (i) metabolites and immune markers (n = 45), (ii) experimental design (n = 12), and (iii) diet (n = 110) was conducted in R (Legendre and Legendre, 2012). A step-down procedure was adopted for each group of variables to test for univariate association of variables with the structure of microbial communities and co-correlated variables were removed from further analyses. This yielded a smaller set of variables in three explanatory matrices significantly associated with microbial communities (n_permutations = 5000; p < 0.05). Variation partitioning was used
to determine most important factors recorded in metadata associated with the dispersion of butyrate producing community OTUs and the extent of explained variation in the structure of communities.

3.3 MICROBIAL 16S RRNA AMPLICON SEQUENCING

In addition to the first publication (Sket et al., 2017a), in the second one (Sket et al., 2017b) the dynamics and diversity of the gut microbiome based on 16S rRNA of archaeal and bacterial genes were investigated together with additional variables spanning electrical conductivity, fecal polyphenols and sterols and various dissolved organic matter (DOM) spectral characteristics (Figure 11).

3.3.1 Establishment of the PlanHab database

Clinical, metabolic, inflammation, immune, human physiology and nutrition data next to experimental design and characteristics of the individual participants were integrated into a novel in-house database comprising all measured variables in the PlanHab experiment (n = 231; Table S2) (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a; Strewe et al., 2017). Together over 12,000 entries of examined factors were compiled and critically assessed. A comprehensive PlanHab database with entries corresponding to samples used in this study was used for data normalization, extraction and interpretation of statistical significant features. In addition to parameters recorded within the past PlanHab substudies (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a; Stavrou et al., 2016; Strewe et al., 2017) such as body mass and composition, menu composition, water intake, micronutrients, miRNA expression profiles, morning resting heart rate, capillary oxyhemoglobin saturation, ratings of perceived appetite, daily dietary intake, inflammation markers (n = 167), new data were measured. In second study 64 variables spanning electrical conductivity, fecal polyphenols and sterols and their diversity, as well as analyses of various dissolved organic matter (DOM) spectral characteristics were obtained and integrated within the PlanHab database (Figure 11) (Sket et al., 2017b).
Figure 11. Schematic outline of the PlanHab experiments and analysis workflow described in second part of our study (Sket et al., 2017b), where dynamics and diversity of the gut microbiome based on 16S rRNA of archaeal and bacterial genes were studied and linked to previous characterization in first part of our study (Sket et al., 2017a) as well an additional characterization of fecal samples performed in second part of our study.

3.3.2 Intestinal electrical conductivity as measure of fecal ionic strength

Samples (10 g) were suspended in distilled water (25 ml) and homogenized at 4 °C. Measurements took place after samples equilibrated at 28 °C using WTW electrode Cond 330i (WTW, Germany). External standard solution (1 M KCl) and series of its 10 fold dilutions were prepared. Aliquots of prepared standard solution and its dilution series were immersed in the same water baths and their conductivity determined in triplicates ($R^2 = 0.998$). In addition, conductivity of standard solutions were measured after each five samples in order to check for electrode fluctuations. The aliquots of standard solution used for control measurements with samples, were measured again and compared to unused aliquots of standard solution. Intestinal electrical conductivity (IEC) served as a measure of ionic strength of intestinal environment that is a measure of effects of innate immune system, influencing the complex interplay between mucus thickness, porosity, its crosslinking and hydration state.

3.3.3 Fecal polyphenols and sterols as determined by HPLC

Methanol (Sigma) was used to extract polyphenols and sterols from fecal dry matter in (vol. to wt) ratio 10 to 1. Resulting extracts (1 mL aliquots) were filtered through 0.22 μm filters into 2.5 mL HPLC vials (Varian) and stored at -20 °C until HPLC analysis. The HPLC method was performed under isocratic conditions at 30 °C on Waters HPLC system (2995 with 2998 PDA detector). Analyses were performed on a reversed-phase C-18 column: Spherisorb ODS1 RP-18, 5 m, 250 x 4.6 mm from Waters (Milford, USA). Bile acids were separated using mobile phase composed of 0.5 M acetate buffer with 0.02 % sodium azide (Sigma), adjusted to pH 4.3 with o-phosphoric acid. The flow was set to 0.5 ml/min (isocratic conditions) with the detection of PDA detector set at 205 nm. The injection volume was 10 μl. Polyphenolic compounds were separated using the two-component solvent system: A: 1.5 % phosphoric acid and B: methanol (Merck) - acetonitrile (Merck) - water, 1:1:1 (V/V/V). The separation gradient of the two solvents used was the following: 0 min: 100 % A; 0-20 min: 100–60 % A; 20-35 min: 60-0 % A at 1 ml/min and the signal of phenolic compounds was determined at 280 nm using PDA detector. The injection volume for polyphenols was set to 100 μL.

In analogy with fast fingerprinting of microbial communities, separate chemical fingerprinting of sterols and polyphenols was used to determine the total content and relative distribution of peaks within each class of compounds that were further used for calculation of chemical diversity of compounds in samples using program mothur (Schloss et al., 2009).

3.3.4 Deconvolution of dissolved organic matter spectral derivatives of biological importance

Fecal samples were centrifuged at 13.000 g and dilutions (1:10; 1:50; 1:100) of supernatants in MQ were prepared. The 200 μL aliquots of each dilution were transferred into 96 well of
transparent UV resistant microtiter plates (Greiner UV-Star®, Greiner, Germany). Spectra spanning from 200 nm to 800 nm with 5 nm step were recorded using a photometer (Multiscan® Spectrum # 1500; Thermo Fisher Scientific Inc., USA). Absorbance measurements were transformed to Napierian absorption coefficient (a) using equation: 
\[ a = 2.303 \times \frac{A}{l}, \]
where \( l \) represents the length of the beam = 2/3 cm. From the absorption spectra various parameters were calculated according to Helms et al. (Helms et al., 2008) to characterize chromogenic dissolved organic matter. The following parameters were determined: (i) indole level index calculated as the ratio between two characteristic wavelengths of 217 nm and 365 nm and 287 nm and 365 nm (Kumar et al., 2015); (ii) specific ultraviolet absorbance (SUVA254), a measure of the aromatic character of dissolved organic matter, was calculated as a ratio between absorbance at 254 nm and total soluble organic carbon per gram of dry matter in feces (TSOC) (Weishaar et al., 2003); (iii) specific visible absorbance (SViA420), provides a measure of nonaromatic DOM and was calculated as a ratio between absorbance at 420 nm and TSOC (Weyhenmeyer et al., 2014); (iv) cDOM index, was calculated as the ratio between colored DOM (measured as the absorption coefficient at 350 nm) and TSOC (Vantrepotte et al., 2015), as an indicator of anaerobic degradation of plant polymeric substances producing tannin-like compounds stable in the water soluble fraction of DOM; (v) the sum of nine p-hydroxy, vanillyl, and syringyl lignin phenols (TDLP9) calculated using the model 
\[ \ln (TDLP_9) = -2.282 \times \ln (a(350)) - 8.209 \times \ln (a(275)) + 11.365 \times \ln (a(295)) + 2.909 \]
(Fichot et al., 2016). Various phenols exhibit important roles in the initiation and/or progression of intestinal permeability leading to “leaky gut” and increased inflammation.

3.3.5 Amplicon sequencing of microbial 16S rRNA

The extracted genomic DNA (gDNA) was used as template for amplification of the V1-V2 hypervariable regions of the bacterial 16S rRNA gene by PCR using primers S-D-Bact-0008-a-S-16 (27F: 5′AGAGTTTGATCMTGGC 3′) and S-D-Bact-0343-a-A-15 (357R: 5′CTGCTGCCTYCCGTA 3′) (Klindworth et al., 2013) with appropriate Illumina adapter sequence. In addition, second set of primers was used to target V6-V7 hypervariable region of bacteria and archaea: S-D-Arch-0519-a-A-16 (5′-CAGCMGCGCGGTAA-3′) (Klindworth et al., 2013) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) (Takahashi et al., 2014). Primers were first in-silico assessed comparatively with other primer sets for their performance in SILVA (Quast et al., 2013) and Ribosomal Database Project II (RDP II) (Cole et al., 2014) databases and iteratively improved in order to effectively extend their bacterial and especially archaeal coverage in analyses of anaerobic environments.

All PCR reactions were performed in triplicates. Total volume of 25 µL comprised: 10 ng of template DNA; 12.5 µL NEBNext® High-Fidelity PCR Master Mix (New England Biolabs, USA), 0.75 µL (10 pmol) of each primer and DEPC treated water to 25 µL. PCR cycling conditions included a hot-start (98 °C; 5 min), 30 cycles of denaturation (98 °C; 10
s), annealing (60 °C; 30 s) and elongation (72 °C; 30 s) followed by a final elongation step (72 °C; 5 min). After PCR samples were purified using Agencourt® AMPure® XP (Beckman Coulter, Inc.). The correct amplicon size was checked on a Bioanalyzer 2100 instrument (Agilent Technologies, USA) using the DNA 7500 kit (Agilent Technologies, USA). The concentration of the purified samples was measured by the Quant-iT PicoGreen kit (Life Technologies, USA). For library preparation the Nextera XT v2 Index kit set A was used (Illumina Inc., USA). The Indexing PCR was performed in 25 μl reactions containing: 12.5 μl NEBNext® High-Fidelity PCR Master Mix (New England Biolabs, USA), 2.5 μl of each Indexing primer, 10 ng of purified amplicons and 6.5 μl DECP treated water. The amplification procedure included an initial denaturation step (98 °C; 30 s), 8 cycles of denaturation (98 °C; 10 s), annealing (55 °C; 30 s) and elongation (72 °C; 30 s) followed by a final extension step (72 °C; 5 min). The amplicons were first checked on a 1 % agarose gel then purified by cutting from a 1 % agarose gel and DNA was eluted with the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG). Quality and quantity of amplicons were determined as described above. Amplicons were pooled equimolar to 4 nM and sequenced using the MiSeq Reagent kit v3 (600 cycles) (Illumina Inc., USA) for paired end sequencing using an Illumina MiSeq Sequencer. The V1-V2 sequencing run resulted in $5.67 \times 10^6$ high quality reads, whereas V6 - V7 sequencing run resulted in $8.07 \times 10^6$ high quality reads, after chimeric sequences, singletons ($< 0.05 \%$) and sequences shorter than 150 bp were removed. Total count of high quality sequences was equivalent to $105300 \pm 39200$ and $149600 \pm 28200$ (mean ± SD) per sample for V1-V2 and V6-V7 sequencing runs, respectively. All obtained reads were deposited on the MG-RAST database server (Meyer et al., 2008) under accession number (mgp80027; http://metagenomics.anl.gov/linkin.cgi?project=mgp80027).

3.3.6 Bioinformatic and statistical analysis of 16S rRNA sequencing

Correlation between sequencing of V1-V2 and V6-V7 hypervariable regions was conducted using Mantel test based on respective dissimilarity matrices. To follow the general microbiological and ecological notation, the Bray-Curtis and Morisita-Horn distance (Legendre and Legendre, 2012) were used ($R^2 = 0.58$, $R^2 = 0.78$, respectively; both $p < 0.001$). Analyses were conducted utilizing data from all samples (as rows) based on genus level sequence abundances of bacteria (as columns), normalized to the equal number of sequences in all samples (Kozich et al., 2013; Legendre and Legendre, 2012; Schloss, 2008) and 9999 permutations of underlying data matrices.

A number of ecological indices were used to assess α-diversity of samples: Taxa_S, Individuals, Dominance_D, Simpson_1-D, Shannon_H, Evenness_ e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher_α, Berger-Parker, Chao-1 (Figure S2) as implemented in mothur (version 1.35.1) (Schloss et al., 2009). One-way NP-MANOVA with 10,000 permutations was used to determine significant differences between samples and experimental variants relative to baseline data collection.
To explore the differences between groups of samples, the Bray-Curtis (community membership, species abundance and matching zeroes adjustment), ThetaYC (community membership and relative abundance) and Jaccard (Jclass; community membership) indices were adopted. Three methods testing independent hypotheses, i.e. UniFrac (weighted and unweighted), AMOVA (analyses of molecular variance) and HOMOVA (homogeneity of molecular variance) were used to address specific ecological questions concerning differences between microbial communities as described before (Figure 6, Figure 7, Figure S1B) (Kozich et al., 2013; Schloss, 2008).

Multiple-group comparisons were performed using Benjamini-Hochberg false discovery rate (FDR) multiple test correction (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). Permutation tests were conducted using 999 permutations.

The distribution of samples into a defined number of community types (i.e. metacommunities or enterotypes) was assessed using the minimum Laplace value linked with Bayesian and Akaike information criteria (Holmes et al., 2012). A non-parametric tests LEfSe (Segata et al., 2011) and metastats (White et al., 2009) as implemented in mothur were used to identify microbial OTUs or genus level taxa that consistently explained the differences between microbial communities. In addition, classical indicator species analysis was conducted as implemented in mothur (Dufrene and Legendre, 1997).

The Firmicutes to Bacteroidetes ratio was calculated for each type of intervention over time in addition to the recently suggested rate of changes over time (Holmes et al., 2017).

Core microbiomes were characterized as fraction of taxa that are found in varying numbers of samples for different minimum relative abundance using Corbata (CORE MicroBiome Analysis Tools) (Li et al., 2013). To measure the difference between taxonomic profiles of two cohorts, a test statistic Abundance-Weighted Kolmogorov-Smirnov (AWKS) was used in order to capture the difference of ubiquities between cohorts across abundances, for each taxon. Major and minor cores were defined and tested for significant differences using AWKS and presented in Abundance-Variability plots that included ubiquity data (Li et al., 2013).

Characterization of genus *Bacteroides* sequence data was accomplished using two distinct approaches, Picrust tool (Langille et al., 2013) to characterize the predicted genus *Bacteroides* related metagenomes and the Ribosomal Database Project Toolbox (http://github.com/rdpstaff/) k-mer search strategy using SequenceMatch utility. First, for analyses within Picrust, sequence IDs belonging to *Bacteroides* were exported from mothur to biom format (McDonald et al., 2012) and dereplicated using Greengenes 13.5 database (version gg_13_8_99, August 2013) which contains 202,421 bacterial and archaeal sequences. The OTU maps were provided within mothur in order to make the necessary biome file compatible with Picrust tool (Langille et al., 2013) to predict major functions of *Bacteroides*. Second, in addition to Picrust analysis, k-mer search was conducted using only
the high quality sequences of all *Bacteroides* type (T) and cultivated strains. Sequences (length of the 16S rRNA gene > 1200 bp) were retrieved from RDP II database in fasta format. The Ribosomal Database Project Toolbox was used to map *Bacteroides* sequence from this study to sequences of *Bacteroides* retrieved from the databases using k-mer search strategy (n = 7; Sens > 0.87) using SequenceMatch utility. The distribution of identified strains was linked to existing published physiological and medical reports on particular *Bacteroides* strains.

Metastats (White et al., 2009) was used to identify groups of sequences binned into *Bacteroides* species based on k-mers, that differed significantly between experimental variants (p < 0.05).

The variation partitioning approach was used to determine the hierarchy of most important metadata from all measured variables (n = 231; Table S2) associated with the dispersion of bacterial communities at 97 % OTU and genus levels to determine the extent of explained and stochastic variation in microbial structure. Variables were distributed into three groups: (i) metabolites and immune markers (n = 109), (ii) experimental design (n = 12), and (iii) diet (n = 110) and analyzed in R (Legendre and Legendre, 2012). A step-down procedure was adopted for each group of variables to test for univariate association of variables with the structure of microbial communities and co-correlated variables were removed from further analyses to decrease dimensionality. This yielded a smaller set of variables in three secondary explanatory matrices significantly associated with microbial communities (n_permutations = 5000) that were used in variation partitioning. Heatmaps were constructed using heatmap.2 function as implemented in R package gplots v3.0.1 (Warnes et al., 2016).

### 3.4 MICROBIAL METAGENOMES AND INTESTINAL METABOLOMES

The third part of our work (Sket et al., 2018) encompass additional acquired data: (i) shotgun metagenomes of intestinal microbial communities at taxonomic and functional levels, (ii) intestinal nuclear magnetic resonance (1H-NMR and 13C-NMR) metabolomes; (iii) X-ray fluorescence (XRF) spectroscopy of elements, and (iv) Bayesian network analysis of significantly different intestinal parameters and metabolites (Figure 12).
3.4.1 Microbial metagenome sequencing

The extracted genomic DNA was used as a template for whole shotgun sequencing. Approximately 200 ng of microbial DNA was used for shearing of the long DNA fragments with Covaris ultrasonicator. Quantity and quality of DNA were examined using DNA Agilent Bioanalyzer 2100 and Quant-iT™ PicoGreen DNA Fragment Analyzer reagents DNF-473 Standard Sensitivity NGS Fragment Analysis Kit.

Metagenomic libraries were constructed using NEBNext Ultra DNA Library Prep Kit (Illumina Inc., USA) according to the manufacturer's protocol. First end repair of sonicated DNA and ligation of adapters was performed. Then cleaning step and size selection
procedure of adapter ligated DNA were performed using magnetic beads. After that step only fragments of DNA around 650 base pairs long remained for downstream reactions. Furthermore, indexing PCR and amplification of adapter ligated DNA was performed, followed by another cleaning step with magnetic beads. Prepared libraries were diluted to 4 nM, pooled equimolar and shotgun sequenced using Illumina MiSeq platform (Illumina Inc., USA). Sequencing run produced $1.8 \times 10^7$ reads in total, $1.0 \times 10^6 \pm 1.9 \times 10^5$ (mean ± SD) per sample.

### 3.4.2 Bioinformatic and statistical analysis of microbial metagenome

Taxonomical and functional annotation of metagenomic datasets was performed using Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST server) (Meyer et al., 2008). The data was compared to REFSQ database for taxonomical annotation and Subsystems database for functional annotation using a maximum e-value of $1e^{-5}$, a minimum identity of 60 %, and a minimum alignment length of 15 base pairs and amino acids respectively.

To analyze relationship between start versus end of each variant, and between ends of particular variants (NBR, HBR and HAmb), a number of established approaches was used: parsimony, weighted UniFrac, uweighted UniFrac, AMOVA (analyses of molecular variance), HOMOVA (homogeneity of molecular variance), lefse, indicator and metastats tests with 999 permutations were used as implemented in mothur (Schloss 2009). Multiple-group comparisons were performed using Benjamini-Hochberg false discovery rate (FDR) multiple test correction (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). In addition, a test statistic Abundance-Weighted Kolmogorov-Smirnov (AWKS) as implemented in Corbata (CORe microBiome Analysis Tools) (Li et al., 2013) was used for calculations of the difference between variants at the taxonomic or functional profiles. The congruency of results from various tests deployed was schematically summarized (Figure 12) and taken to reflect the signal observed at various levels of microbiome data integrating over the underlying statistical assumptions of particular separate analytical approach adopted in this study. The use of a concerted approach of various statistical approaches enabled us to detect the most evident congruent changes in microbiome.

### 3.4.3 Intestinal metabolome analysis using proton nuclear magnetic resonance

Fecal samples (200 mg of dry matter) were resuspended in 800 μl of NMR phosphate buffer and centrifuged at 10,000 g for 30 min at 4 °C to remove fine particles. Samples were filtered through 0.22 μm HPLC compatible filters (Millipore, Germany), 400 μl aliquots were mixed with 200 μl 1H-NMR buffer as described before (Beckonert et al., 2007) and stored at -25 °C until analysis. Phosphate buffer (pH 7.4) was prepared by weighting 1.443 g NaH2PO4, 0.263 g NaH2PO4, 2 mM TSP and 1 mM NaN3 into 50 mL volumetric flask. 10 mL of D2O was added and filled up to 50 mL with Milli-Q water. Before analysis, samples were thawed...
at room temperature, centrifuged at 12,000 g for 5 min at 4 °C. 550 μL of each sample was transferred into 5 mm NMR tube.

