Gut Mycobiome Dysbiosis Is Linked to Hypertriglyceridemia among Home Dwelling Elderly Danes

Hajar Fauzan Ahmad[1,2], Josue Leonardo Castro Mejia[1], Lukasz Krych[1], Bekzod Khakimov[1], Witold Kot[3], Rasmus Leidesdorff Bechshøft[4,5], Søren Reitelseder[4,5], Grith Westergaard Højfeldt[4,5], Søren Balling Engelsen[1], Lars Holm[6], Karoline Faust[7], and Dennis Sandris Nielsen[1]

[1] Department of Food Science, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark.

[2] Faculty of Industrial Sciences and Technology, Department of Industrial Biotechnology, Universiti Malaysia Pahang, Pahang, Malaysia.

[3]Department of Environmental Science, Aarhus University, Roskilde, Denmark.

[4] Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital, Copenhagen, NV, Denmark.

[5] Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

[6] School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, United Kingdom.

[7] Department of Microbiology and Immunology, Rega Institute, KU Leuven, Leuven, Belgium.

[*] Correspondence to Hajar Fauzan Ahmad (fauzanahmad@ump.edu.my) and Dennis Sandris Nielsen (dn@food.ku.dk)

Email addresses:

HFA fauzanahmad@ump.edu.my

JLC jcame@food.ku.dk

ŁK krych@food.ku.dk

BK bzo@food.ku.dk

WK wk@envs.au.dk

RLB r.bechshoeft@gmail.com

SR s.reitelseder@gmail.com

GWH grith.westergaard.hoejfeldt.01@regionh.dk

SBE se@food.ku.dk

LH l.holm@bham.ac.uk

KF karoline.faust@kuleuven.be

DSN dn@food.ku.dk

ABSTRACT

Gut microbial dysbiosis have been in the etiology of a number of diseases, yet the presence of fungal communities and their possible association with host health are little understood. This study attempts to identify gut microbial fungal associations with the progression of atherogenic dyslipidemia in a population of older adults by investigating the interplay between dietary intake, gut mycobiome composition,

plasma and fecal metabolome and anthropometric/body-composition measurements of 100 Danes aged 65 to 81 (69.57 ± 3.64) years. The gut mycobiome composition were determined by high-throughput sequencing of internal transcribed spacer (ITS2) gene amplicons, while the plasma and fecal metabolome was determined by GC-TOF-MS. The gut microbiome of the subjects investigated is home to three main eukaryotic phyla, namely Ascomycota, Basidiomycota and Zygomycota, with genera *Penicillium*, *Candida*, and *Aspergillus* being particularly common. Hypertriglyceridemia was associated with fewer observed fungal species, and Bray-Curtis dissimilarity matrix-based analysis showed significant ($P<0.05$) clustering according to fasting levels of circulating plasma triglycerides (Tg) and very low-density lipoprotein (VLDL) cholesterol fasting levels, respectively. Interestingly, neither hypertriglyceridemia nor elevated VLDL levels were reflected in the prokaryotic component of the gut microbiome as determined by 16S rRNA gene amplicon sequencing. Higher levels of Tg and VLDL cholesterol significantly associates with increased relative abundance of genus *Penicillium*, possibly mediated by a higher dietary fat intake (ANOVA, $P<0.05$), and *Aspergillus* and *Guehomyces* were positively associated with SCFAs groups. Collectively, these findings suggest that in older adults' gut mycobiome dysbiosis is associated with hypertriglyceridemia, a known risk factor for development of cardiovascular disease.

Keywords: older-adults, hypertriglyceridemia, dysbiosis, gut mycobiome, host metabolome, triglyceride, VLDL and dietary fat intake

INTRODUCTION

Some of the major challenges in healthy ageing is the deterioration of body and functional capabilities, frailty, and metabolic health. Gut microbiota (GM) dysbiosis has previously been found to be associated with age-related frailty and declines in the physiology of the gastrointestinal tract due to ageing in elderly people as well as being a risk factor for metabolic disorders [1]–[6]. Thus, maintaining a diverse core gut microbiome has been proposed as a possible signature of healthy ageing [7]–[9].