1H NMR spectra were acquired on an Agilent Technologies DD2 600 MHz NMR spectrometer equipped with 5 mm HCN Cold probe. 2D experiments were measured on Agilent Technologies (Varian) VNMRS 800 MHz NMR spectrometer equipped with 5 mm HCN Cold probe. All experiments were measured at 25 °C. 1H NMR spectra of the samples were recorded with spectral width of 9.0 kHz, relaxation delay 2.0 s, 32 scans and 32 K data points. Water signal was suppressed using Double-pulsed field gradient spin echo (DPFGSE) pulse sequence. Heteronuclear single quantum coherence spectrum (HSQC) was acquired with spectral widths of 9.0 kHz and 40 kHz for 1H- and 13C-dimensions, respectively, 1536 complex points for 1H-dimension, relaxation delay 1.5 s, 160 number of transients and 128 time increments. Total correlated spectrum (TOCSY) was measured with 1H spectral widths of 7.0 kHz, 4096 complex points, relaxation delay 1.5 s, 32 number of transients and 144 time increments. The 1H and 2D spectra were apodized with an exponential function and a cosine-squared function, respectively, and zero filled before Fourier transform. NMR spectra were processed and analyzed using VNMRJ (Agilent/Varian) and Sparky (UCSF) software and MestReNova.

The resulting spectra were consequently analyzed in two complementary ways: (i) human expert chemometric untargeted metabolomics, including 2D spectra, and (ii) targeted quantitative metabolomics using Chenomx NMR Suite version 8.3 (Chenomx, Inc). For the latter, all spectra were randomly ordered for spectral fitting using ChenomX profiler. Metabolites were thus independently identified using 2D NMR spectroscopy (Figure S3, Table S4) and with the support of Chenomx Compound Library extended by Human Metabolome Data Base (Wishart et al., 2013), giving access to chemical shift profiles of 674 compounds used in analyses.

Three different approaches to asymmetric sparse matrix data analysis were adopted (Legendre and Legendre, 2014). Each compound concentration obtained was (i) normalized by dividing the measured concentration into the total concentration of all metabolites in that sample; (ii) Box-Cox or log2 transformed; (iii) corrected for the total dissolved organic matter concentration (TSOC) determined as described before (Kolbl et al., 2016). The significance of difference in the metabolic characteristics of various groups of samples was tested using NP-MANOVA (with Benjamini-Hochberg multiple corrections), expressed as an overlap in non-metric multidimensional scaling (nm-MDS) trait space using Gower and Euclidean distance measures, the dimensionality reduction selected through stress function and inspection of Shepard’s plots of correspondence between target and obtained ranks.

3.4.4 X-ray fluorescence spectrometry of intestinal metal content

Sample (10 g) was dried at 60 °C, homogenized and 100 mg of subsamples were compressed using a pellet die and hydraulic press. Element analysis was performed by X-ray florescence
spectrometry focusing on the following ten elements: P, S, K, Ca, Mn, Fe, Cu, Zn, Rb and Sr (Figure S4). XRF spectrometer based on Rh anode (35 kV) with 5 mm beam was used to irradiate the samples. XRF signal was detected with silicon drift diode (SDD) (Amptek). Spectra were analyzed in LabView and quantitative analysis was performed as described before (Kump et al., 1996). Dark matrix (the non-responsive elements) were determined by emission transmission method (Nečemer et al., 2008). Quality assurance for the element analysis was performed using standard reference materials: NIST SRM 1573a (tomato leaves, homogenised powder); CRM 129 (hay powder); and OU-10 (geological sample of Longmyndian greywacke, GeoPT24). All statistical analyses were performed as described above.

### 3.4.5 Bayesian network modelling

To explore the relationships between the immune, intestinal and environmental parameters associated significantly with the distribution of microbial communities in experimental variants over time observed in our whole study (Sket et al., 2017a, 2017b, 2018), Bayesian network analysis was conducted using the C++ utility (Ziebarth et al., 2013). A number of parameters ($n = 231$) was monitored over the course of the PlanHab experiment and a database containing these data was interrogated (Sket et al., 2017b). Only the intestinal parameters that differed significantly over the course of the PlanHab experiment were used (Figure S5). First, the structure of a network model that best explains the data was learned, and second, the model was used to make predictions about the interactions between the variables in the model. Global structure learning settings included (i) Maximum number of parents ($n = 4$) as the number of immediate parents for every node in the network as it has an impact structure learning in two main ways. First, limiting the maximum number of parents can dramatically increase the speed of structure learning for larger networks. Second, this limit also helps avoiding over-fitting a network model, as it prevents a variable from being directly influenced by a large number of the other variables in the network; (ii) Number of high-scoring networks to include in model averaging ($k = 100$) to increase the performance of the structure learning search; (iii) Model averaging selection threshold of directed edges to be included in the network given their posterior probabilities after model averaging. All directed edges with posterior probabilities greater than the threshold were included in the network ($t = 0.8$); (iv) Number of tiers in the network which can then be used to specify structure learning constraints was not assigned in order to perform the structure learning from the data. The significant parameters identified in first and second part of our study (Sket et al., 2017a, 2017b) were inspected and segmented according to the recorded time of significant deviation. The arranged data were superimposed next to the calculated Bayes network in order to check for the correspondence between the experimentally observed and calculated hierarchy governed by time-frame of appearance.
4 RESULTS

4.1 BUTYRATE PRODUCING MICROBIAL COMMUNITY

4.1.1 Inactivity affects fecal consistency

Significant changes were observed in consistency of fecal material relative to BDC within and between variants (Figure 13A). A pronounced decrease in fecal BSS towards constipation was observed for both bedrest variants (Figure 13B). Faster and more pronounced progression towards constipation was observed in the HBR than in the NBR participants, although this difference was not significant ($p = 0.14$). In addition, the apparent weekly retention time (as time between defection events) was significantly higher in NBR and HBR in comparison to HAmb (Figure 13B). Relatively limited extent of daily physical activity in HAmb provided an effective protection towards constipation when the same type of controlled diet was used, despite hypoxia.

![Figure 13. Changes in Bristol stool scale values (BSS) (A) and retention time (as time between particular defections) (B) during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a).](image)

Slika 13. Spremembe v Bristol lestvici konsistence vzorcev blata (BSS) (A) in časom zakasnitve rednega iztrebljanja blata (B) v obdobju privajanja (1 teden) in sledečemu tri-tedenskemu poskusu (Sket in sod., 2017a).

4.1.2 Inactivity and hypoxia aggravate the state of gut inflammation but not permeability

Four immunological markers, mapping the tight junction leakage (zonulin), mucosal integrity (A1AT), on site inflammation (EDN) and pro-inflammatory cytokine pathways/lipid metabolism (BA) were determined over time for all participants (Figure 14). The two markers describing gut leakage, zonulin (chemical flow through tight junctions into
blood stream) and A1AT (mucosal integrity and mucosal permeability in direction from the host towards intestinal lumen), were not significantly different between experimental variants over time, suggesting that a healthy epithelium and mucosal integrity were maintained over the course of the experimental period, irrespective of inactivity or hypoxia.

Figure 14. Changes in immunological markers present in fecal samples: zonulin (A), α1-antitrypsin (B), eosinophil-derived neurotoxin (EDN) (C) and bile acids (D) during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a).

Slika 14. Spremembe v imunoloških kazalcih v fecesu: zonulin (A), α1-antitrypsin (B), nevrotoksin eozinofilcev (EDN) (C) žolčnih kislinah (D) v obdobju privajanja (1 teden) in slededečemu tri-tedenskemu poskusu (Sket in sod., 2017a).

In contrast, clear shifts after exposure were detected for EDN and BA (Figure 14). EDN was highest in HBR, followed by NBR, with limited fluctuations in HAmb. BA increased most in NBR, but was not significantly higher than in HBR, whereas in fecal samples from participants of the HAmb treatment BA was not significantly increased until the end of experiments despite hypoxia. The results show that inactivity clearly increased fecal BA
content, which is an indicator for increased local inflammation in the intestinal tract and higher cytotoxic activity towards microbiota, whereas hypoxia had no measurable negative effect under the chosen experimental setup.

4.1.3 Inactivity and hypoxia did not affect gut metabolic markers

No significant changes \( (p = 0.19) \) were detected between experimental variants in a number of measured gut metabolites and physicochemical parameters despite the observed changes in stool consistency levels based on BSS (Figure 15-17). Increased fecal pH in both hypoxic conditions (week 2) coincided with hypoxia-related hyperventilation and blood alkalosis (Figure 15B).

![Figure 15. Variation in water content (A), pH (B), and total soluble organic carbon (TSOC) in fecal samples (C) during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a).](image)

In addition, the concentrations and ratios of butyrate and next two most important SCFA acetate and propionate were also variable and were not associated neither with time, inactivity or hypoxia, but showing response to differences in ingested food composition (Figure 16, Figure 17).
Figure 16. Changes in concentration of C1–C6 short chain fatty acids (SCFA) present in fecal samples during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a): total SCFA (A), acetic acid (B), propionic acid (C), iso-butyric acid (D), n-butyric acid (E), iso-valeric acid (F), n-valeric acid (G), and n-capric acid (H).

Slika 16. Spremembe v koncentraciji C1-C6 kratko-verižnih hlapnih maščobnih kislin (SCFA) v fekalnih vzorcih v obdobju privajanja (1 teden) in sledečemu tri-tedenskemu poskusu (Sket in sod., 2017a): celokupne SCFA (A), ocetna kislina (B), propionska kislina (C), izo-maslena kislina (D), n-maslena kislina (E), izo-valerinska kislina (F), n-valerinska kislina (G) in n-caprilna kislina (H).
Figure 17. Changes in molecular weight indices (MWI) of fecal samples during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a): Sr = ratio of absorption slopes between 275–295 and 350–400 nm slope (A), E2:E3 = ratio between absorption coefficients at 250 nm and at 365 nm (B), a (300 nm) = Napierian absorption coefficient at 300 nm (C), a (255 nm) = Napierian absorption coefficient at 255 nm (D), Total a = integrated absorbance between 250 nm and 450 nm (E) and S = spectral slope of absorbance from 300 to 700 nm (F).

Slika 17. Spremembe v indeksih molekulske mase (MWI) v obdobju privajanja (1 teden) in sledečemu tri-tedenskemu poskusu (Sket in sod., 2017a): Sr = razmerje absorpcijskih nagibov med 275-295 in 350-400 nm (A), E2:E3 = razmerje med absorpcijskimi koeficienti pri 250 nm in pri 365 nm (B), a (300 nm) = Napierov koeficient absorpcije pri 300 nm (C), a (255 nm) = Napierov absorpcijski koeficient pri 255 nm (D), celokupni a = integrirana absorbanca med 250 nm in 450 nm (E) in S = spektralni nagib absorpcije od 300 do 700 nm (F).

4.1.4 Diversity and abundance of butyrate producing microbial community was not influenced by bedrest and hypoxia

Detailed sequence analysis of but and buk genes also showed that butyrate producing microbial communities were comparatively diverse in all experimental variants (Figure 18, Figure 19). There was no significant difference in α-diversity estimates (p > 0.39) of but and buk genes between BDC and experimental time points, suggesting no detectable effect of the 21 day hypoxia and inactivity on the structure and diversity of butyrate microbial communities.
Figure 18. A schematic representation of closest matches of *buk* (A) and *but* (B) gene sequences describing butyrate producing communities in PlanHab samples (Sket et al., 2017a).

Slika 18. Shematična predstavitev ujemanja sekvenc genov *buk* (A) in *but* (B) mikrobnih združb, ki proizvajajo masleno kislinno (Sket in sod., 2017a).
Figure 19. NM-MDS ordination showing a host-specific grouping of butyrate communities based on combined but and buk gene datasets for all participants in experimental variants (Sket et al., 2017a): NBR (blue), HBR (red), HAmb (yellow). The time spent in experiment is designated by darker colors. Stress$_{\text{buk}+\text{but}} = 0.18$. Only OTUs represented by at least 50 sequences were used in analyses. The numbers designate sample groupings according to participants.

Based on band intensity the more abundantly represented butyrate producing community harboured but genes, however, the abundance of but and buk genes was rather dynamic and within the same range in all samples (Figure 20). This is in line with the observation that the concentration of butyrate or other SCFA in samples did not change significantly over time within and between experimental variants.
Figure 20. Abundance of butyrate producing community members as estimated by two approaches, qPCR (A, B), and band intensities (C, D) during run-in (week 1) and subsequent 3-week experimental phase (mean ± SD) (Sket et al., 2017a). Units are scaled to represent % of total bacterial microbial community (A, B). Band intensities of PCR products (C, D) were categorized according to band intensity classes (0–5) (Vital et al., 2015).

The abundance of Roseburia/E. rectale and F. prausnitzii based on but gene qPCR analysis was comparable through the course of experiments, with the exception of week 3 of HAmb when the highest levels were transiently detected. The effects of experimental variant and time were not significant in two-way NP-MANOVA (p > 0.46) after the correction for multiple comparisons.
The observed fluctuations in abundance of butyrate producing community were correlated to menu composition, where butyrate producing community responded to fluctuations in dietary fiber content within the expected limits exhibiting its healthy physiological status.

4.1.5 Correlative analysis

To determine the association between the data matrices recorded in this study (metabolites, immunological markers, experimental setup, diet) (n = 167; Table S2) and OTUs of butyrate producing communities, variation partitioning was conducted. A step-down procedure identified a smaller subset of variables out of all recorded metadata that were significantly associated with dispersion of butyrate producing microbial communities. A subset of all metabolic and immunological variables (butyrate, isoValerate, acetate, total SCFA, BA, EDN, TSO, nCapreate, A1AT), experimental data (participant characteristics (age, height, BMI), activity level, experimental variant) as well as diet related parameters (histidine, Chloride ions, rare bacterial OTUs, ingested fat and water, sucrose, pantothenic acid (vitamin B5)) were ranked in descending order of importance within each, respectively (Table S5).

In general, variation in butyrate producing microbial community structure was significantly explained by experimental setup (13.4 %), experimentally structured metabolites (12.8 %), gut metabolite content and levels of immunological markers (11.9 %). Despite the introduction of numerous variables into analyses (n = 167) many metadata variables were found to be correlated and hence were removed as redundant during the step-down procedure and excluded from further analyses. Consequently, 61.9 % of variation in butyrate producing microbial community data remained unexplained and attributed to a combination of experimental and unknown, but real sources of variation, next to random noise.

4.2 MICROBIAL 16S RRNA AMPLICON SEQUENCING

4.2.1 General microbial diversity and composition are largely unaffected by 21-day inactivity and hypoxia

Unweighted unifrac metrics based either on Bray-Curtis, ThetaYC and Jaccard indices at the level of 97 % OTU or genus did not result in significant differences in the microbiome composition between NBR, HBR and HAmb participants of the PlanHab experiment over time (p = 0.16). There were also no significant differences in microbial diversity over time based on analysis of numerous ecological diversity indices (p = 0.44) (Figure S2). There were no significant differences in enterotype assignment, the Firmicutes to Bacteroidetes ratio nor in the rate of change in that ratio over 21 days of experiment. The phylogenetic tests used in this study congruently showed that microbial membership at the level of 97 % OTU or genus did not differ significantly between experimental variants over the course of the PlanHab study.
Weighted unifrac, AMOVA and HOMOVA congruently identified that the significant differences in relative abundance, group centroids and group variance size existed between specific groups at the end of experiment after the correction for multiple comparisons (p < 0.01, p < 0.01 and p < 0.004 of tests, respectively). All three tests confirmed that microbiomes of HBR participants became significantly different from NBR and HAmb by the end of the study (p = 0.01, p = 0.01, respectively) (Figure 21). However, microbiomes of NBR and HAmb participants did not differ significantly over the course of PlanHab study (p = 0.11 and p = 0.31, respectively) over multiple time points.

Figure 21. Schematic overview of the detected changes in microbial communities. The congruency of four statistical tests was used to detect significant differences in the structure of microbial community (Sket et al., 2017b). The additional four statistical tests were used to congruently identify the first responding taxa over the course of the PlanHab experiment. P-values on the lines are used in the context of null hypothesis testing. The end-points of the PlanHab experiment with the significant changes are shown (p < 0.05; FDR corrected). NBR – normoxic bed rest, HBR – hypoxic bed rest, HAmb – hypoxic ambulatory.

Slika 21. Shematski pregled sprememb v mikrobeni združbi. Skladnost štirih statističnih testov je bila zahtevana za odkrivanje pomembnih razlik v strukturi mikrobenih skupnosti (Sket in sod., 2017b). Dodatni štirje statistični testi so bili uporabljeni za soglasno potrditev pojavnosti prvih taksonomskih skupin v času trajanja PlanHab poskusa. P-vrednosti na črtah so uporabljene v kontekstu preizkušanja ničelnih hipotez. Prikazane so končne točke PlanHab poskusa s pomembnimi spremembami (p < 0.05; upoštevana stopnja lažnega odkrivanja (FDR)). Ležanje v normoksičnih pogojih (NBR), ležanje v hipoksičnih pogojih (HBR), gibanje v hipoksičnih pogojih (HAmb).

Further, the Corbata, LEfSe, metastats and indicator species analyses (Figure 21) congruently identified that members of the genus Bacteroides, encompassing many
uncultivated strains, became significantly enriched in HBR participants relative to NBR or HAmb ($p = 0.0003$) in the last week of experiment (Figure 22). However, a large proportion of *Bacteroides* species (75.7%) was shared between experimental groups (Figure S7) and also matched the physiologically characterized strains described in published literature exhibiting many detrimental and inflammagenic characteristics related to mucin degradation, bile acid resistance, intestinal tract inflammation, opportunistic infections or those preceding autoimmune disorders in type 1 diabetes in youngsters (Table S6).

Figure 22. Heatmap plot of the genus *Bacteroides* sequences (Sket et al., 2017b). NBR, HBR and HAmb sequences were classified at species level and their number normalized to their mean at week four (the end of PlanHab study). The specific and significant increase in the levels of various *Bacteroides* species in HBR can be seen ($p < 0.05$).

Slika 22. Toplotni diagram na osnovi prisotnosti sekvenc rodu *Bacteroides* (Sket in sod., 2017b). NBR, HBR in HAmb sekvence so bili razvrščene na nivoju vrste in njihova številčnost normalizirana glede na povprečje v četrtem tednu (konec PlanHab poskusa). Opazno je statistično značilno povečanje bakterij vrst *Bacteroides* v HBR ($p < 0.05$).
Picrust tool was used to impute genus *Bacteroides* related metagenomes based on the availability of 16S rRNA sequences from sequenced genomes. *Bacteroides* collection of full genomes is available at Joint Genome Institute Integrated Microbial Genomes and Microbiomes (JGI IGM/M; http://img.jgi.doe.gov/cgi-bin/m/main.cgi) \((n_{\text{Genus}} = 275\text{ genomes JGI/IGM})\). Most of the listed species \((n = 61)\) contained up to two genomes, in contrast to the few most well and easiest to culture \((n = 16)\), that covered 72.7\% of all *Bacteroides* genomes. In addition, 33.8\% of *Bacteroides* genomes were represented by single species whereas 60\% of genomes were represented by 6 *Bacteroides* species only. The obtained Picrust tool derivatives describing the imputed *Bacteroides* metagenomes were not informative, i.e. did not differ significantly, reflecting the low coverage of *Bacteroides* intragenomic diversity within the genome sequence databases.

The composition of major and minor cores of microbiomes as identified by abundance-variability-ubiquity analysis in Corbata (Figure 23) largely corresponded to the microbiome composition observed in Human Microbiome Project (HMP) (The Human Microbiome Project Consortium, 2012). The limited detection of Archaea by V6-V7 prokaryote primer set (archaea : bacteria ratio < 1 : 10^4) in this study further supports this observation. The most abundant phyla were Firmicutes and Bacteroidetes, with the family of *Prevotellaceae* showing the highest variability. Other bacterial taxa apparently responded to inactivity and hypoxia over the 21-day experiment (Figure 21). However, because of inter-individual variation over the 21-day study, these shifts did not reach statistical significance with all four statistical tests \((p > 0.05)\) (Figure 21) used for identification of taxa that responded differently between the variants (Figure 11). For example, members of the genus *Eubacterium*, a commensal causing opportunistic infections of soft tissues were increased in HBR participants, whereas mucus dwelling *Akkermansia* were reduced. Opportunistic, pro-inflammatory members of *Dialister* characteristics of pre-type 2 diabetes were apparently enriched in NBR participants. In contrast, members of the probiotic genus *Bifidobacterium* were enriched in phenotypically healthy HAmb over time.
Figure 23. The overview of core microbiomes at the start-up and endpoint of the PlanHab experiment (Sket et al., 2017b). The Corbata plots of abundance (x-axis), variability (y-axis) and ubiquity (circles). Each circle represents a single taxon with its size proportional to the ubiquity of the taxon, thus the larger the circle, the more ubiquitous the taxon across the cohort. Taxa were classified and divided into major (green) and minor (blue) core microbiomes next to other bacterial taxa unclassified as either major or minor core (black). Taxa towards the top of the plot have greater variation. Abundance increases towards the right of the plot. (A) Microbiomes at start-up of PlanHab experiment; (B) hypoxic ambulatory (HAmb); (C) hypoxic bed rest (HBR); and (D) normoxic bed rest (NBR).
4.2.2 Intestinal environmental parameters are congruent with human physiology markers

A number of novel parameters not considered before were explored in this study to describe gut environment and its biochemical characteristics over the course of a 21-day experiment and their relationship to existing data from past PlanHab sub-studies was explored. The frequency of defecation as the number of defecation events per week progressively decreased over the course of bed rest in NBR and was more pronounced in HBR under hypoxia (Figure 24). The defecation rates were also highly correlated to Bristol stool scale (BSS) and weekly retention time in addition to bile acid content ($R^2 > 0.85$) reported in first part of our study for the same participants (Figure 13) (Sket et al., 2017a).

![Figure 24. Weekly defecation rates as a direct measure of constipation due to increased retention times of organic matter in intestinal system throughout the PlanHab experiment (mean ± SD) (Sket et al., 2017b).](image)


IEC of fecal matter, a robust measure of ionic strength and hence of innate immune system activity, increased within the first week of bed rest in HBR and NBR participants for 67% and 32%, respectively (Figure 25A) ($p < 0.05$). In contrast, IEC of HAmb participants remained stable despite the synchronized and controlled diet in all variants. The increased
values of IEC were also highly correlated to a number of negative human physiology symptoms recorded before (Debevec et al., 2014; Strewe et al., 2017) (e.g. insulin insensitivity) and intestinal parameters such as constipation (BSS), weekly retention time, bile acids (BA) and eosinophil derived neurotoxin (EDN) ($R^2 > 0.83$) (Figure 13, Figure 14).

Figure 25. Fluctuations in the intestinal parameters over the course of PlanHab experiment (mean ± SD) (Sket et al., 2017b). (A) Electrical conductivity of fecal matter as a measure of ionic strength of intestinal environment; (B) total quantities of sterols, and (C) polyphenols; (D) indole level index; (E) diversity of sterol and (F) polyphenol peaks; (G) the sum of nine p-hydroxy, vanillyl, and syringyl phenols; (H) specific ultraviolet absorbance (SUVA) of dissolved organic matter (DOM) as a measure of overall DOM aromaticity; and (I) specific visual absorbance (SViA) index as a measure of colored DOM.

Slika 25. Spremembe v črevesnih parametrih med trajanjem PlanHab poskusa (Sket in sod., 2017b). (A) električna prevodnost fekalne vsebine kot merilo ionske moči črevesnega okolja; (B) celokupne količine sterolov in (C) polifenolov; (D) indolnokolični indeks; (E) raznolikost sterolov in (F) raznolikost polyfenolov; (G) vsota devetih p-hidroksih, vanilil in syringil fenolov; (H) specifična ultravijolična
absorbanca (SUVA) raztopljene organske snovi (DOM) kot merilo aromatičnosti DOM; in (I) specifično vizualna absorbanca (SViA) kot merilo barvnega spektra DOM.

The concentration and chemical diversity of sterols and polyphenols reported in this study were not significantly affected over time based on NP-MANOVA (p > 0.25) (Figure 25). Total polyphenol content was only transiently increased in NBR participants with large fluctuations between participants whereas remained largely stable in HBR and HAmb. Detailed analyses of dissolved organic matter (DOM) spectral data revealed that the sum of nine p-hydroxy, vanillyl, and syringyl phenols (TDLP<sub>9</sub>), levels of DOM aromaticity (specific ultraviolet absorption (SUVA)) and colored DOM (specific visual absorption (SViA)) next to the index of anaerobic production of tannin-like compounds were also not significantly different between the experimental variants over time. In contrast, the levels of indole, a microbial derivative of essential aromatic amino acid tryptophan ((2S)-2-amino-3-(1H-indol-3-yl)propanoic acid) and a precursor for simple indole alkaloids such as melatonin and serotonin, were found to increase significantly over time in HAmb in comparison to NBR and HBR (Figure 25D).

In order to integrate variables recorded in this and past sub-studies of all participants throughout the run-in and experimental phase an in-house PlanHab database was established in our study (n = 231; Table S2). This enabled us to identify parameters that differed significantly between the experimental variants over the course of experiment and link them to bacterial community structure. Ten parameters out of 231 describing diet, intestinal metabolites, immune and chemical parameters (Figure 26) and 36 markers describing human physiology were significantly different between NBR, HBR and HAmb before the week four of PlanHab experiments (p < 0.05; Figure 27). Covariation of the two datasets increased progressively with the time spent in the PlanHab experiment (linear regression R^2 = 0.73) confirming that the two datasets were highly correlated (Mantel test, R^2 = 0.978; p < 0.003).
Figure 26. Heatmap plot showing the relationship between parameters describing intestinal environment that differed significantly by week four at the end of PlanHab experiments (n = 10; p < 0.05; FDR corrected) (Sket et al., 2017b). See online supplementary material for details on all measured variables describing diet, experimental conditions, intestinal environment, metabolites and immune parameters (n = 231) that are part of the new in-house PlanHab database (Table S2) (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a; Stavrou et al., 2016; Strewe et al., 2017).

Slika 26. Toplotni diagram spremenljivk opisa črevesnega okolja, ki so se statistično značilno razlikovale na koncu PlanHab poskusa (n = 10; p < 0,05; upoštevana stopnja lažnega odkrivanja (FDR)) (Sket in sod., 2017b). Informacije o vseh izmerjenih spremenljivkah, ki opisujejo prehrano, eksperimentalne pogoje, črevesno okolje, metabolite in imunske parametre (n = 231), so del nove podatkovne zbirke PlanHab poskusa (Preglednica S2) (Debevec in sod., 2014, 2016; Rittweger in sod., 2016; Simpson in sod., 2016; Sket in sod., 2017a, 2017b, 2018; Stavrou in sod., 2016; Strewe in sod., 2017).
Figure 27. Heatmap plot showing the relationship between parameters describing human physiology that differed significantly by week four at the end of PlanHab experiments \((n = 36; p < 0.05; \text{FDR corrected})\) (Debevec et al., 2014, 2016; Rittweger et al., 2016; Simpson et al., 2016; Sket et al., 2017a, 2017b, 2018; Stavrou et al., 2016; Strewe et al., 2017).

Slika 27. Toplotni diagram sprememljiv opisa človeške fiziologije, ki so se statistično značilno razlikovale na koncu PlanHab poskusa \((n = 36; p < 0.05; \text{upoštevana stopnja lažnega odkrivanja (FDR)})\) (Debevec in sod., 2014, 2016; Rittweger in sod., 2016; Simpson in sod., 2016; Sket in sod., 2017a, 2017b, 2018; Stavrou in sod., 2016; Strewe in sod., 2017).

4.2.3 Identification of the key structuring parameters

To determine the association between microbiome data and the three data matrices describing (i) diet, (ii) experiment, (iii) intestinal metabolites, immune and chemical parameters assembled in the PlanHab database \((n = 231; \text{Table S2})\), two variation partitioning analyses were conducted at two different levels of resolution (97 % OTU and Genus). Despite the introduction of numerous variables into the two analyses, many of the
metadata variables were found to be correlated, and were hence removed during the stepdown procedure, or were at the threshold of association of a completely random test variable, and were hence excluded from further analyses. A smaller subset of variables significantly associated with the dispersion of bacterial communities was identified as the key parameters explaining significantly the distribution of microbial taxa over time and experimental setup (Table S7). The important overarching parameters were characteristics of individual participants and interactive effects of hypoxia and inactivity (experiment), short chain fatty acids and immune system variables (intestinal parameters) next to dietary water, fats and proteins. The three matrices ((i) intestinal metabolite and inflammation markers, (ii) PlanHab experimental setup, and (iii) experimentally structured metabolites) used in variation partitioning at the level of 97 % OTU, explained 27.4 %, 17 % and 7.1 % of variability, respectively. Hence, 48.5 % of variation in microbial community data remained unexplained. In contrast, variation partitioning using the same three matrices at the genus level showed that the same categories explained slightly lower proportion of variability (21.4 %, 12.8 % and 1.7 %, respectively), leaving 65.8 % of variability unexplained. The extent of unexplained variation at 97 % OTU and genus levels was consequently attributed to a combination of: (i) experimental sources of variation, (ii) unknown real sources of variation and (iii) random noise. The progressively increasing extent of phylogenetic resolution (phylum to species levels, or 80 to 99 % OTU) also increased the extent of explained variation, providing a rationale for detailed functional metagenomic analyses targeting subspecies genomic diversity.

4.3 MICROBIAL METAGENOMES AND INTESTINAL METABOLOMES

4.3.1 Taxonomic annotation of bacteria, archaea and fungi

Altogether $2.68 \times 10^7$ taxonomic hits were assigned of which $2.66 \times 10^7$ hits belonged to bacteria. The most abundant bacterial phyla were Firmicutes ($59.6 \pm 13.3$ %) and Bacteroidetes ($29.7 \pm 14.2$ %), followed by Actinobacteria ($3.87 \pm 2.02$ %), Proteobacteria ($3.80 \pm 1.27$ %), Fusobacteria ($0.52 \pm 0.12$ %) and Spirochaetes ($0.48 \pm 0.14$ %), in line with the general assembly of human microbiome (The Human Microbiome Project Consortium, 2012) and our past observations on the same samples (Sket et al., 2017b).

No significant differences were observed between initial and endpoint bacterial microbiomes at the level of bacterial taxonomic distribution using parsimony and unweighted unifrac metrics based either on Bray-Curtis, ThetaYC and Jaccard indices. The group centroids (AMOVA) and dispersion (HOMOVA) between initial and endpoint experimental variants were at the margin of significance ($p = 0.09$) after the correction for multiple comparisons. Lefse, indicator species and metastats tests showed that Eubacterium ventriosum ($p = 0.028$), Bacteroides sp. (multiple species) and Proteobacteria (Escherichia coli ($p = 0.026$), Shigella sp. ($p = 0.029$), Veillonella sp. ($p = 0.042$)) were significantly enriched at the end of HAmb, HBR and NBR variants, respectively (Figure 28A).
Figure 28. Schematic representation of the significant changes in the taxonomic annotation of metagenomes (Sket et al., 2018). Annotations are shown at the level of bacterial (A), archaeal (B) and fungal (C) microbial communities.

Slika 28. Shematična predstavitev pomembnih sprememb v taksonomskem opisu metagenomov (Sket in sod., 2018). Opisi so prikazani na ravni bakterijskih (A), arhejskih (B) in glivnih (C) mikrobnih skupnosti.
The members of *Eubacterium* that were enriched at the end of HAmb are intensively involved in anaerobic degradation of various plant polyphenols, e.g. flavonoids, through deconjugation of various sugars obtained through diet into derivatives with important physiological bioactivity for the host (Rowland et al., 2018; Schneider and Blaut, 2000). However, it is evident from several studies, that the complete metabolism of polyphenol glycosides in the intestinal tract requires active involvement of complex consortium of microbes (Rowland et al., 2018).

Closer examination showed that the members of the genus *Bacteroides* were significantly enriched at the end of HBR variant (*B. xylanisolvens* (*p* = 0.012), *B. finegoldii* (*p* = 0.015), *B. ovatus* (*p* = 0.015), *B. sp. D22* (*p* = 0.018), *B. thetaiotaomicron* (*p* = 0.021), *B. fragilis* (*p* = 0.033), *B. caccae* (*p* = 0.036), *B. cellulosilyticus* (*p* = 0.04), *B. capillosus* (*p* = 0.045), *B. dorei* (*p* = 0.048)). In addition, *B. eggerthii* (*p* = 0.02) and *B. pectinophilus* (*p* = 0.049) were decreased at the end of NBR and HAmb variants. The results are in line with analyses at the level of 16S rRNA amplicon sequencing (Figure 21) that identified a significant increase in the presence of the members of the genus *Bacteroides* in HBR participants that belonged to species, previously linked to various dysbioses in humans (e.g. intestinal tract inflammation, obesity, insulin resistance, hyperglycemia, metabolic syndrome, T2D (Sket et al., 2017b). The elevated levels of the members of the genus *Bacteroides* were associated with a poor postprandial glucose response in HBR and NBR participants and other negative physiological symptoms (Simpson et al., 2016; Sket et al., 2017b). However, not all members of the genus *Bacteroides* interacted with their host in the same way, showing that different but highly related species can have very different effects on host metabolism, further suggesting that the key effectors of improved metabolism of the host in HAmb could be microbial metabolome, proteome and lipidome (Johnson et al., 2016).

On the other hand, the increase in *Proteobacteria* in NBR falls in line with past observations of potential metabolic endotoxemia (Kamada et al., 2013). Altered intestinal conditions may facilitate the overgrowth of potentially harmful subsets of indigenous bacteria within the intestine as some pathogens more efficiently utilize common resources under novel conditions established due to physical inactivity. Another important strategy utilized by bacterial pathogens to further acquire a growth advantage over bacterial commensals is to promote host intestinal inflammation that additionally impedes commensal survival, but increases competitive advantages of pathogenic behaviour in terms of nutrient acquisition.

Archaeal diversity and composition was also assessed in the same samples (Figure 28B). The majority of all archaeal hits (1.3 × 10^5 sequences) was mostly assigned to phylum *Euryarchaeota* (93.4 ± 1.01 %), followed by *Crenarchaeota* (5.46 ± 0.78 %), *Thaumarchaeota* (0.54 ± 0.29 %), *Korarchaeota* (0.53 ± 0.19 %) and *Nanoarchaeota* (0.04 ± 0.06 %). No significant differences in the taxonomic distribution at various levels of archaea were identified using all of the above statistical tests between initial and end-point samples between experimental variants. Last, fungal diversity and composition was
described using $1.7 \times 10^4$ fungal hits that were distributed between phylums *Ascomycota* ($80.2 \pm 5.7\%$), *Basidiomycota* ($15.5 \pm 6.5\%$) and *Microsporidia* ($4.3 \pm 2.5\%$) (Figure 28C). Similar to the archaea, there were no significant differences in the fungal taxonomic distribution at various levels of between initial and end-point samples of experimental variants.

### 4.3.2 Functional annotation of metagenomes

Altogether $1.2 \times 10^7$ functional hits were assigned to four functional levels. Parsimony and unweighted unifrac metrics analyses based either on Bray-Curtis, ThetaYC and Jaccard indices did not result in significant differences between initial and end-point experimental variants at the gene level (Table S8). The AMOVA and HOMOVA tests were also not significantly different ($p = 0.13$, $p = 0.11$) between initial and end-point experimental variants after correction for multiple comparisons. In contrast, comparison at the functional level 1 showed that gene groups at the end of HBR variant differed significantly from the end of HAmb variant ($p = 0.038$). Genes coding for proteins involved in iron acquisition and metabolism, cell wall, capsule, virulence, disease and defense were enriched at the end of HBR in comparison to the end of HAmb variant. Although the differences after the correction for multiple comparisons between the start of experiment and the end of HBR variant were on the margin of significance ($p = 0.09$) and hence deemed not significant, the increase in abundance of gene groups for virulence, disease and defense have been observed ($p = 0.036$) (Figure 29, Figure S7). These results suggest that with additional exposure to inactivity (e.g. in ESA 60 day bedrest experiments) significant differences at the level of the genes for cell wall, capsule, virulence, disease and defense could be anticipated. This shows that physical inactivity (lack of activity) per se effectively modified the physiological responses of microbial communities towards modified interaction with the host (Johnson et al., 2016; Sket et al., 2017a, 2017b) that eventually resulted in modified microbial community structure and distinct distributions of functional genes. However, no significant differences in richness of microbial functional genes over the course of the PlanHab experiment were detected at any level ($p > 0.05$).
Figure 29. Schematic representation of the significant changes in the functional annotation of microbiomes (Sket et al., 2018). Annotations are shown at the functional level (A), level 1 (B) and the category of genes involved in mucin degradation (C) (Table S8).

Slika 29. Shematična predstavitev statistično značilnih sprememb v funkcionalnem opisu mikrobnih združb (Sket in sod., 2018). Prikazan je popis na funkcionalni ravni (A), ravni 1 (B) in skupini genov, vključenih v razgradnjo mucina (C) (Preglednica S8).
In line with second part of our study (Sket et al., 2017b) mucin degradation genes differed significantly between endpoints of HAmb and HBR variants (p = 0.04), NBR and HBR (p = 0.027) variants, and between the start and end of HBR (p = 0.032) variants as well. Genes for beta-galactosidase (EC 3.2.1.23) as well for α-L-fucosidase (EC 3.2.1.51), Sialidase (EC 3.2.1.18) and α-N-acetylglucosaminidase (EC 3.2.1.50) were uniformly increased at the end of HBR variant. These enzymes are largely present in the Bacteroides genomes, the members of the genus that were significantly enriched at the end of HBR variant (Sket et al., 2017b). The co-identification of significant enrichments for genes coding for capsule proteins and mucin degradation genes are not surprising as these two gene categories are coregulated in Bacteroides species (Martens et al., 2009; Sket et al., 2017b) such as B. thetaiotamicron, B. fragilis and B. vulgatus at the timescale of minutes in response to the environmental characteristics (Johnson et al., 2016). In addition, the environmental polymer distribution (ratios between plant cell wall, microbial exopolysaccharides and host mucin polymers) and temporal (un)availability (constipation, diet shifts) of chemically diverse environmental polymers were shown to affect the preferential use of substrates and hence gene expression (Martens et al., 2009).

Further, ion milieu affects availability, transport and uptake of nutrients of Bacteroides and is considered the first sign of innate immune response activity (Martens et al., 2009; Ravcheev et al., 2013). Further, Bacteroides are capable of effective switching between the three carbon sources although there exists a preferential use of plant polymers over the host mucus under nonconstipated conditions. In addition, the effects of other commensal exopolysaccharides on Bacteroides thetaiotamicron gene expression, such as Bifidobacteria, were clearly elucidated (Rios-Covian et al., 2013, 2016). Bacteroides thus have the capacity to swiftly alter gene expression to match changing substrate availability and environmental fluctuations (Johnson et al., 2016), whereas remarkably few Bacteroidetes were shown to be influenced by host genetics. For the majority of the members of this phylum, environmental factors determine their metabolic activities leading to increased abundance, making them good targets for therapeutic interventions to fine tune their abundance and metabolic activities.

Genes involved in aerobic and anaerobic respiration have regularly been implicated as those responsible for metabolic endotoxemia of microbial communities in response to increased levels of reactive, oxygen, nitrogen and sulfur species during inflammatory response in obesity related syndromes (Johnson et al., 2016). However, genes for aerobic and anaerobic respiration pathways were not significantly different between the initial and end-point samples in PlanHab experimental variants.