To date, research on the GM of elderly has focused on the bacterial component largely ignoring fungi, archaea and viruses [3], [10]. However, recent studies show that fungi have significant effects in the gut milieu despite their small proportion in number as compared to bacteria [11], and gut mycobiome dysbiosis has been associated with irritable bowel disease (IBD) [12], obesity [13], and carotid atherosclerosis vascular disease [14]. The fungal component of the gut microbiome of healthy individuals has been reported to be dominated by the yeast genera *Saccharomyces*, *Malassezia*, and *Candida* [15].

Age is known as the dominant cardiovascular disease (CVD) risk factor due to dyslipidaemia in both men and women older than 65 years, as compared to younger individuals [16]. Further, elevated triglycerides (Tg) and very low density level (VLDL) cholesterol levels have been associated with subclinical atherosclerosis and dubbed as independent risk factors for CVD [17]. Several large studies suggest that hypertriglyceridemia is related to increased levels of remnant lipoproteins in promoting atherogenesis [18], [19]. The possible mechanisms for this association include excessive free fatty acid release, production of proinflammatory cytokines, coagulation factors, and impaired fibrinolysis [20]. Similarly, Tg are also synthesized

from free fatty acids and glycerol in hepatocytes and then, together with apoB, they form VLDL particles [21].

Here, we report the gut fungal composition, dietary intake, fecal and plasma metabolome, and anthropometric/body-composition measurements among 100 older adult Danes aged 65-81 years and relate this to hypertriglyceridemia (Tg > 1.70 mmol/l). We observed that the fecal mycobiome distribution is strongly associated with variations in Tg and VLDL cholesterol plasma levels.

MATERIALS AND METHODS

Study Design and Participants Recruitment

Participants for this study consisted of 100 older adult Danes from the Counteracting Age-related Loss of skeletal Muscle mass (CALM) cohort. The details about the inclusion criteria has been described elsewhere [22]. All experiments were performed in accordance with the Declaration of Helsinki II and approved by The Danish Regional Committees of the Capital Region (number H-4-2013-070) and with informed consent from all participants, registered at ClinicalTrials.gov (NCT02034760), and data protected under Danish Data Protection Agency 2012-58-0004 – BBH-2015-001 I-Suite.

Sample Collection and Processing

Fecal samples were collected at admission into the cohort. Every sample was placed in an insulated bag with freezer elements until delivery at Bispebjerg Hospital, Copenhagen, Denmark, within 24 hours. The container was stored at -60°C until analysis. In brief, the fecal samples were thawed at 4°C, re-suspended in autoclaved Milli-Q water (1:2 feces/water) prior homogenization for 1 min at high speed (Lab Seward, BA7021). The homogenized fecal samples were aliquoted in 2 mL vials for

usage in this study [22]. For gut microbiome characterization, 200 mg of the fecal pellet was recovered for DNA extraction using the standard protocol from the PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) supplemented with a bead beating step (FastPrep) to enhance cell lysis. Quality and concentration of isolated DNA was measured using NanoDrop 1000 Spectrophotometer (Thermo-Fisher, DE, USA), and was stored at − 20 °C until later use.