Butyrate synthesis pathways genes at the end of NBR differed significantly from those at the end of HBR (p = 0.018). Genes for butyrate kinase were uniformly increased at the end of HBR (p = 0.039) whereas butyryl-CoA dehydrogenase gene were increased at the end of NBR. These results are in contrast to the results of no significant difference reported in first part of our study (Ske et al., 2017a). The main difference stems from the fact that qPCR and
amplicon sequencing were used to analyze *buk* and *but* genes, focusing mostly on the most widely reported members belonging to the Firmicutes (Sket et al., 2017a). As shot-gun untargeted metagenomics was used in this study, this shows that additional butyrate producing bacteria could be detected and established under the conditions of HBR in addition to those covered by the primers used before (Vital et al., 2013; Sket et al., 2017a). Anyhow, the elevated levels of functional genes were not reflected at the level of increased butyrate concentration in the same samples (Sket et al., 2017a) and 1H-NMR metabolomes in this study. The steady state concentrations of C1-C6 short chain fatty acids (SCFA) in feces were also not significantly different between the variants in experiment, thus the exact flux from feces towards the host intestinal cells remains unknown. This is especially intriguing as HBR and NBR participants exhibited significant constipation levels based on Bristol Stool Scale classification (Sket et al., 2017a). A simulation taking into account production and consumption rates from the same diet at different exercise levels (Figure S8) showed the relationship between actual concentration and potential removal rates from fecal matter, congruent with observations of increased SCFA content in obese groups described before.

4.3.3 Microbial metabolome in the intestinal environment

The analyses of 1H-NMR intestinal metabolomes revealed no significant difference between variants over time in response to hypoxia or inactivity (Figure 30). These findings are in-line with our previous measurements of numerous parameters that revealed insignificant changes in the concentration of SCFA, TSOC, sterols and polyphenols, various aromatic compounds through TSOC spectral deconvolution and reducing sugar measurements in the same samples (Sket et al., 2017a, 2017b).
Figure 30. Schematic representation of the relationships between microbial fecal 1H-NMR metabolomes (Sket et al., 2018).

Slika 30. Shematična predstavitev mikrobnih fekalnih 1H-NMR metabolomov (Sket in sod., 2018).

On the other hand, a small set of intestinal parameters (Figure S5) was significantly modified over the course of 21-day experimental phase in NBR, HBR and HAmb variants: Bristol Stool Scale (BSS), EDN, BA, IEC and indole (Sket et al., 2017a, 2017b). As indole is microbial metabolite and the other are host derived this shows that the physiological changes primarily take place at the level of the host and cascade further down to the microbial subsystem that responds by adjusting particular metabolic activities. The predominant lack of change at the level of fecal 1H-NMR metabolome points towards the importance of other levels of interaction between host and microbiome, most probably at the level of proteome, its glycosylation and lipid modification cycles in relation to the negative physiological and psychological symptoms observed in HBR and NBR variants in comparison to HAmb (Debevec et al., 2014; Simpson et al., 2016; Sket et al., 2017a, 2017b; Stavrou et al., 2016; Strewe et al., 2017)

4.3.4 Electrical conductivity governs intestinal metal availability

XRF measurements of elements and metals in fecal matter revealed no significant difference between variants and time spent in experiment (Figure 31). Elemental and metal content in intestinal tract is of key importance to microbial metabolism, virulence and pathogenesis, their protein synthesis and activity on one hand, and host physiology on the other. On this line of proteomic interaction between the microbiome and the host, microbial siderophores play an important role in sequestering iron and other metals in intestinal tract (Ahmed and
Holmström, 2014). In nature, Fe has to compete not only against free protons for the siderophore binding sites but also against other metal ions such as divalent cations, including Cd²⁺, Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺; trivalent cations, such as Mn³⁺, Co³⁺ and Al³⁺; and actinides, such as Th⁴⁺, U⁴⁺ and Pu⁴⁺. There are several studies that have shown that siderophores have an impact on the mobility of these metal ions in the environment. Many siderophores are non-ribosomal peptides in addition to the catecholates (phenolates), hydroxamates and carboxylates (e.g. derivatives of citric acid). Overall binding rate of Fe ions to siderophores decreased by 2–11-fold as electrical conductivity (EC) as a measure of ionic strength increased from approximately 0.01 to 0.5 M (Ahmed and Holmström, 2014). The increased IEC in NBR and HBR as part of innate immune system indicates the attempts of the host to systematically decrease availability of metals to microbes. Over the course of the PlanHab experiment NBR and HBR participants exhibited elevated IEC (Sket et al., 2017b) at comparable metal contents in their diet as observed in this study, coinciding with the increased inflammatory responses (Simpson et al., 2016).

Figure 31. Schematic representation of the relationships between trace metal compositions based on X-ray fluorescence (XRF) spectroscopy (Sket et al., 2018).

Slika 31. Shematična predstavitev sestave kovin v sledovih, glede na rezultate rentgenske fluorescenčne spektrometrije (XRF) (Sket in sod., 2018).

In addition to promoting microbial growth by binding metals, there is emerging evidence of clinical relevance that microbial siderophores can effectively modulate the host response. It was previously shown that siderophores secreted by enteric pathogens have the capacity to cause hypoxia-dependent activation of HIF-1 in the Peyer's patches and in human epithelial
and endothelial cells, a transcription factor that plays pivotal roles during infection (Behnsen and Raffatellu, 2016; Sket et al., 2017a, 2017b).

4.3.5 Bayesian network as a model of interactions at systems level

A number of parameters ($n = 231$) was monitored over the course of the PlanHab experiment and a database containing these data was interrogated (Sket et al., 2017b). Only the intestinal parameters that differed significantly over the course of the PlanHab experiment were used to derive a model of human physiology responses (Figure 12, Figure 32; Figure S5) establishing hierarchy in the measured parameters for the first time.

Figure 32. Bayesian network analysis (Sket et al., 2018). The intestinal parameters that differed significantly over the course of the PlanHab experiment were used to derive a model of human physiology responses establishing hierarchy in the measured parameters.

Slika 32. Bayesova analiza omrežja (Sket in sod., 2018). Parametri črevesja, ki so se bistveno razlikovali med trajanjem PlanHab poskusa, so bili uporabljeni za izpeljavo hierarhije v izmerjenih parametrih in modela odzivov človeške fiziologije, ki jih ti parametri določajo.
Exploration of the network showed that changes in the parameter levels resulted in reproduction of the responses observed within the PlanHab study (Debevec et al., 2014; Simpson et al., 2016). For instance, decrease in fecal electrical conductivity resulted in increased BSS (no constipation) and reduced bile acids (BA) levels. Secondly, increased indole concentrations at retained lower electrical conductivity resulted in lowered intestinal inflammation (EDN) levels. On the other hand, if BA levels were increased within the network, EDN and conductivity were increased as well, whereas BSS levels declined further towards constipation and reduced indole production. However, this link between microbial indole production and inflammation marker EDN suggested that a non-linear responses take place over the network as small changes in indole levels exhibited unexpectedly large effects on BA content.
5 DISCUSSION

5.1 CHANGES IN HUMAN PHYSIOLOGY WITHIN THE PLANHAB PROJECT

The real-time 21-day exposure of healthy participants to three different settings induced significant adaptations in human physiology (Figure 27). On the one hand the importance of exercise is linked to many-faceted physiological responses to physical activity: the vertical hydrostatic gradients within body (Debevec et al., 2014), electrolyte losses from sweating, posture energy consumption (Miles-Chan and Dulloo, 2017), significant changes in the host muscle and circulating metabolome, that all take place at the time scale of minutes following resistance and or aerobic exercise (Figure 4) (Berton et al., 2016). On the other hand systemic hypoxia was associated with ≈10 % reduction of SpO₂ in HAmb and HBR (Keramidas et al., 2016). However, only the combination of hypoxia and inactivity (HBR) resulted in general signs of congestion such as 10 % increased mean arterial pressure, 15 % increased heart rate, three times larger decrease in plasma volume and concomitant 3-fold increase in mature blood cells, hemoglobin and hematocrit concentrations in comparison to either NBR or HAmb. It has to be noted that systemic hypoxia could have thus also aggravated the inactivity-induced local tissue hypoxia in the HBR. General fatigue, tiredness, tension, recovery delay, negative affective responses, induced participativeness (i.e., extent of induced willingness to join in an activity) were also largest in HBR, followed by NBR, whereas almost undetectable at HAmb despite equal hypoxia levels as in HBR (Stavrou et al., 2016). Increased postprandial glucose concentration and reduced insulin sensitivity were reported in both inactive variants NBR and HBR (Simpson et al., 2016), suggesting the onset of obesity-related symptoms. At the same time, HAmb exhibited numerous positive attributes of human physiology such as decreased fasting insulin concentration, retained insulin sensitivity, unchanged postprandial serum insulin concentration and increased fasting fat oxidation rate, c-peptide response, c-peptide/insulin ratio (fed or fasted) (Figure 27) (Sket et al., 2017b). Lastly, the negative effects, observed in human physiology, were reported to be dose-dependent on the extent of time each participant was subjected to inactivity (NBR) or inactivity and hypoxia (HBR) (Simpson et al., 2016).

5.2 INTESTINAL ENVIRONMENT

Experimental physical inactivity effectively modified intestinal conditions through increased constipation (Figure 13, Figure 24), modified intestinal parameters (Figure 25) and autonomous shift in diet (Figure 26), over the course of the PlanHab experiment (Debevec et al., 2014). During prolonged and increasing constipation the luminal content became more rigid by absorption of nutrients and water. As a consequence, pushing, mixing and separating chime into segments by peristaltic waves (i.e., circular constrictions) can inflict intestinal abrasions. The derived abrasions are highly amenable for further microbial colonization, leading to increased local inflammation on the long run. This is especially true for locations with a thin or modified mucus layer (e.g., small intestine, abrasions, reduced mucus...
thickness, increased porosity and modified mucus glycosylation pattern) (Glover et al., 2016). Moderate activity on daily basis in HAmb variant effectively precluded the development of constipation and intestinal inflammation, despite the concomitant negative effects of systemic hypoxia. As especially the benefits of exercise, that are well established for cardiovascular and metabolic health, extend to other organ systems, including the gut (Cronin et al., 2016), the lack of exercise also reached and affected the gut on a short time scale of 21 days (NBR) and was additionally aggravated by systemic hypoxia (HBR).

Numerous characteristics of intestinal environment (Table S2) that were measured in our study (Sket et al., 2017a, 2017b, 2018), revealed that many variables were either correlated and also covaried or did not significantly change over the course of the experiment. Such variables were SCFA, sterol and polyphenol content and diversity next to DOM spectral derivatives. The lack of change in parameters of the colon such as SCFA concentrations, epithelial and mucosal integrity and permeability recorded in this study are in line with reports that physiologic hypoxia predominates in colon (Glover et al., 2016). Hence its health status and oxygenation are under the control of SCFA, not postprandial hyperaemia as was observed in small intestine (Zheng et al., 2015). This indicates that the on-site inflammation observed through EDN is probably taking place in the small intestine that is more susceptible to microbial overgrowth under the conditions of slowed chime transport due to high-energy (Western) diet intake, physical inactivity and associated hypoxia, increasing constipation and associated fluctuations in oxygen availability. Dietary polyphenols are a major source of antioxidants consumed by humans. Polyphenols possess not only antioxidant properties but also antiviral, antibacterial, anti-inflammatory and anti-carcinogenic effects, as well as the ability to modulate certain signaling pathways, such as nuclear factor-κB activation.

Sterols as lipids have important biological functions in humans depending on the interaction between the diet of the host (that determines the relative quantities of sterol precursors) and the complex metabolic transformations of sterols by intestinal microbiota to their secondary derivatives with potent biological effects (Barton et al., 2018; Cronin et al., 2016; van Dijk et al., 2012; Egan and Zierath, 2013; Pham et al., 2012).

On the other hand, positive effects of the energy-consumption-through-exercise lifestyle in HAmb are reflected as healthy levels of IEC (Figure 25), BA, lack of constipation (Figure 13), lack of intestinal inflammation (EDN) and increased indole levels (Figure 26) (Skej et al., 2017b) and are in accordance with lower blood glucose, insulin sensitivity, reduced body fat, postprandial glucose, fasting serum total cholesterol, and lipoprotein cholesterol observed before in HAmb participants (Figure 27) (Simpson et al., 2016). Listed above indicated a healthy physiological state in HAmb despite the negative effects of hypoxia, evident in inactive HBR participants (Skej et al., 2017a). Increased EDN levels in NBR and HBR pointed to progressive development of intestinal inflammation. Increased inflammatory responses are in line also with tissue (NBR) and combination of tissue and systemic hypoxia (HBR) in inactive variants that were recently linked to central inflammatory mediators nuclear factor kappa B (NF-kB) and hypoxia inducible factor 1
(HIF-1α) (Glover et al., 2016; Zeitouni et al., 2016). Those factors modulate cell transcription, shape nutritional-immunity status of the gut and induce the release of reactive oxygen and nitrogen species (Faber and Bäumler, 2014). These reactive chemical species can act as additional electron acceptors for microbes, creating new niches for facultatively anaerobic bacteria and imposing a new selective force on microbial growth, their activity patterns and microbial cross-talk. From this it follows that the observed systemic inflammation levels in NBR and HBR were not produced by direct external microbial infection as the HAmb participants would become infected as well within the same experimental facility, receiving the same food and drinks, microbial burden, and aerosols. Increased BA levels observed in NBR and HBR originate from increased liver BA excretion into the small intestine in an attempt of the host to increase gut motility and decrease small-intestine overgrowth by commensal microbiota. In addition, the facts that BA are known to be potent vasodilators (Ward et al., 2014; Zheng et al., 2015) and play a key role in intestinal hyperaemia point again to the small intestine as the starting location of tissue hypoxia under physical inactivity and consequent associated inflammation in response to microbial overgrowth activities. The increasing levels of indole as a microbe-generated signal substance in HAmb variant coincided with healthy host physiology in HAmb. This shows that the positive effects of rather limited bouts of exercise exerted on host as well as the microbiome were linked. Indole was shown to function as a quorum-sensing signal that regulates the virulence and biofilm formation of enterohemorrhagic E. coli, Pseudomonas and other commensal bacteria, but also strengthens the barrier function of the mucous membrane by repairing tight junctions (Table S6). Hence, it is plausible that most relevant changes for the host physiological status took place at the level of molecular and chemical cross-talk between microbes and the host.

Constipation (Figure 13, Figure 24) in response to inactivity effectively changed IEC that is linked to the effectiveness of microbial nutrient transport (Figure 25), mucin characteristics (folding, thickness and porosity) and consequently surface availability of tripartite complex of glycans derived from diet (plant polymers), host (mucin-O-glycans) and microorganisms (extracellular polymeric substances) (Johnson et al., 2016; Rios-Covian et al., 2013, 2016). This points to one important link between exercise and constipation (i.e., decreased gut motility) (Figure 13, Figure 24) for the pathologies in NBR and HBR observed in this study and apparent increase in intestinal anoxic ischemia (Glover et al., 2016; Haglund, 1994). The positive effects of exercise for reducing the negative effects of tissue hypoxia observed in NBR and exacerbated by systemic hypoxia in HBR were confirmed as even passive resistive vibration exercise decreased systemic inflammation observed in 60-day normoxic bedrest (Hoff et al., 2015).

5.3 INTESTINAL MICROBIOTA

PlanHab experimental setup was tuned to control the effects of factors, that have been identified to contribute to changes in microbial community structure: host lifestyle and diet.
The diet supplied to participants in this study was balanced and synchronized to provide feeding habit continuation and resembled the habitual menu composition of participants (Debevec et al., 2014), contrary to an abrupt change from plant-based to animal-based diet and back, leading to observed rapid and reproducible changes in human gut microbiome (David et al., 2014b). Despite that, exercise clearly led to modified food preference in HAmb (Figure 26). The amounts of ingested iron, proteins, fat and Ca\(^{2+}\) were 9 %, 10 %, 6 % and 7.5 % significantly higher in HAmb in comparison to HBR and NBR (Debevec et al., 2014), an observation mirroring the diet composition of professional athletes reported recently in a study employing end-point groups without controlled diet (Clarke et al., 2014). Hypoxia alone did not induce significant differences in feeding behavior between NBR and HBR (Sket et al., 2017b). In order to incorporate the difference in food preference between different campaigns diet intake data was used as co-variable matrix in explaining the variability of measured parameters (Sket et al., 2017a; 2017b). Differences in cyclic circadian oscillations, known to induce transkingdom control of microbiota diurnal oscillations that promote metabolic homeostasis (Thaiss et al., 2014), were minimized through the adoption of 16/8 daylight regime in this study (Debevec et al., 2014). In addition, the timing of food delivery was kept constant in order to minimize variation in flow-rate of material through the gut that was previously shown to influence microbial and host metabolic activities (Debevec et al., 2014; Gilbert and Alverdy, 2016). The prescreening of participants according to NASA/ESA SOP yielded healthy microbiomes (Figure 23A) that corresponded to those described as healthy in HMP (The Human Microbiome Project Consortium, 2012). Consequently, the PlanHab study enabled us to monitor the effects of inactivity and hypoxia over time on many vital subsystems of human body, including intestinal microbiome.

The significant changes in a number of intestinal parameters that were observed during the second week through increased constipation (lower defecation frequency) (Figure 13, Figure 24) and modified intestinal parameters e.g. IEC (Figure 25)) or in third week e.g. indole (Figure 25), EDN, BA (Figure 14)) and autonomous shift in diet (Figure 26) over the course of PlanHab experiment (Debevec et al., 2014), corroborate dose dependent response of human physiology to cessation of exercise (Sket et al., 2017a).

Despite the increased stool consistency and prolonged retention times observed in NBR and HBR variants, no significant difference in butyrate concentration or alpha-diversity of butyrate producing communities (but and buk) was observed between experimental variants (Sket et al., 2017a). The lack of directed changes in butyrate producing communities suggests that the microbial intestinal system exhibited possibly an evolutionary resilience toward short-term modifications due to inactivity or hypoxia in this study. However, the same microbial components might be responding to tissue hypoxia, inactivity and additional systemic hypoxia derived inflammation responses by adjusting their metabolic activities, supporting the development of host pathologies on the long run (i.e., > 21 days; (Faber and}
Bäumler, 2014). On the other hand, bacterial members belonging to Firmicutes identified as the major butyrate-producing group (Sket et al., 2017a; Vital et al., 2014) left a possibility that major changes in intestinal microbiome occurs in other bacterial taxa.

To address total microbial diversity one can use 16S rRNA sequencing as it was done in second part of our study (Sket et al., 2017b). Multiple 16S rRNA hypervariable regions used in our study showed similar bacterial taxonomic distribution, as shown before where in particular V2 and V6 regions showed considerable sequence variability and were able to distinguish among most bacterial species (Chakravorty et al., 2007). The same was confirmed with more detailed analysis using WGS in third part of our study (Sket et al., 2018).

Exploring microbial distribution, calculated bacterial alpha diversity indices and beta diversity comparisons did not indicate significant changes between experimental groups. Lack of directed changes in taxonomic composition of intestinal microbiome, defined with 16S rRNA sequencing and confirmed with WGS over the course of the PlanHab experiment, points to a lag in response of microbial communities or possibly to an evolutionary resilience in microbial intestinal system (Shade et al., 2012) towards short-term inactivity (Sket et al., 2017a, 2017b) despite accompanying significant changes in intestinal environmental parameters. This was further confirmed based on detected changes in microbiota composition that occur in the fourth week of PlanHab experiment exploring predicted genus on 16S rRNA sequencing data (Sket et al., 2017b) and WGS (Sket et al., 2018).

A major factor shaping the balance between different human bacterial lineages is their ability to compete efficiently for complex nutrients delivered to the intestinal system and adapt to different external stimuli. The assembled data in our study (Sket et al., 2017b, 2018) and supported by others (Clarke et al., 2014; Johnson et al., 2016; Wexler, 2007) identified genus Bacteroides as a major bacterial group capable of such rapid adaptations, where the same microbial taxa responded to modified environmental conditions provided by the host by flexibly adjusting their metabolic activities through different gene expression, leading to significant increase in their relative abundance and internal diversity. HAmb exhibited a healthy human physiology phenotype (Debevec et al., 2014; Simpson et al., 2016; Sket et al., 2017a), despite the fact that most of the Bacteroides species observed in HBR were also present in NBR and HAmb, although at lower levels (Figure 22). In addition, HAmb, NBR and HBR groups of PlanHab study contained at least 28.8 %, 35.8 % and 21.2 % lower fraction of Bacteroidetes, respectively, than the athletes, low BMI and high BMI controls, reported before, respectively (Clarke et al., 2014). This clearly shows that related species and microbes can have multiple, very different or even opposing effects on host metabolism and health (Johnson et al., 2016). However, inferring metabolic roles by taxonomic classification alone by making the association of specific functional roles to entire taxonomic groups is difficult, because phylogenetically closely related organisms might be very different in their metabolism (Bauer et al., 2015), and hence could produce rather distinct metabolic fingerprints (Beaumont et al., 2017). Although the host inactivity resulted in
significant modification of some of the environmental parameters of intestinal tract, this has apparently primarily directed microbial physiology towards degradation of host mucin polymers and concomitant upregulation of inflammagenic capsid proteins at the same time (Martens et al., 2009). The increased abundance of mucin degradation and capsule-related genes in HBR metagenomes (Sket et al., 2018) supports this observation. The increased BA and IEC levels in feces point towards host attempts to counteract microbial activities, overgrowth and production of metabolites. Although Bacteroides are tolerant to BA, their increased abundance in HBR was associated with the abnormal host glucose metabolism and insulin resistance before (Karlsson et al., 2013; Qin et al., 2012; Zeevi et al., 2015). In parallel, the same positive correlation between insulin resistance and BA as observed in HBR, was also present in NBR devoid of significant community change.

As the increase in IEC decreases availability of iron in intestinal system by default, this forces Bacteroides to activate hemolysin production in order to perforate host cells to provide haem inflow (Robertson et al., 2006). Second, capsule genes code for proteins that bind major clotting proteins from human plasma and prolong the intrinsic coagulation time or prevent clot formation (Murphy et al., 2011), whereas accompanying polysaccharide A activates immune system leading to increased blood inflow (Elhenawy et al., 2014). Capsule and mucin degradation genes are coregulated, and the latter have the potential to decrease mucin thickness, crosslinking, structure, porosity, influencing the diffusion gradients and immune contacts (Martens et al., 2009), especially under constipated conditions, observed in HBR and NBR. Third, glycosidases, proteases, polysaccharide A and polysaccharide B, outer membrane proteins, phospholipids, sphingolipids, exotoxins are preferentially packed into outer membrane vesicles (OMV) released by many Bacteroides, targeting complex polysaccharides from diet or human origin, such as mucus glycans, allowing Bacteroides to modulate host pathways. Lastly, inactivity and hypoxia lead to suboptimal mitochondrial function and release of reactive oxygen species that effectively damage microbial cells, releasing LPS (lipid A). These are taken up by host cells under high BA and IEC conditions through leaky tight junctions, microbial perforations, chylomicrons responsible for dietary lipid uptake, or even OMV (Elhenawy et al., 2014), resulting in the initialization of inflammation-based processes linked with insulin resistance (Boulangé et al., 2016; Rowland et al., 2018), a condition observed in HBR and NBR participants. Consequently, the expression of inflammatory molecules is triggered by increased systemic levels of endotoxins, including nuclear factor kB (NF-kB) (Simpson et al., 2016; Sket et al., 2017a, 2017b). In humans, the circulating endotoxin levels increase by 20 % in individuals with glucose intolerance in comparison to healthy individuals (Boulangé et al., 2016; Cani et al., 2007; Li et al., 2011). This is in line with the observation that increased BA and IEC in HBR and NBR variants gave rise to increased intestinal translocation into plasma, causing the observed negative inflammatory dose dependent physiological symptoms in HBR and NBR (Debevec et al., 2014; Simpson et al., 2016).
Exploring archaea composition using 16S rRNA sequencings did not provide high numbers of archaeal sequences, indicating high ratio between bacteria and archaea in these systems which was also confirmed with WGS sequencing in third part of or study (Sket et al., 2018). Results obtaining with WGS identified no significant differences in the taxonomic distribution at various levels of archaea. These results are in-line with past observations of the significant difference in the microbial community of archaea between the obese and healthy control groups (Bojanova and Bordenstein, 2016; Pimentel et al., 2012). However, archaeal methane production in humans has been epidemiologically and clinically associated with constipation related diseases (Triantafyllou et al., 2014), a trait observed in NBR and HBR variants of PlanHab but not HAamb (Sket et al., 2017a). Methane producers also had greater serum glucose content than non-methane subjects suggesting impaired glucose tolerance and higher susceptibility to hyperglycemia when challenged (Mathur et al., 2014), the latter trait also observed in NBR and HBR (Simpson et al., 2016). Methane decreased peristaltic velocity and increased ileum contraction amplitude significantly in laboratory animals (Jahng et al., 2012), supporting the observed eosinophil-derived neurotoxin (EDN) increase and micro injuries at the site of small intestine in NBR and HBR variants (Sket et al., 2017a). Slow transit time would thus assist methanogens in the gut due to their slow growing nature to increase their metabolic activity and adjust their community structure in the long run, resulting in the reported differences between obese and healthy volunteers (Pimentel et al., 2012). The observed relationship between the methane production and the pathogenesis of constipation as well as obesity related syndromes, points to the fact that despite no significant change in archaeal microbial community was observed in this study, their modified metabolic activity could well be one of the missing parts of the negative manifestations observed in NBR and HBR.

Fungi, yet with no significant differences in their taxonomic distribution in our study (Sket et al., 2018), received least attention so far in other studies, although many of the species have the potential to conferment various substrates, metabolism of the fungal cell wall in the gut might influence the growth of E. coli and other bacteria, while the production of extracellular substances inhibits the growth or yeast-to-hyphae transition of pathobionts, such as C. albicans (Bojanova and Bordenstein, 2016; Sam et al., 2017). Microbiome and commensal fungi interaction was suggested to be a balancing act; when the microbiome is disrupted, the normally commensal elements in the mycobiome might be unchecked and turn pathogenic in the long run, further aggravating the observed negative physiological spiral observed in the PlanHab study (Simpson et al., 2016). The observed significant differences in archaea and fungal communities (Mar Rodriguez et al., 2015) in obese and healthy individuals therefore appear to take place at later phases of progressed dysbiosis in rather dose dependent manner on the extent of time spent in inactivity.
5.4 REVERSIBILITY OF PHYSIOLOGICAL SYMPTOMS AS A PART OF INBUILT MAMMALIAN PHYSIOLOGY

Evolutionary adaptations, such as inactivity of mammals during winter hibernation bouts, have the capacity to induce seasonal cyclic changes in insulin resistance, lipid metabolism, increased constipation, triglyceride and glucose concentration, total cholesterol, increased red blood cells, hemoglobin and hematocrit and increased neutrophils, lymphocytes and monocytes, C-reactive protein, elevated levels of genus Bacteroides and other (Carey et al., 2013; Sommer et al., 2016). These characteristics corresponded surprisingly well to inactive phenotype observed in 21-day PlanHab experiment in HBR and NBR variants. In contrast, other negative physiological characteristics of NBR and HBR matched those observed during seasonal overeating (hyperphagia) in the same mammalian species (e.g. increased bile acid content, adiposity). This indicates that physical inactivity and food availability acted as two separate overarching signals governing gene regulation and expression of the mammalian host (Villanueva-Cañas et al., 2014). Adiposity in humans, however, has been shown to be associated with reduced insulin sensitivity and accompanied by constant daily overeating and inactive sedentary lifestyle with limited or no exercise as observed in captive primates (Clayton et al., 2016; Moeller et al., 2014). The fact that mammalian hibernators returned to normal physiological status (including insulin sensitivity) during the physically active part of seasonal cycle despite overeating (Carey et al., 2003, 2013; Sommer et al., 2016), is complementary to the alleviation of inactivity-generated negative physiological and psychological symptoms observed in NBR and HBR over 5 and 14 days, respectively, during the PlanHab wash-out period (Debevec et al., 2014; Simpson et al., 2016; Sket et al., 2017a). This period was characterized by increased (voluntary) exercise, (re)establishment of hydrostatic gradients within body (Debevec et al., 2014; Strewe et al., 2017), posture maintenance, leading to reintroduction of abdominal splanchnic circulation (Uva et al., 2016), significantly increased physiologic demands of the host and alleviated constipation (Debevec et al., 2014), indicating that signals from exercise and muscle turnover reached intestinal tract as suggested before for athletes (Barton et al., 2018), animal models (Campos-Rodriguez et al., 2016; Matsumoto et al., 2008; Nichol et al., 2008; Rodriguez-Castaño et al., 2017) and potentially primed functional capacity of healthy microbiome (this study (IEC, indole)). Short-term inactivity thus, appears to be completely reversible as part of inbuilt mammalian physiology (Debevec et al., 2014; Strewe et al., 2017). However, interactive and dose dependent responses to extensive physical inactivity and overeating, previously not encountered in the human evolution, lead to progressively significant system deconditioning, physiologic, immune and psychologic comorbidities observed in primates in captivity and humans indulging in Western lifestyle over prolonged periods (Figure 5) (Clayton et al., 2016; Moeller et al., 2014).
5.5 HOST AND MICROBIOTA DIALOGUE

Microbial production of SCFA as explored also in our study (Sket et al., 2017a) and their metabolic properties in regulating inflammatory response, glucose-tolerance and energy balance in other studies, remained most explored linkage between activity of gut microbes and modification of host cell metabolism (Maslowski et al., 2009; Serino, 2016). More examples of human-microbe linkage conditioned by the diet of the host are: (i) degradation of various plant polyphenols, which arrive almost intact in to the colon and are subjected to microbial fermentation that resulted in absorbable metabolites with potential effects on host health, (ii) and metabolism of sterols by intestinal microbiota to their secondary derivatives with potent biological effects (Barton et al., 2018; Cronin et al., 2016; van Dijk et al., 2012; Egan and Zierath, 2013; Pham et al., 2012; Saura-Calixto et al., 2010). Considering the diet influence, no observed significant effects in above listed “communications” between host and microbiota in our study (Sket et al., 2017b), could be result of individually tailored, standardized and controlled diet that was used throughout all three different interventions.

On the other hand, more direct mechanism (i.e. less dependent on food intake) providing missing link in explaining bacteria-to-cell interaction in intestinal tract and in other by microbiota dysbiosis targeted metabolic tissues (e.g. liver, adipose tissue, pancreas, skeletal muscle), could be micro RNAs (miRNA) molecules. miRNA are often differentially expressed in the presence of bacteria and can even be released and taken up by them. (Serino, 2016; Walters et al., 2014; Williams et al., 2017). miRNA mediated post-transcriptional gene silencing via translation repression or degradation of messenger RNAs (mRNAs) affects metabolism either in host eukaryotic cells, or within extracellular vesicles mediates inter-species gene regulation between host and microbe and as such facilitates host control of the intestinal microbiota and vice versa microbiota controlled miRNA expression in intestine (Liu et al., 2016; Serino, 2016). miRNA-mediated gene regulations are involved in reducing the colonic inflammation in response to intestinal microbiota via lowering the uptake of inflammatory stimulating bacterial peptides, as well in amelioration the disruptions of the tight junctions, induced by pathogenic microbiota (Dai et al., 2015; Veltman et al., 2012).

On the other hand, lack of mature fecal miRNA during intestinal colitis resulted in dysbiosis with specific increase of Bacteroidaceae family (Liu et al., 2016). The fact that members of the genus Bacteroides were identified as the first responders, and that tight junctions and mucin layer were affected in our study in most detrimental variant (HBR) (Sket et al., 2017a, 2017b, 2018) suggest that by invoking miRNA capabilities could allow explaining how dysbiosis is converted into metabolic outcomes. Adding the miRNA next to all other known messenger molecules and studying their complex interplay leading to human physiologic disorders or wellbeing should definitely be pursued as a perspective for future direction of our study, where the presence and dynamic of miRNA in fecal samples of PlanHab participants should be investigated. This observation also suggests that novel mechanisms await to be discovered and that miRNAs as mechanism of intergenomic/interkingdom communication are far from being the last “new kid on the block”.

71
5.6 SYNTHESIS AND STUDY SIGNIFICANCE

Null hypotheses (H0) in our study stated that among the groups of subjects there were no differences. Numerous characteristics of intestinal environment such as zonulin, A1AT, SCFA, sterols, polyphenols, DOM spectral derivatives, total metabolites, metal content or general descriptions of intestinal microbiota targeting butyrate synthesis pathways, 16S rRNA bacterial and archael genes or total microbial genepool were: (i) either correlated or also covaried over the course of experiment, indicating a substantial and potentially hierarchical codependence between parameters within intestinal environment, (ii) or did not change significantly, indicating that the extent of experimental disturbance (inactivity, time, hypoxia) and their interactive effects were not sufficient to induce significant changes (Sket et al., 2017a, 2017b, 2018). The successful evolutionary shaping of the mammals and humans as species support this observation.

On the other hand, significant adaptations in human physiology and transition from healthy physiological state towards the developed symptoms of low magnitude obesity-related syndromes as observed before (Debevec et al., 2016; Simpson et al., 2016; Strewe et al., 2017), together with identified parameters in our study (Sket et al., 2017a, 2017b, 2018) such as BSS, IEC, indole, BA and changes in microbiota composition and function at the end of most detrimental variant HBR, indicate the response to inactivity and hypoxia that were further elaborated in the context of our four alternative hypotheses (H1.1-4):

First (H1.1), parameters listed above and their identification as significant over the course of the experiment, confirmed that their variability between subjects within the same experimental group was smaller than the difference due to the influence of either bedrest or hypoxia or combined.

Second (H1.2 and H1.3), the predicted effects of inactivity and hypoxia, due to crossover design of the PlanHab experiment without normoxic ambulatory group as a control, are explained mutually. Lack of exercise reached and affected the gut on a short time scale of 21 days (NBR), its effects were additionally aggravated by systemic hypoxia (HBR), but significantly alleviated by exercise, despite hypoxia (HAmb). This relationship showed the influence of both inactivity and hypoxia and identified inactivity as a factor having greater impact than hypoxia.

Last (H1.4), responses to the four levels (human physiology, metabolites, immune status and microbiota) are in fact interconnected as shown in Bayesian network analysis (Figure 32) and far more complex than initially suggested. Metabolic (BA), proteomic (EDN), ionic (EC), systemic (BSS) adaptations observed before (Colgan, 2013; Mach and Fuster-Botella, 2016; Sket et al., 2017a, 2017b) take place in parallel with physiological symptoms (Debevec et al., 2016; Simpson et al., 2016; Strewe et al., 2017), but precede the dysbiosis with the time window of 2-3 weeks. We hypothesize that observed microbiome persistence has evolved to act as a “buffer” contributing to the stability of metabolic homeostasis over
prolonged periods of time, by preventing overly fluctuating metabolic responses to incidental nutritional or environmental signals (Beaumont et al., 2017; Thaiss et al., 2016).

As such, the present study design provides a unique insight, and moreover extends our understanding of the relevant medical and physiological adaptations of the quadripartite relationship between human physiology, immunological state of intestinal tract (hence broad immune status), intestinal metabolites and intestinal microbiota that occur as a response to decrease of physical activity especially in people who are inactive and/or bedridden as a consequence of long-term clinical condition (chronic hypoxia due to respiratory insufficiency, congestive heart failure, obesity). In addition, the tiers within the Bayes network corresponded to the weeks within metabolic, proteomic, ionic and systemic adaptations within the experiment and provided for the first time a model describing the initial steps within the human microbiome and its host system in response to the onset of acute inactivity: (i) decision to decrease the level of physical activity gave rise to a cascade-like modifications in human physiology over time; (ii) changes in host physiology preceded changes in human microbiome structure; (iii) changes in host physiology and intestinal parameters guided human microbiome physiology and metabolic activity (i.e. the same microbes started to behave differently); (iv) prolonged changes in host physiology supported the expression of distinct microbiome metabolic activities and resulted in modified microbiome structure next to aggravated physiological status of the host via the produced metabolites and proteomes and RNA-omes; and (v), the introduction of a lifestyle change in the form of the exercise during the wash-out period at the end of 21-day experiment effectively resulted in alleviation of the developed negative symptoms in human physiology, also in a dose-dependent manner, (vi) finally, the acquired knowledge and understanding in our study contributed significantly to FP7 PlanHab project’s scientific objectives to better understand human body adaptations to the life in space planetary habitats in the future. The life sciences contribution of the present work stems from the understanding the initial aspects of medically relevant conditions, such as non-communicable diseases on Earth, by deriving the first hierarchical model of initial inactivity mediated deconditioning steps over time. The generalization of the model with other mammalian data suggests that the yo-yo dieting and active/inactive lifestyle, along with its beneficial/ill effects, is not a human peculiarity but rather a common evolutionary adaptation of mammals to survive food shortage and seasons rotation.
6 CONCLUSIONS

- The transition from healthy physiological state towards the developed symptoms of low magnitude obesity-related syndromes was dose dependent on the extent of time spent in inactivity.
- Increasing progressive decrease in defecation frequency (constipation), gut inflammation (increase in IEC, EDN) appear shortly after the onset of physical inactivity (lack of physical exercise in NBR) that were potentially exacerbated by systemic hypoxia (during HBR) and significantly alleviated by exercise, despite hypoxia (HAmb), characterizing HBR variant with the most severe symptoms.
- Structure and abundance of butyrate producing microbial communities or taxonomic and functional rearrangements in total bacterial, archaeal and fungal microbial communities and their metabolomes in the colon did not change significantly, suggesting that the intestinal system apparently exhibited a resilience toward short-term modifications in host exercise levels or systemic hypoxia.
- Significant changes in bacterial community structure were delayed until week four in HBR only where the first significantly enriched taxa were inflammagenic and mucin degrading members of the genus Bacteroides.
- The fact that the genus Bacteroides and proteins involved in iron acquisition and metabolism, cell wall, capsule, virulence and mucin degradation were enriched, indicating a shift towards host mucin degradation and harmful immune crosstalk and elucidates for the first time the initial mechanism of step-by-step development of the observed negative physiological symptoms of the host.
- The onset of the wash-out period in the PlanHab experiment corresponded to a profound life-style change that resulted in immediate amelioration of the negative physiological symptoms, indicating that exercise acted as an important parameter apparently downplaying microbial physiology and metabolic activities involved in host mucin degradation and pro-inflammatory immune crosstalk.
- In addition, the onset of wash-out period is rather symmetric to the end of hibernation bout as largely the same negative symptoms in other studies were alleviated after reintroduction of exercise irrespective of mammalian species, suggesting that the yo-yo dieting and active/inactive lifestyle, along with its effects, is not a human peculiarity but rather a common evolutionary adaptation of mammals to survive food shortage and seasons rotation.
- Features of microRNA molecules are providing missing link in explaining bacteria-to-cell interaction in intestinal tract and should be considered as a perspective for future direction of our study.
7 SUMMARY (POVZETEK)

7.1 SUMMARY

Most studies thus far focused on the beneficial effects of re-introduction of exercise to obese population to alleviate these negative effects on one side and to understand the effects of athletic on the other. However, there is an obvious lack of data on the initial changes in human microbiome and physiology during acute cessation of exercise. To improve our understanding regarding the pathophysiological consequences of acute and prolonged inactivity and hypoxia, this study took advantage of the PlanHab project experimental setup.

We explored the assembly of intestinal microbiota in healthy male participants during the run-in (5 day) and experimental phases (21-day normoxic bed rest (NBR), hypoxic bedrest (HBR) and hypoxic ambulation (HAmb) in a strictly controlled laboratory environment, balanced fluid and dietary intakes, controlled circadian rhythm, microbial ambiental burden and 24/7 medical surveillance. The fraction of inspired $O_2$ ($F_iO_2$) and partial pressure of inspired $O_2$ ($P_iO_2$) were 0.209 and 133.1 ± 0.3 mmHg for NBR and 0.141 ± 0.004 and 90.0 ± 0.4 mmHg for both hypoxic variants (HBR and HAmb; ~4000 m simulated altitude).

Our work was divided into three parts, corresponding to three separate publications (Sket et al., 2017a, 2017b, 2018). First the abundance, structure and diversity of butyrate producing microbial community using the two primary bacterial butyrate synthesis pathways, butyryl-CoA: acetate CoA-transferase ($but$) and butyrate kinase ($buk$) genes and number of parameters linked to intestinal transit spanning Bristol Stool Scale, defecation rates, zonulin, $\alpha_1$-antitrypsin, eosinophil derived neurotoxin, bile acids, reducing sugars, short chain fatty acids, total soluble organic carbon, water content, diet composition and food intake were assessed (Sket et al., 2017a). Then structure and diversity of bacterial microbial community using 16S rRNA amplicon sequencing, along with measurements of intestinal electrical conductivity, sterol and polyphenol content and diversity, indole, aromaticity and spectral characteristics of dissolved organic matter were investigated (Sket et al., 2017a, 2017b). And lastly, the metagenomic analyses at various taxonomic and functional levels using shot-gun metagenome sequencing, nuclear magnetic resonance of metabolomes and X-ray fluorescence spectrometry of metals were performed (Sket et al., 2018). New in-house PlanHab database was established to integrate all measured variables on host physiology, diet, experiment, immune and metabolic markers.

A number of negative physiological symptoms related to obesity and metabolic syndrome have been observed to a large extent in the PlanHab study in a dose dependent manner over the course of 21-day experimental period in HBR and NBR, but were absent from HAmb variant.

The observed progressive decrease in defecation frequency and concomitant increase in intestinal electrical conductivity suggested that the transition from healthy physiological state towards the developed symptoms of low magnitude obesity-related syndromes was
dose dependent and primarily driven by the onset of inactivity (lack of exercise in NBR) that were exacerbated by systemic hypoxia (HBR) and significantly alleviated by exercise, despite hypoxia (HAmb).

Changes in human physiology and intestinal environment preceded or took place in absence of significant rearrangements in microbial parameters such as butyrate producing microbial community and the general bacterial and archaeal microbial community. Significant changes in bacterial community were delayed until week four in HBR only, where members of the genus *Bacteroides* and proteins involved in iron acquisition and metabolism, cell wall, capsule, virulence and mucin degradation were enriched.

Supporting that hypotheses, Bayesian network analysis linked relevant parameters in metabolic (bile acids), proteomic (eosinophil-derived neurotoxin), ionic (intestinal electrical conductivity) and systemic (constipation) adaptations with preceding the dysbiosis with the time window of 2-3 weeks and derived the first hierarchical model of initial inactivity mediated deconditioning steps over time.

On the other hand the PlanHab wash-out period corresponded to a profound life-style change (i.e. reintroduction of exercise) and resulted in stepwise amelioration of the negative physiological symptoms, indicating that exercise apparently prevented the crosstalk between the microbial physiology, mucin degradation and pro-inflammatory immune activities in the host. Using the observed patterns of deterioration and its amelioration after reintroduction of exercise in our study, and their comparison to matching multiscale patterns reported to take place in other mammalian species within comparable timeframes in nature, it seems that the yo-yo dieting and active/inactive lifestyle, along with its effects, is not a human peculiarity but rather a common evolutionary adaptation of mammals born out of necessity to survive food shortage and change of seasons.

Finally, features of microRNA molecules showing possibilities to provide missing link in explaining bacteria-to-cell interaction in intestinal tract and should be considered as a perspective for future direction of our study.
7.2 POVZETEK

Hiter tempo življenja, stres, nepravilna prehrana ter premalo gibanja predstavljajo del vsakdana razvitega zahodnega sveta, ki ima lahko za posledico pojav srčno-žilnih bolezni, s pljučnimi boleznimi povezane respiratorne insufficence, debelostnega sindroma in ostalih motenj. Zgoraj omenjene alternacije človekovega zdravja zmanjšujejo kvaliteto življenja posameznika in so kot takšne deležne veliko pozornosti. Kljub temu, pa ni celovitega odgovora na vprašanje, kaj se med tem dogaja v človeškem prebavnem traktu in kakšna je vloga črevesne mikrobiote pri razvoju teh alternacij. Vzroki so v kompleksnosti mikrobiote, ki je prisotna v človeškem prebavnem traktu in hkrati veliki variabilnosti v njeni sestavi med različnimi posamezniki. Ko k temu prištejemo še veliko število notranjih in zunanjih dejavnikov, ki prav tako vplivajo na sestavo črevesne mikrobiote, v večini primerov do sedaj dobimo nejasen odgovor na zgoraj zastavljeno vprašanje.


Za lažjo opredelitev vloge mikroorganizmov pri določenem bolezenskem stanju je potrebno najprej opredeliti sestavo mikrobiote v črevesju zdravega človeka, kot je cilj raziskav v okviru projekta človeškega mikrobioma (angl. Human Microbiome Project) in MetaHIT (angl. Metagenomics of the Human Intestinal Tract) (Belkaid in Hand, 2014; Lloyd-Price in

Pomanjkanje gibanja ali dalje mirovanje povzroči spremembe v fiziologiji človeškega telesa, kot so znaki atrofije skeletnih mišic (Agostini in sod., 2010; Biolo in sod., 2008; Iovino in sod., 2013; Pišot in sod., 2008), demineralizacija kosti (Berg in sod., 2007; Frings-Meuthen in sod., 2013; Rittweger in sod., 2009), popuščanje srca (Levine 1997), znižanje krvnega tlaka (Eiken in sod., 2008), sistemske vnetje, inzulinsko neravnovesje (Hamburg in sod., 2007; Mazzucco in sod., 2010), zaprtje (Iovino in sod., 2013). Kljub jasnemu vplivu mirovanja na humano fiziologijo, je odnos med črevesno mikrobioto in gibanjem ali pa pomanjkanjem gibanja še zmeraj nejasen (Clarke in sod., 2014). Za preučevanje dinamike spremnjanja črevesne mikrobiote v primeru mirovanja je potrebno naprej izvesti poskus, kjer zdravi preiskovanci določen čas mirujejo (angl. bed rest study). Nadalje je potrebno, da bi lahko vpliv mirovanja povezali z dinamiko spremnjanja mikrobiote, čim bolj zmanjšati vpliv zunanjih dejavnikov. Pri tem je pomemben dejavnik homogena sestava populacije preiskovancev, uravnotežena in enaka prehrana, enako splošno okolje, medicinska oskrba ter s tem zmanjšanje tveganje za bakterijske, virusne ali kakšne druge okužbe, ki bi povečale disbiozo oz. drastične spremembe v sestavi črevesne mikrobiote. Pokaže se, da je takšno študijo možno izvesti v nadzorovanem okolju ob stalnem nadzoru za to usposobljenih oseb in seveda z dovoljenjem etične komisije.

V našem delu smo tako uporabili vzorce pridobljene tekom eksperimentov na zdravih prostovoljcih PlanHab projekta (Planetary Habitat Simulation project EU FP7-space; št. projekta 284438; http://cordis.europa.eu/project/rcn/104127_en.html; PI: Igor Mekjavič, IJS: Inštitut Jožef Štefan, Ljubljana) (Debevec in sod., 2014), kjer smo raziskovali ločene in skupne učinke fizične neaktivnosti, zmanjšanja gravitacije zaradi horizontalne imobilizacije (hidrostatski tlak) in zmanjšanja parcialnega kisika (hipoksijski) na človekove fiziološke sisteme pri zdravih ljudih, ki so pod medicinskim nadzorom mirovali v kontroliranih pogojih 21 dni. Našteti dejavniki vplivajo na stanje prehranjenosti, uravnnavanje telesne sestave in
splošno zdravstveno stanje (Debevec in sod., 2014). Našteto sproži odzive gostitelja na različnih nivojih, kar vodi do sprememb v gastrointestinalnem mikrookolju in posledično vpliva na črevesno mikrobioto (Rhee in sod., 2009). Prav zaradi kontroliranega okolja (medicinski pred-test preiskovancev, identično standardizirano okolje, tipizirana prehrana, spremljanje fizioloških odzivov, medicinski nadzor 24/7), je PlanHab poskus ponujal edinstveno priložnost za preučevanje dinamike človeške črevesne mikrobiote in asociranih parametrov med daljšim mirovanjem.

Namen naše raziskave je bil ugotoviti odziv (i) strukture črevesne mikrobiote, (ii) črevesnih metabolitov in (iii) imunskega statusa pri preiskovancih v eksperimentu mirovanja v horizontalnem položaju ter hipoksi (n=28 dni; »pred« (-7 dni); »po« (+21 dni)), (iv) reformatirati in uskladiti podatke o spremembah humane fiziologije iz podatkovne baze partnerjev PlanHab (IJS) v ustrezno obliko; ter (v) raziskati štiristranski odnos med odzivi gostitelja (humana fiziologija), imunskim statusom, metaboliti ter črevesno mikrobioto tekom eksperimentov na zdravih preiskovancih. Naše zastavljene hipoteze so bile:

- Ho: Med obravnavanimi množicami spremenljivk ni razlik.
- H1.1: Razlike v metabolitih, imunskem statusu ter sestavi mikrobiote zaradi medosebne variabilnosti med preiskovanci znotraj iste eksperimentalne skupine bodo manjše od razlik v sestavi mikrobiote zaradi vpliva bodisi mirovanja bodisi hipoksije bodisi obojega.
- H1.2: Mirovanje vodi do signifikantnih sprememb v metabolitih, imunskem statusu ter sestavi mikrobiote.
- H1.3: Hipoksi vodi do karakterističnih sprememb v metabolitih, imunskem statusu ter sestavi mikrobiote.
- H1.4: Odzivi na štirih nivojih (humana fiziologija, metaboliti, imunski status, mikrobiota) so medsebojno povezani. Spremembe v humani fiziologiji so večje od sprememb v statusu mikrobiote, metabolitov in imunskem statusu.

Ekperimentalni del PlanHab poskusa, ki je vključeval preiskovance, je potekal v Olimpjskem športnem centru v Planici v Sloveniji med junijem 2012 in januarjem 2014. Študijo je odobril Nacionalni odbor za medicinsko etiko, Ministrstva za zdravje Republike Slovenije. Odobreni so bili vsi eksperimentalni postopki, ki so potekali v skladu s priporočili in protokoli Evropske vesoljske agencije (ESA) za izvedbe imobilizacijskih študij (Standardization of bed rest study conditions 1.5, August 2009), in so skladni s smernicami Helsinške deklaracije. Sodelujoči v študiji so podpisali soglasje. Vse v raziskavi zbrane podatke smo obravnavali v skladu s pravili kot zaupno medicinsko dokumentacijo. Rezultati v obliki za objavo ne izdajajo identitete preiskovancev. Dovoljenje etične komisije hrani Inštitut Jožef Štefan (IJS).

V naš del študije je bilo izmed vseh preiskovancev PlanHab projekta, vključenih 9 preiskovancev moškega spola, ki so bili izbrani iz širše populacije zdravih ljudi (pri katerih je bil opravljen celovit klinični pregled) (Preglednica SI). Vključitev v študijo je potekala
glede na priporočila ESA. Izključeni so bili vsi, pri katerih so v obdobju dveh mesecev pred začetkom študije odkrili akutne ali kronične bolezni srca, gastrointestinalne bolezni ali kakršnakoli imunološka in druga zavedena stanja. Celoten poskus so sestavljale tri kampanje, ki so trajale po 21 dni. V vsako kampanjo so bili vključeni po tri preiskovanci. Pred začetkom kampanj so bili preiskovanci teden dni v nadzorovanem okolju. Med sabo so se kampanje razlikovale po tem: (i) ali so preiskovanci med 21 dni trajajočim poskusom mirovali v ležečem položaju ali so se lahko prosto gibali v nadzorovanem okolju (ii) ali je bilo okolje normoksično in je ustrezo deležu parcialnega kisika na nadmorski višini 400 m ali je bilo okolje hipoksično in je ustrezo deležu parcialnega kisika na nadmorski višini ~4,000 m. V prvi kampanji so preiskovanci ležali v normoksičnem okolju (NBR; angl. normoxic bed rest). V drugi kampanji so se preiskovanci med potekom študije prosto gibali in zmerno telovadili 2 x na dan po pol ure pod hipoksičnimi pogoji (HAMB; angl. hypoxic ambulatory confinement). V tretji kampanji so preiskovanci ležali pod hipoksičnimi pogoji (HBR; angl. hypoxic bed rest) (Slika 8, Slika 9). Med poskusom so bili preiskovanci deležni individualno prilagojene, standardizirane in nadzorovane prehrane. Med trajanjem poskusov so bili odvzeti vzorci fecesa preiskovancev. Zaradi posledic imobilizacije (konstipacija oz. zaprtje) so bili v analizah uporabljeni vzorci v dneh -5 ter -1 pred začetkom poskusov in 3, 10, 18 ter 21 dan poskusa. Vsega skupaj 54 vzorcev je bilo razdeljenih v tri paralelke po 10 g v sterilne kontejnerje in zamrznjenih pri -25 ° C (Slika 8).

Yekutieli, 2001). Statistična analiza rezultatov sekvencioniranja genov za masleno kislino je vključevala (i) izračun kazalcev raznolikosti znotraj posameznih vzorcev oz. skupin vzorcev (angl. alfa diversity), ki smo jih analizirali s programskim orodjem mothur (Schloss in sod., 2009) in (ii) na podlagi prisotnosti, odsotnosti ter številčnosti operativnih taksonomskih enot (OTU) v vsakem vzorcu izračun matrike razdalj z uporabo podobnosti Bray-Curtis ter grafični prikaz vzorcev na dvodimenzionalnem grafu z nesimetričnim večdimenzionalnim lestvičenjem (NM-MDS) (Oksanen J., 2014). Nadalje smo izvedli analizo virov variabilnosti ter opredelili, kateri parametri značilno prispevajo k razlikam v sestavi mikrobiote, ki proizvaja masleno kislino (Stres in sod., 2013). Skupno 167 parametrov (Preglednica S2) smo razdelili v skupine: (i) metabolitov in imunskih označevalcev (n = 45), (ii) parametre poskusa (n = 12) in (iii) prehrano (n = 110) (Legendre in Legendre, 2012) (Slika 10).

V sklopu druge objave (Sket in sod., 2017b) smo preiskovali raznolikost črevesne mikrobiote na taksonomskem nivoju s sekvenciranjem arhejskih in bakterijskih genov za ribosomalno RNA (16S rRNA) (Slika 11). Sekvencirali smo visoko variabilni regiji V1-V2 bakterijske 16S rRNA (Klindworth in sod., 2013) ter visoko variabilni regiji V6-V7 16S rRNA bakterij in arhej (Klindworth in sod., 2013; Takahashi in sod., 2014). Za analizo pridobljenih sekvenc smo uporabili programsko orodje MOTHUR (Schloss in sod., 2009) ter razvili postopke analize, analoge standardnim operativnim postopkom (SOP), uporabljenim pri projektu človeškega mikrobioma (HMP; angl. Human Microbiome Project) (The Human Microbiome Project Consortium, 2012). Ugotavljali smo sestavo bakterijske združbe znotraj posameznih vzorcev oz. skupin vzorcev (angl. alfa diversity) in jo primerjali med skupinami vzorcev (angl. beta diversity) (Kozich in sod., 2013) z različnimi metodami (Unifrac, AMOVA, HOMOVA, AWKS (Abundance-Weighted Kolmogorov-Smirnov) test). Pri tem smo upoštevali popravke za testiranje mnogoterih povezav (Benjamini-Hochberg) (Stres in sod., 2013). Prav tako smo identificirali mikrobne skupine, s katerimi smo razložili razlike med združbami v vzorcih (LEfSe, metasts, Corbata (CORe MicroBiome Analysis Tools), indicator) (Dufrene in Legendre, 1997; Li in sod., 2013; Segata in sod., 2011; White in sod., 2009). Izvedli smo analizo virov variabilnosti na nivoju 97 % podobnosti operativnih taksonomskih enot (OTU) in na nivoju rodu (Stres in sod., 2013), kjer smo 167 parametrom iz prvega dela študije, dodali še 64 parametrov (n = 231) (Slika 11, Preglednica S2), ki so obsegali meritve električne prevodnosti, polifenolov in sterolov v fecesu ter dodatne analize spektrov raztopljenih organskih snovi (DOM). Dodatni spektri kot npr: (i) kazalec indola v fecesu (Kumar in sod., 2015), (ii) specifična ultravijolična absorbancija (SUVA_{254}) (Weishaar in sod., 2003), (iii) specifična vidna absorbancija (SViA_{420}) (Weyhenmeyer in sod., 2014), (iv) cDOM indeks in (v) vsota devetih p-hidroksi, vanilil in syringil lignin fenolov (TDL_{9}, kažejo pomembno vlogo pri začetku in / ali napredovanju prepustnosti črevesa, ki vodi v povečano vnetje.

Tretji del naše študije (Sket in sod., 2018) je zajemal sekvenciranje celokupne mikrobiobne DNA in popis bakterijske, arhejske in glivne mikrobiote na taksonomskem ter funkcionalnem nivoju. Dodatno smo izvedli še analizo črevesnih metabolomov.

(def: celokupni popis mikrobnih in humanih črevesnih metabolitov) z jedrsko magnetno resonanco (NMR) in rentgensko fluorescenčno (XRF) spektrometrijo elementov (Slika 12). Taksonomski in funkcionalni opis smo analizirali s strežnikom MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) (Meyer in sod., 2008). Statistična analiza metagenomskih podatkov je potekala kot opisano pri sekvenciranju 16S rRNA (Šket in sod., 2017b). Med številnimi spremljanimi parametri (n = 231) (Preglednica S2) smo upoštevali tiste, ki so se v času trajanja PlanHab poskusa bistveno razlikovali in so bili statistično značilno povezani z razpršenostjo mikrobnih združb (Slika S5), ter jih uporabili za izpeljavo hierarhičnega modela odzivov človeške fiziologije na neaktivnost z uporabo Bayesove analize omrežja (Slika 31).


Rezultati preučevanja črevesnega okolja so pokazali izrazito zmanjšanje Bristol kazalca konsistence fecesa (BSS) in tako pokazali znake zaprtosti pri obeh različicah neaktivnosti (HBR in NBR), medtem ko se je v HAmb gibanje pri isti prehrani pokazalo kot učinkovita zaščita proti zaprtju, navkljub hipoksiji (Slika 13B). Navkljub spremembam v BSS, so bile številne preiskovane značilnosti črevesnega okolja (Preglednica S2) v naši študiji, v času trajanja poskusa, bodisi korelirane ali pa se niso bistveno spremenile (Šket in sod., 2017a; 2017b; 2018). Primeri so kratkoverižne hlapne maščobne kisline (SCFA), steroli, polifenoli, spektralni derivati raztopljenega organskega ogljika, celokupni metaboliti in vsebnost kovin. Po drugi strani so se pozitivni učinki telesne aktivnosti v HAmb odražali v: (i) nespremenjeni stopnji črevesne električne prevodnosti (IEC) (Slika 25), ki služi kot kazalec ionske moči črevesnega okolja in je merilo učinkovitost prirojenega imunskega sistema, kateri je med drugim odvisen od debeline mukoznega sloja črevesja, njegove poroznosti, zamreženosti in
hidratacije, (ii) zdravi ravni žolčnih kislin (BA), (iii) odsotnosti zaprtja (Slika 13), (iv) odsotnosti vnetnega odziva črevesja (EDN) in (v) povečanju indola, ki krepi zaščitno funkcijo sluznice (Slika 26) (Sket in sod., 2017b). Ti rezultati so skladni z nižjo koncentracijo glukoze v krvi, nespremenjeno občutljivost na inzulin, zmanjšano telesno maščobo, glukozo in celokupnim holesterolom, ki so jih opazili pri HAmb preiskovancih (Slika 27) (Simpson in sod., 2016) in kljub hipoksijski kažejo zdravo fiziološko stanje v HAmb v primerjavi z HBR (Sket in sod., 2017a). Povečane ravni EDN (kazalec lokalnega vnetja) v NBR in HBR kažejo na postopen razvoj vnetja črevesja, povezanega z osrednjim vnetnim faktorjem (NF-kB; angl. nuclear factor kappa B) (Glover in sod., 2016; Zeitouni in sod., 2016), ki vpliva na celično transkripcijo, oblikuje imunost črevesja in povzroča sproščanje reaktivnih kisikovih in dušikovih spojin (Faber in Bäumler, 2014).}

Med PlanHab poskusom je zaradi fizične aktivnosti v HAmb prišlo do spremenjene preference za prehrano (Slika 26). Tako so bile količine zaužitega železa, beljakovin, maščob in Ca^{2+} za 9%, 10%, 6% in 7,5% višje v HAmb v primerjavi s HBR in NBR (Debevec in sod., 2014) in smo jih upoštevali pri analizi virov variabilnosti kot kofaktorje pri pojasnjevanju razlik v sestavi mikrobiote. 

pokazala primerljivo bakterijsko taksonomsko porazdelitev, kar sovпадa z ugotovitvami, da sta regiji V2 in V6 omogočili razlikovanje med večino bakterijskih vrst (Chakravorty in sod., 2007). Enako smo potrdili s podrobnejšo analizo z sekvenciranjem celotnih metagenomov v tretjem delu naše študije (Sket in sod., 2018). Raziskava mikrobrov porazdelitve z izračunani kazalci alfa in beta raznovrstnosti niso pokazali sprememb med eksperimentalnimi skupinami. Kljub statistično značilno pomembnim spremembam nekaterih izmerjenih črevesnih parametrov, pomanjkanje sprememb v taksonomski sestavi črevesne mikrobiote, opredeljenih s 16S rRNA sekvenciranjem in potrjenih s sekvenciranjem celotnih metagenomov, kaže na zaostajanje odziva mikrobne skupnosti, oziroma na evolucijo odpornost črevesne mikrobiote na vplive kratkotrajne telesne fizične neaktivnosti (Shade in sod., 2012; Sket in sod., 2017a; 2017b). Odpornost mikrobiote na hitre spremembe smo potrdili, ko so se spremembe v njeni sestavi zgodile šele v četrtem tednu PlanHab poskusa (Sket in sod., 2017b; 2018).

Nadalje smo preiskovali sestavo in funkcijo arhejske in glivne združbe, kjer pa nismo opazili sprememb. Ugotovljeno nakazuje, da se odziv arhej in gliv na neaktivnost ter posledične spremembe v njihovi sestavi in funkciji, opisane pri primerjavi debelih in suhih preiskovancev (Mar Rodríguez in sod., 2015), zgodijo še kasneje, kot spremembe v bakterijski združbi (Sket in sod., 2018). Če pri zgoraj navedenem upoštevamo še funkcionalni odziv mikrobiote, se celotna kaskada sprememb začne najprej z odzivom na funkcionalnem nivoju, šele nato se spremeni taksonomska sestava bakterij, kasneje pa še arhej in gliv.

Na začetku naše študije smo v ničelni hipotezi (H0) predpostavili, da med preiskovanimi skupinami ne bo razlik. Številne preiskovane lastnosti črevesnega okolja (zonulin, A1AT, SCFA, steroli, polifenoli, spektralni derivati DOM, skupni metaboliti, vsebnost elementov, splošni opisi črevesne mikrobiote na osnovi genov za masleno kislino, na osnovi 16S rRNA bakterijskih in arhejskih genov ali pa sekvenciranja celokupnih metagenomov) so bile: (i) bodisi soodvisne, kar kaže na znatno hierarhično soodvisnost med parametri v črevesnem okolju, (ii) ali pa se niso bistveno spremenile, kar kaže, da vpliv poskusa (neaktivnost, čas, hipoksija in njihovi interaktivni učinki) ni bil zadosten, da bi povzročil spremembe teh preiskovanih lastnosti (Sket in sod., 2017a; 2017b; 2018).

Po drugi strani, pomembne prilagoditve v človeški fiziologiji in prehod iz zdravega fiziološkega stanja v simptome povezane z debelostjo, kot o požnjo že prej (Debevec in sod., 2016; Simpson in sod., 2016; Strewe in sod., 2017), skupaj z ugotovljenimi spremembami v naši študiji (Sket in sod., 2017a; 2017b; 2018), kot so zaprtost, vnetje, ionski potencial, indol, žolčne kisline ter spremembe sestave mikrobiote in funkcije na koncu HBR, kažejo odziv na neaktivnost in hipoksijo, ki smo ga nadalje pojasnili na osnovi štirih alternativnih hipotez (H1.1-4). Prvič (H1.1), variabilnost zgoraj navedenih statistično značilno spremenjenih parametrov med preiskovanci znotraj iste eksperimentalne skupine je bila manjša od razlik med skupinami, ki jo je povzročil bodisi vpliv fizične neaktivnosti bodisi hipoksije ali kombinacija obojega. Drugič (H1.2 in H1.3), predvideni učinki fizične neaktivnosti in hipoksije, zaradi križne zasnove PlanHab poskusa (brez kontrolne skupine, kjer bi preiskovanci ležali v normoksičnih pogojih), so pojasnjeni vzajemno. Pomanjkanje gibanja negativno vpliva na črevesni sistem v kratkem časovnem obdobju 21 dni (NBR), te negativne učinke še dodatno poslabša sistemska hipoksija (HBR), vendar navkljub hipoksiji (HAmb), gibanje vse negativne učinke izniči. To nakazuje vpliv tako neaktivnosti, kot hipoksije in hkrati potrjuje neaktivnost kot glavni dejavnik, ki v zasnovanem poskusu vpliva na črevesni sistem. Zadnja hipoteza (H1.4), potrjuje, da so odzivi humane fiziologije, metabolitov črevesja, imunskega odziva v črevesju in črevesne mikrobiote zares medsebojno povezani, kot je razvidno iz analize Bayesovega omrežja (Slika 32) in veliko bolj zapleteni, kot prvotno predlagano. Prilagoditve na nivoju metabolitov (BA), proteomov (EDN), ionskega ravnovesja (EC) in sistemskih (BSS) prilagoditve (Colgan, 2013; Mach in Fuster-Botella, 2016; Sket in sod., 2017a; 2017b; 2018) potekajo vzporedno s človeškimi fiziološkimi spremembami (Debevec in sod., 2016; Simpson in sod., 2016; Strewe in sod., 2016).
2017), vendar prehitijo spremembe v mikrobi združbi in funkciji v časovnem oknu od dveh do treh tednov. Tako predpostavljamo, da opažena vztrajnost mikrobiote, prispeva k stabilnosti metabolne homeostaze v črevesju in posledično preprečuje prehitro in prekomerno nihanje presnovnih odzivov na prehranske ali okoljske signale (Beaumont in sod., 2017; Thaiss in sod., 2016).

Na osnovi pridobljenih rezultatov smo tako sklepali:

- Prehod iz zdravega fiziološkega stanja v smeri simptomov, povezanih z debeloostjo, je odvisen od trajanja fizične neaktivnosti.
- Zaprtost in vnetje črevesja (povečanje prevodnosti, nevrotoksina eozinofilcev in žolčnih kislin) se pojavita kmalu po začetku fizične neaktivnosti (pomanjkanje telesne vadbe v NBR), ki se v hipoksiji še poslabšata (HBR) in bistveno izboljšata s fizično aktivnostjo, kljub hipoksiji (HAmb). Slednje potrjuje fiziološko najslabše stanje v HBR.
- Struktura in številčnost mikrobiote, ki proizvaja masleno kislino, ali taksonomske in funkcionalne preurejnosti v velikih bakterijskih, arhejskih in glivnih združbah ter njihovi metaboliti v debelem črevesju preiskovancev, se niso bistveno spremenili, kar kaže, da je črevesni sistem očitno pokazal odpornost proti kratkotrajnim spremembam.
- Znatne spremembe v koncentraciji bakterijskih skupnosti so se odrazilo kot površina vnetnih in mukus razgrajajočih vrst Bacteroides v četrtem tednu v HBR.
- Povečanje rodu Bacteroides in obogatitev genov, vključenih v prihajanje železa, celično steno, kapsulo, virulentnost in razgradnjo mukoznih polimerov, nakazuje na razgradnjo mukoznega sloja črevesja gostitelja in posledično imunski odziv ter med prvimi razkriva začetni mehanizem razvoja ugotovljenih negativnih fizioloških simptomov gostitelja.

PlanHab poskus omogoča edinstven vpogled v razumevanje ustreznih medicinskih in fizioloških prilagoditev štiristranega odnosa med človeško fiziologijo, imunska stanje, črevesna prevodnost in črevesna mikrobiota, ki se pojavijo kot odziv na zmanjšanje fizične telesne aktivnosti, kljub ljudem, ki so zaradi dolgotrajne hude bolezni imobilizirani (hibrične hipoksiji zaradi dihalne insuficience, kongestivno srčno popuščanje, debeloost). Tako smo z izvedbo naše raziskave povezali pomembne parametre metabolnih (žolčne kisline), proteomskih (nevrotoksina eozinofilcev), ionskih (črevesna električna prevodnost) in sistemskih (zaprtje) prilagoditev ter dvotedensko oz. tritedensko zakasnitev sprememb v črevesni mikrobioti ter oblikovali prvi hierarhični model negativnih vplivov zaradi trajajoče telesne fizične neaktivnosti. Ponovna telesna fizična aktivnost, po koncu poskusa PlanHab, je privedla do takojšnjega izboljšanja negativnih fizioloških simptomov, kar še dodatno potrjuje njen vpliv. V naši študiji opažene negativne lastnosti zaradi neaktivnosti, se odražajo tudi v drugih vrstah sesalcev med zimskim spanjem oz. neaktivnostjo. Če k temu prištejemo še njihovo aktivnost in prekomerne energijske vnose zaradi intenzivnega hranjenja poleti, ki so vključene...
z bogato človeško prehrano v razvitem svetu, potem ugotovimo, da drastične spremembe v prehrani (angl. yo-yo dieting) in aktiven / neaktiven življenjski slog, skupaj s pripadajočimi učinki, ni človeška posebnost, temveč skupna evolucijska prilagoditev sesalcev, razvita kot posledica preživetja pomanjkanja hrane in prilagajanja na spremembe v letnih časih. Nazadnje, prehajanje mikroRNA molekul med gostiteljem in mikrobi ter nazaj, hkrati z njihovo sposobnostjo uravnavanja izražanja genov v obeh sistemih, predstavlja manjkajoči člen pri pojasnjevanju interakcije med mikrobi v črevesnem traktu in celicami gostitelja, ki bi ga bilo smiselno upoštevati in podrobneje raziskati v prihodnjih usmeritvah naše študije.
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Last but not least, my family and friends, thank you for your priceless support!
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SUPPLEMENT B: JOURNALS PERMISSIONS TO REPRODUCE PUBLISHED ARTICLES

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SUPPLEMENT C: LIMITATIONS OF THE PLANHAB STUDY

A few limitations and concepts of our study need to be considered as well (Sket et al., 2017a, 2017b, 2018). Firstly, the sample size in this study was relatively small, but well within the limits of recent detailed studies (David et al., 2014a, 2014b; Thaiss et al., 2014). Second, the limited statistical power and accompanying potential for type-II error was at least partly alleviated by the fact that the test participant population was prescreened for healthy young males according to SOP used by ESA/NASA and the study executed according to SOP for NASA bed rest studies. Nevertheless, a larger study sampling random population longitudinally as participants experience variations in intestinal transit times might be able to find additional and more significant effects. Third, the inclusion of female participants would definitely add a much needed layer of complexity. Last, the current study represents a proof of principle that numerous variables are in fact implicated into development of systemic inflammation and that transdomain systems biology research on the same participants within the same experiments deploying a diversity of research objectives, subsystem monitoring and tools (physiology, body traits, body composition, immunology, psychology, neuroendocrine, nutrients, appetite, microbial communities, constipation, water intake, temperature control, sleep architecture, antioxidant defenses) is feasible and could be integrated into future studies adopting multiomics approaches. Future experiments utilizing conditions without hypoxia and inactivity may be a good addition to understand potential interaction between tissue-hypoxia and exercise over prolonged monitoring periods, e.g. > 60 days.

SUPPLEMENT D: ETHICAL ASPECTS OF THE PLANHAB STUDY

The study protocol was approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia. All experimental procedures were conducted according to the European Space Agency recommendations for bed rest protocols (Standardization of bed rest study conditions 1.5, August 2009) and conformed to the principles of the Declaration of Helsinki. Participants gave written informed consent after receiving detailed information regarding the study protocol and all experimental procedures. All collected data will be treated as confidential medical documentation. Results in the publication will not impersonate the identity of the subjects. Ethics Committee permission is held by Jožef Stefan Institute.
SUPPLEMENT E: ADDITIONAL FIGURES OF OUR SUB-STUDY CONDUCTED WITHIN THE PLANHAB PROJECT (Sket et al., 2017a, 2017b, 2018)

Figure S1. (Continues below)
Figure S1. (Continued) Schematic representation of experimental outline used in PlanHab study (http://cordis.europa.eu/project/rcn/104127_en.html). (A) The parameters (hypoxia and exercise) and the resulting status of host physiology after 21 days in controlled experiment. (B) Human systems biology exploration space mapping exercise with oxygen saturation levels and human population diversity (Clarke et al., 2014; Debevec et al., 2014; Liao et al., 2016; Sket et al., 2017a). (C) The difference between accumulation and short-term real-time experiments. The inset shows a tentative scheme of dose dependent benefits derived from various levels of exercise (Bermon et al., 2015; Cronin et al., 2016; Egan and Zierath, 2013; Ringseis et al., 2015; Sket et al., 2017a).
Figure S2. (Continues below)
Figure S2. (Continues below)
Figure S2. (Continues below)
Figure S2. (Continued) Ecological indices used to assess α-diversity microbiomes at the start-up and endpoints of the PlanHab experiment: Taxa_S, Individuals, Dominance_D, Simpson_1-D, Shannon_H, Evenness_e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher_α, Berger-Parker, Chao-1 (Sket et al., 2017b).
Figure S3. 2D $^{1}H$-$^{13}C$ NMR HSQC spectrum of sample S12_1A measured at 25 °C on 800 MHz spectrometer (A), and three zoomed sections showing aliphatic (B and C) as well as aromatic regions (D). HSQC was recorded in multiplicity-edited mode, where correlation signals in black correspond to CH and CH$_3$ groups, whereas signals in red represent CH$_2$ groups. Identified metabolites with assigned NMR chemical shifts are shown in Table S4 (Sket et al., 2018)
Figure S4. Fluorescence spectrum of intestinal metal content measured by X-ray fluorescence spectrometry (XRF) (Sket et al., 2018).

Figure S5. A schematic representation of the significant changes in measured parameters of intestinal tract during the 21-day PlanHab experiments in NBR, HBR and HAmb variants (Sket et al., 2017a, 2017b, 2018).
Figure S6. (Continues below)
Figure S6. (Continued) Strain level deconvolution of the genus *Bacteroides* sequences found in NBR, HBR and HAmb variants at the end of the PlanHab experiments. The overall significant increase in various strains of *Bacteroides* at the end of PlanHab experiment in HBR is shown (p < 0.05) (Sket et al., 2017b)
Figure S7. Statistically significantly increased genes at the functional level at the end point of NBR, HBR and HAmb variants, as described in Figure 29A (Sket et al., 2018).
Figure S8. A conceptual framework for schematic comparison between the observed concentration of SCFA in feces (A) and the potential rates of SCFA removal into host tissue (B). These results are in line with the generally elevated SCFA concentrations in the obese population (Sket et al., 2018).
SUPPLEMENT F: ADDITIONAL TABLES OF OUR SUB-STUDY CONDUCTED WITHIN THE PLANHAB PROJECT (Sket et al., 2017a, 2017b, 2018)

Table S1. Baseline demographic and clinical characteristics for each experiment group (mean ± SD) in the PlanHab experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sex</th>
<th>Nationality</th>
<th>Age (years)</th>
<th>Height (m)</th>
<th>Weight (Kg)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>NBR</td>
<td>32.7</td>
<td>6.2</td>
<td>1.79</td>
<td>0.01</td>
<td>69.9</td>
<td>3.7</td>
</tr>
<tr>
<td>HAmb</td>
<td>26.7</td>
<td>0.5</td>
<td>1.85</td>
<td>0.06</td>
<td>82.8</td>
<td>8.7</td>
</tr>
<tr>
<td>HBR</td>
<td>23.0</td>
<td>2.9</td>
<td>1.77</td>
<td>0.01</td>
<td>72.7</td>
<td>11.8</td>
</tr>
</tbody>
</table>
Table S2. Overview of new in-house Planhab database. The three data matrices containing (i) experimental, (ii) diet, and (iii) metabolite community datasets in this study.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Diet</th>
<th>Environment: metabolite and immunological parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant (n = 9)</td>
<td></td>
<td>trp - tryptophan (g) pH</td>
</tr>
<tr>
<td>Hypoxia (n = 2)</td>
<td></td>
<td>tyr - tyrosine (g) reducing sugars - Colorimetric detection of reducing sugars content</td>
</tr>
<tr>
<td>Inactivity (n = 2)</td>
<td></td>
<td>val - valine (g) %source feces - Water content in feces (%)</td>
</tr>
<tr>
<td>Sample (n = 54)</td>
<td></td>
<td>k - Potassium (mg) Acetic - Acetic acid (gL)</td>
</tr>
<tr>
<td>Time in experiment (n = 6)</td>
<td></td>
<td>vii - Vitamin A (µg) Propionic - Propionic acid (gL)</td>
</tr>
<tr>
<td>Experimental variant (n = 3)</td>
<td></td>
<td>viib - Vitamin B12 (µg) isoval - iso-Val (no acid) (gL)</td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td>vtc - Vitamin C (mg) nVal - n-Valeric acid (gL)</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td></td>
<td>mg - Magnesium (mg) water - Water (g)</td>
</tr>
<tr>
<td>BMI - Body mass index</td>
<td></td>
<td>zn - Zinc (mg) tSOCTA - Total short chain fatty acids (gL)</td>
</tr>
<tr>
<td>cho - Total carbohydrates (g)</td>
<td></td>
<td>Magnesium (mg) toSCTA - Total short chain fatty acids (gL)</td>
</tr>
<tr>
<td>chot - Total carbohydrates (%)</td>
<td></td>
<td>mm - Manganese (µg) BA - Bile acids (fold)</td>
</tr>
<tr>
<td>chot_p - Total carbohydrates (%)</td>
<td></td>
<td>mg - Manganese (mg) zonulin - Zonulin (fold)</td>
</tr>
<tr>
<td>cal - Chlorine (mg)</td>
<td></td>
<td>fapun3 - Total omega 3 acids (g) a1aT - Alpha 1 anti-trypsin (fold)</td>
</tr>
<tr>
<td>color - Color</td>
<td></td>
<td>fapun6 - Total omega 6 acids (g) EDN - Eosinophil derived neurotoxin (fold)</td>
</tr>
<tr>
<td>cr - Chromium (µg)</td>
<td></td>
<td>GI - Glutamatic acid (g) BSS - Bristol stool scale</td>
</tr>
<tr>
<td>cu - Copper (µg)</td>
<td></td>
<td>GL - Glycemic load (µg) retention time - Time between particular defecations (days)</td>
</tr>
<tr>
<td>cyste - Cysteine (mg)</td>
<td></td>
<td>BA - Bile acids (fold) dedication frequency - Number of defecations during experiments</td>
</tr>
<tr>
<td>edible - &quot;no name&quot;</td>
<td></td>
<td>BI - Bile acid (µg) Se - Ratio of absorption slopes between 275-295 nm slope and 350-400 nm slope</td>
</tr>
<tr>
<td>essen - Energy (kcal)</td>
<td></td>
<td>E2.23 - Ratio between absorption coefficients at 230 nm and at 365 nm</td>
</tr>
<tr>
<td>fib4.0 - Myristic fatty acid (g)</td>
<td></td>
<td>a25 - Absorption coefficient at 255 nm</td>
</tr>
<tr>
<td>fib6.0 - Palmitic fatty acid (g)</td>
<td></td>
<td>a300 - Absorption coefficient at 300 nm</td>
</tr>
<tr>
<td>fib8.0 - Stearic fatty acid (g)</td>
<td></td>
<td>Total.a.250.450 - Total soluble organic carbon per gram of dry matter in feces</td>
</tr>
<tr>
<td>fib10.0 - Oleic fatty acid (g)</td>
<td></td>
<td>S.300.700 - Total soluble organic carbon per gram of dry matter in feces</td>
</tr>
<tr>
<td>fib2.0 - Linolenic fatty acid (g)</td>
<td></td>
<td>totalPolyphenols (area under curve) Sn</td>
</tr>
</tbody>
</table>
Table S3. Comparison of observed trends in four immunological markers after the completion of experiments relative to BDC values. NBR-normobaric normoxic bedrest, HBR – normobaric hypoxic bedrest, HAmb – normobaric hypoxic ambulation. Please see details in extended captions.

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>NBR</th>
<th>HBR</th>
<th>HAmb</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zonulin</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>low is good</td>
</tr>
<tr>
<td>increases tight junction degradation and permeability of epithelia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A1AT</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>high is good</td>
</tr>
<tr>
<td>signals damaged mucosal integrity and increased permeability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EDN</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+ &gt; 200 %</td>
<td>+ &gt; 300 %</td>
<td>0</td>
<td>low is good</td>
</tr>
<tr>
<td>signals location of inflammation, site of tissue damage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BA</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+ &gt; 200 %</td>
<td>+ &gt; 150 %</td>
<td>0</td>
<td>low is good</td>
</tr>
<tr>
<td>marker of FFA metabolism, precursor for secondary BA, exhibits toxicity for bacteria, increases pro-inflammatory cytokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Zonulin binds to a specific receptor on the surface of intestinal epithelia and triggers a cascade of biochemical events which induces tight junction disassembly and a subsequent permeability increase of the intestinal epithelia, allowing some substances to pass through and activate immune reactions. It is suggested that increased levels of zonulin are a contributing factor to the development of intestinal disorders and other autoimmune disorders.

<sup>2</sup>α<sub>1</sub>-antitrypsin represents the majority of serine protease inhibitors and protects tissues from protease damages during inflammation. The protein is synthesized primarily in the liver but also to a small extent in intestinal macrophages, monocytes, and intestinal epithelial cells. Since α<sub>1</sub>-antitrypsin is relatively resistant against enzymatic digestion, the secreted amount in stool reflects the internal concentration of the protein. An elevated α<sub>1</sub>-antitrypsin stool concentration is therefore a widely recognized marker for intestinal protein loss and for an increased mucosal permeability. Intestinal protein loss is a serious consequence of various systemic or local gastrointestinal diseases (e.g. allergies, chronic inflammation, malignancies). These pathologies damage the mucosal integrity and/or cause lymphostasis, thereby leading to an increased transfer of plasma proteins into the bowel lumen.

<sup>3</sup>EDN (eosinophil-derived neurotoxin, eosinophil protein x, EPX), a cationic glycoprotein, which is released by activated eosinophils, has strong cytotoxic characteristics and plays a significant role in the prevention of virus infections. It is released by the eosinophil granules in places where eosinophils are mainly found: in the skin, lungs, urogenital and gastrointestinal tract, that is, in the organs acting as an entry point for pathogens. The accumulation of EDN in the intestine is associated with inflammation and tissue damage. Measuring of EDN in stool can serve as an objective parameter for a current clinical or sub-clinical chronic inflammation located in the gastrointestinal area. In the case of Colitis ulcerosa and Crohn’s disease, the EDN measurement enables the evaluation of a disease’s activity and the prediction of a relapse.

<sup>4</sup>Bile acids are produced in the liver as end-products of cholesterol metabolism. Together with other components of the liver bile, such as cholesterol, bilirubin, phospholipids and proteins, bile acids are secreted into the duodenum. Important functions of bile acids are the excretion of cholesterol, absorption of fatty acids and fat-soluble vitamins in the small intestine as well as stimulation of intestinal motility. The majority of the secreted bile acids are reabsorbed in the terminal ileum and returned to the liver via the portal venous system for eventual recirculation in a process known as enterohepatic circulation; only a small proportion (3-5%) are excreted into the feces. If the enterohepatic recycling of bile acids fails, excess amounts of bile acids enter the colon and are lost with the feces; this condition is called bile acid malabsorption.
Table S4. $^1$H and $^{13}$C NMR chemical shifts of metabolites identified in sample S12_1A (in ppm).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H (ppm)</th>
<th>$^{13}$C (ppm)</th>
<th>Metabolite</th>
<th>$^1$H (ppm)</th>
<th>$^{13}$C (ppm)</th>
<th>Metabolite</th>
<th>$^1$H (ppm)</th>
<th>$^{13}$C (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Glucose</td>
<td>3.841</td>
<td>63.958</td>
<td>L-Isoleucine</td>
<td>3.673</td>
<td>62.175</td>
<td>L-Tyrosine</td>
<td>3.934</td>
<td>59.169</td>
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<td>Acetate</td>
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<td>19.144</td>
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<td>68.852</td>
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<td>1.327</td>
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Table S5. Variables significantly associated with the distribution of butyrate producing microbial community in the PlanHab experiment.

<table>
<thead>
<tr>
<th>Butyrate producing microbial community</th>
<th>Environment</th>
<th>Experiment</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-butyric acid</td>
<td></td>
<td>age</td>
<td>histidine</td>
</tr>
<tr>
<td>iso-valeric acid</td>
<td></td>
<td>height</td>
<td>chloride ion</td>
</tr>
<tr>
<td>acetatic acid</td>
<td></td>
<td>activity</td>
<td>rare bact. OTUs</td>
</tr>
<tr>
<td>total SCFA</td>
<td></td>
<td>experimental variant</td>
<td>ingested fat</td>
</tr>
<tr>
<td>bile acids</td>
<td></td>
<td>individual</td>
<td>ingested water</td>
</tr>
<tr>
<td>EDN</td>
<td></td>
<td>BMI</td>
<td>sucrose</td>
</tr>
<tr>
<td>TSOC</td>
<td></td>
<td></td>
<td>vitamin B5</td>
</tr>
<tr>
<td>n-capric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1AT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Characteristics of identified *Bacteroides* species. Identification of *Bacteroides* species at the end of PlanHab experiment with their mucin degrading and inflammogenic characteristics of medical relevance reconstructed from published literature. The k-mer search strategy (RDPTools; n = 7) with Sab score > 0.87 to type and cultivated strains with reported relevance for dysbiotic medical conditions and described metabolic characteristics was used in this analysis (Bakir et al., 2006; Bloom et al., 2012; Brook, 1995; Chow and Lee, 2008; Davis-Richardson et al., 2014; Jakobsson et al., 2015; Jousimies-Somer et al., 2003; Miyamoto and Itoh, 2000; Salyers et al., 1977; Tailford et al., 2015; Wexler, 2007).

<table>
<thead>
<tr>
<th><em>Bacteroides</em> species</th>
<th>1 Degradation of mucin</th>
<th>2 α-Fucosidase activity</th>
<th>3 Indole production</th>
<th>4 Aesculin hydrolysis</th>
<th>5 Associated with the following clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. acidifaciens</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td><em>B. caccae</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td><em>B. dorei</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td><em>B. ovatus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>type 1 diabetes, inflammation</td>
</tr>
<tr>
<td><em>B. thetaiotamicron</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>enteric infections, inflammation</td>
</tr>
<tr>
<td><em>B. vulgatus</em></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ulcerative colitis</td>
</tr>
</tbody>
</table>

1 *Bacteroides* derive nutrients from either diet or host derived or commensal derived polymers. Overutilization of host derived polymers (e.g. during constipation) results in thinning of mucus layer and modified barrier functioning (Jakobsson et al., 2015; Tailford et al., 2015).

2 De-glycosylation of mucin components leads to degradation mucus properties and degradation of collocated immune compounds (IgA). Free fucose is a danger signal to human intestinal epithelial cells and is directly utilized in fermentations by facultatively anaerobic enteropathogenic *E.coli* and other bacteria, affecting the mucosal physicochemical characteristics (Chow and Lee, 2008).

3 Indole is a microbe-generated signal substance that has positive effects on its host as well as the microbiome. Indole functions as a ‘quorum-sensing’ signal that regulates the virulence and biofilm formation of EHEC, *Pseudomonas* and other commensal bacteria. Indole strengthens the barrier function of the mucous membrane by repairing ‘tight junctions’ (Berstad et al., 2015; Shimada et al., 2013).

4 Aesculin hydrolysis *in-vivo* generates secondary derivatives from the family of coumarins that exert potent positive pharmacological effects on the host (Ding et al., 2009).

5 The same commensal *Bacteroides* species observed in numerous healthy fecal samples undergo significant metabolic changes associated with the clinical manifestations under specific environmental conditions.

The *underlined species* were recently identified as those exhibiting extensive genomic variants with markedly different metabolic capacities in patients (Vineis et al., 2016).
Table S7. Variables significantly associated with the distribution of bacterial community structure in PlanHab experiment at the level of 97% OTUs and genus. The data were retrieved from the newly established in-house PlanHab database and used in variation partitioning. Please see Materials and methods next to Table S2 for additional information. A1AT – Alpha1 antitrypsin; TSOC – total soluble organic carbon, EDN – eosinophil derived neurotoxin; BSS – Bristol stool scale; polyphenol a1, b1, a3 – polyphenol peaks with currently unknown chemical structure.

<table>
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<th>97% OTU</th>
<th>Environment</th>
<th>Experiment</th>
<th>Variable</th>
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<td>iso-valeric acid</td>
<td>hypoxia</td>
<td>cholesterol</td>
<td></td>
</tr>
<tr>
<td>n-butyric acid</td>
<td>individual</td>
<td>histidine</td>
<td></td>
</tr>
<tr>
<td>n-capric acid</td>
<td>experimental variant</td>
<td>f18_3cn3 (alpha-linolenic acid)</td>
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</tr>
<tr>
<td>AIAT</td>
<td>body traits (mass, height)</td>
<td>water</td>
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<tr>
<td>n-valeric acid</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TSOC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water content</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>acetic acid</td>
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<td></td>
<td></td>
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<tr>
<td>bile acids</td>
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<td></td>
</tr>
<tr>
<td>reducing sugars</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Genus</th>
<th>Environment</th>
<th>Experiment</th>
<th>Variable</th>
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<tbody>
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<td>experimental variant</td>
<td>protein</td>
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</tr>
<tr>
<td>n-butyric acid</td>
<td>hypoxia</td>
<td>fat</td>
<td></td>
</tr>
<tr>
<td>iso-valeric acid</td>
<td>time (day in experiment)</td>
<td>f18_2 (linoleic acid)</td>
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<tr>
<td>n-capric</td>
<td>individual</td>
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<tr>
<td>AIAT</td>
<td>body traits (height, BMI)</td>
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<tr>
<td>bile acids</td>
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<td>sucrose</td>
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<td>BSS</td>
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<td>water</td>
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### Table S8. Groups of annotated genes used for statistical analyses in Figure 29: (A) Subsystem database level 1; (B) Degradation of host mucins; (C) Butyrate synthesis pathways; (D) Aerobic and anaerobic respiration capacities.

**(A) Subsystem database level 1:** Amino Acids and Derivatives; Carbohydrates; Cell Division and Cell Cycle; Cell Wall and Capsule; Clustering-based subsystems; Cofactors Vitamins Prosthetic Groups Pigmements; DNA Metabolism; Dormancy and Sporulation; Fatty Acids Lipids and Isoprenoids; Iron acquisition and metabolism; Membrane Transport; Metabolism of Aromatic Compounds; Miscellaneous; Motility and Chemotaxis; Nitrogen Metabolism; Nucleosides and Nucleotides; Phages Prophages Transposable elements Plasmids; Phosphorus Metabolism; Photosynthesis; Potassium metabolism; Protein Metabolism; Regulation and Cell signaling; Respiration; RNA Metabolism; Secondary Metabolism; Stress Response; Sulfur Metabolism; Virulence Disease and Defense.

**(B) Degradation of host mucins:** Sialidase (EC 3.2.1.18); Beta-galactosidase (EC 3.2.1.23); Beta-galactosidase (EC 3.2.1.23) LacA family; Beta-galactosidase (EC 3.2.1.23) LacZ family; Beta-galactosidase 3; Beta-galactosidase large subunit (EC 3.2.1.23); Beta-galactosidase small subunit (EC 3.2.1.23); Alpha-N-acetylgalactosaminidase; Alpha-L-fucosidase (EC 3.2.1.51); Beta-hexosaminidase (EC 3.2.1.52); Alpha-galactosidase (EC 3.2.1.22); Alpha-galactosidase precursor (EC 3.2.1.22).

**(C) Butyrate synthesis pathways:** Butyrate kinase (EC 2.7.2.7); Butyrate-acetoacetate CoA-transferase subunit A (EC 2.8.3.9); Butyrate-acetoacetate CoA-transferase subunit B (EC 2.8.3.9); Butyryl-CoA dehydrogenase (EC 1.3.99.2); D-beta-hydroxybutyrate dehydrogenase (EC 1.1.1.30); D-beta-hydroxybutyrate permease; 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55); 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157); 2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29); Acetyl-CoAacetoacetyl-CoA transferase. alpha subunit (EC 2.8.3.8); Lysine 2.3-aminomutase (EC 5.4.3.2); L-beta-lysine 5.6-aminomutase alpha subunit (EC 5.4.3.3); L-beta-lysine 5.6-aminomutase beta subunit (EC 5.4.3.3); 3-keto-5-aminoheptanoate cleavage enzyme; 3-amino butyryl-CoA ammonia lyase (EC 4.3.1.14); Phosphate butyryltransferase (EC 2.3.1.19); 3-ketoacyl-CoA thiols (EC 2.3.1.16).

**(D) Aerobic and anaerobic respiration capacities:** 2.4-dienoyl-CoA reductase (NADPH) (EC 1.3.1.34); 2-dehydropropionate 2-reductase (EC 1.1.1.169); 2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60); 2-polypropylphenol hydroxylase and related flavodoxin oxidoreductases; 3-oxoacyl-(acyl-carrier protein) reductase (EC 1.1.1.100); 3-oxoacyl-(acyl-carrier protein) reductase paralog (EC 1.1.1.100) in cluster with unspecified monosaccharide transporter; 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2); 5.10-methylene tetrahydrofolic acid reductase (EC 1.5.1.20); 5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193); 5-keto-D-gluconate 5-reductase (EC 1.1.1.69); Acetoacetyl-CoA reductase (EC 1.1.1.36); Adenylylsulfate reductase alpha-subunit (EC 1.8.99.2); Adenylylsulfate reductase beta-subunit (EC 1.8.99.2); Aldo-keto reductase family 1 member B10 (EC 1.1.1.-); Alkyl hydroperoxide reductase protein C (EC 1.6.4.-); Alkyl hydroperoxide reductase protein F (EC 1.6.4.-); Alkyl hydroperoxide reductase subunit C-like protein; Altronate oxidoreductase (EC 1.1.1.58); Anaerobic dimethyl sulfoxide reductase chain A (EC 1.8.99.-); Anaerobic dimethyl sulfoxide reductase chain B (EC 1.8.99.-); Arsenate reductase (EC 1.20.4.1); Benzoyl-CoA reductase subunit BadE (EC 1.3.99.15); Benzoyl-CoA reductase subunit BadF (EC 1.3.99.15);
Benzoyl-CoA reductase subunit BadG (EC 1.3.99.15); CoA-disulfide reductase (EC 1.8.1.14); Cob(II)alamin reductase; Cob(II)alamin reductase; Cobalt-precorrin-6x reductase (EC 1.3.1.54); CoB--CoM heterodisulfide reductase subunit A (EC 1.8.98.1); CoB--CoM heterodisulfide reductase subunit B (EC 1.8.98.1); CoB--CoM heterodisulfide reductase subunit C (EC 1.8.98.1); CoB--CoM heterodisulfide reductase subunit D (EC 1.8.98.1); Coenzyme A disulfide reductase; Cytochrome c nitrite reductase. small subunit NrfH; Cytochrome c-type biogenesis protein DsbD. protein-disulfide reductase (EC 1.8.1.8); Dihydrolipicolinate reductase (EC 1.3.1.26); Dihydroflavonol-4-reductase (EC 1.1.1.219); Dihydrofolate reductase (EC 1.5.1.13); Dissimilatory sulfite reductase (desulfoviridin). alpha and beta subunits; D-mannonate oxidoreductase (EC 1.1.1.57); D-proline reductase. 45 kDa subunit (EC 1.21.4.1); D-proline reductase. 23 kDa subunit (EC 1.21.4.1); dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133); Enoyl-(acyl-carrier-protein) reductase (FMN) (EC 1.3.1.9); Enoyl-(acyl-carrier-protein) reductase (NADH) (EC 1.3.1.9); Ferredoxin reductase; Ferredoxin reductase subunit B (EC 1.3.1.9); Ferredoxin reductase subunit C (EC 1.3.1.9); Glutamyl phosphate reductase (EC 1.2.1.41); Glutamyl-tRNA reductase (EC 1.2.1.70); Glycine reductase component B alpha subunit (EC 1.21.4.2); Glycine reductase component B beta subunit (EC 1.21.4.2); Glycine reductase component B gamma subunit (EC 1.1.1.77); L-lysine deazaguanine reductase (EC 1.7.1.68); Dihydrofolate reductase (EC 1.5.1.3); Methionine adenosyltransferase; Mercuric ion reductase; N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38); NAD(P)H oxidoreductase YRKL (EC 1.6.99.-); NADH ubiquinone oxidoreductase chain A (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain B (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain C (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain D (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain E (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain F (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain G (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain H (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain I (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain J (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain K (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain L (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain M (EC 1.6.5.3); NADH ubiquinone oxidoreductase chain N (EC 1.6.5.3); NADPH-dependent 7-cyano-7-deazaguanine reductase (EC 1.7.1.1-); Nitric oxide reductase activation protein NorD; Nitric oxide reductase activation protein NorQ; Nitrite reductase (NAD(P)H) large subunit (EC 1.7.1.4); Nitrite reductase probable (NAD(P)H) subunit (EC 1.7.1.4); Nitrite reductase probable electron transfer 4Fe-S subunit (EC 1.7.1.4); Nitrogenase (molybdenum-iron)
reductase and maturation protein NifH; Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11); Peptide methionine sulfoxide reductase MsrB (EC 1.8.4.12); PF00070 family. FAD-dependent NAD(P)-disulphide oxidoreductase; Polyferredoxin NapH (periplasmic nitrate reductase); Predicted L-lactate dehydrogenase. Fe-S oxidoreductase subunit YkgE; Probable electron transfer flavoprotein-quinone oxidoreductase FixC (EC 1.5.5.-); Probable thiol oxidoreductase with 2 cytochrome c heme-binding sites; PUA-PAPS reductase like fusion; Putative oxidoreductase linked to yggC; Pputative oxidoreductase YdjL; Pyrroline-5-carboxylate reductase (EC 1.5.1.2); Pyruvateferredoxin oxidoreductase. alpha subunit (EC 1.2.7.1); Pyruvateferredoxin oxidoreductase. beta subunit (EC 1.2.7.1); Pyruvateferredoxin oxidoreductase. delta subunit (EC 1.2.7.1); Pyruvateferredoxin oxidoreductase. gamma subunit (EC 1.2.7.1); Pyruvateferredoxin oxidoreductase. alpha chain (EC 1.7.99.4); Ribonucleotide reductase of class Ia (aerobic). alpha subunit (EC 1.17.4.1); Ribonucleotide reductase of class Ia (aerobic). beta subunit (EC 1.17.4.1); Ribonucleotide reductase of class Ib (aerobic). alpha subunit (EC 1.17.4.1); Ribonucleotide reductase of class Ib (aerobic). beta subunit (EC 1.17.4.1); Ribonucleotide reductase of class II (coenzyme B12-dependent) (EC 1.17.4.1); Ribonucleotide reductase of class III (anaerobic). activating protein (EC 1.97.1.4); Ribonucleotide reductase of class III (anaerobic). large subunit (EC 1.17.4.2); Ribonucleotide reductase transcriptional regulator NrdR; Superoxide reductase (EC 1.15.1.2); Thioldisulfide oxidoreductase related to ResA; Thioredoxin reductase (EC 1.8.1.9); Trimethylamine-N-oxide reductase (EC 1.6.6.9); UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)