The internal transcribed spacer 2 (ITS2) Amplification and Sequencing

The gut mycobiome composition was determined using Illumina MiSeq based sequencing of ITS2 gene region amplicons with adapters compatible for the Nextera Index Kit® (Illumina, CA, USA). For ITS2, the primers used were ITS3_F: 5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GCA TCG ATG AAG AAC GCA GC -3' and ITS4_R: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA TGC -3' [23]. The 1st PCR reaction was performed on a SureCycler 8800 (Agilent Technologies, Santa Clara, USA) using the following temperature profile: denaturation at 95°C for 5 min; 33 cycles of 95°C for 20 s, 56°C for 30 s and 68°C for 45 s; followed by final elongation at 68°C for 5 min, while barcoding (2nd PCR) was performed at 98°C for 1 min; 12 cycles of 98°C for 10 s, 55°C for 20 s and 72°C for 20 s; elongation at 72°C for 5 min. Amplicon concentrations was determined using Qubit® dsDNA BR Assay Kit (Life Technologies, CA, USA) using a Varioskan Flash Multimode Reader (Thermo Fischer Scientific, MA, USA) at 485/530 nm. Samples were pooled in equimolar concentrations and sequenced on a MiSeq platform (Illumina, CA, USA) using the V3, 2x250bp MID pair-ended kit chemistry.

Blood Clinical Parameters, Stool Metabolome and 16S rRNA Gene Amplicon High Throughput Sequencing Data

Phenotypic and blood clinical parameters, stool and plasma metabolome, 3-days weighted dietary records, and 16S rRNA gene amplicon sequencing have been reported previously [24] but here, we integrated these data with gut mycobiome compositional data.

Bioinformatics and Statistical Analysis

For the ITS2 amplicons, the raw dataset containing forward reads with corresponding quality scores were trimmed using USEARCH (*v*6.1) [25]. High quality sequences were subsequently de-replicated, filtered from chimeric reads and *de novo* Operational Taxonomic Units (OTU), with 97% similarity were constructed using the UPARSE pipeline [26]. UNITE was used as reference database for ITS2 amplicons [27]. The Unassigned taxa were then manually re-checked for the best hit as referred to the NCBI nucleotide collection (nr/nt) database using BLAST [28]. Furthermore, the OTUs belonging to plants and Agaricomycetes [29] were manually filtered out as they were identified as common in diet.

Samples were rarefied to 1427 reads per sample, unless otherwise noted, based on rarefaction analysis to optimize the number of sequences per sample without losing too many samples from the dataset (25 samples had less than 1427 reads after removing plant DNA and were thus discarded). Downstream analyses of alpha- and beta-diversity were carried out using QIIME (*v*1.9 and *v*1.8) [30].

The relative distribution of the mycobiome genera registered in 100 samples was calculated, unified and summarized in genus level OTU tables. Alpha diversity measures were expressed as observed species, PD whole tree, and chao1 (sequence similarity 97% OTUs) computed for rarefied OTU tables using the alpha rarefaction

175 workflow. Differences in alpha diversity were determined using a *t*-test-based
176 approach employing the non-parametric (Monte Carlo) method (999 permutations)
177 implemented in the compare alpha diversity workflow. Bray-Curtis dissimilarity
178 matrix were calculated and visualized via Principal Coordinate Analysis (PCoA) as
179 previously described and ADONIS was used to evaluate group differences [31], [32].
180 Additionally, analysis and visualization of microbiome communities was conducted
181 in R version 3.4.3. Plots were made using ggplot2 package version 2.2.1. Significant
182 differences in the level of Tg between the groups were assessed using Welch's test.
183 Correlation between the variables was computed by Spearman Rank correlation.
184 Differentially abundant taxa were determined by LEfSe analysis [33]. Only
185 functional categories with log LDA scores of >2.0, and alpha values of < 0.05 for the
186 factorial Kruskal-Wallis test among classes and pairwise Wilcoxon test between
187 subclasses were considered as differential signatures discriminating between groups.
188 A redundancy analysis (RDA) model was used to estimate the amount of variation
189 among the most abundant mycobiome communities uniquely explained by dietary
190 patterns after controlling for Tg status (Normal or Hypertriglyceridemia). The
191 matrices were Hellinger-transformed using the "decostand" function followed by the
192 "rda" function of the "vegan" package in R [34]. Significance levels determined by
193 ANOVA and the R^2 values were generated by the "RsquareAdj" function in R [35],
194 [36]. Correlation of anthropometric/body-composition data, fecal and plasma
195 metabolome, and gut mycobiome associations were investigated by sparse Partial
196 Least Squares (sPLS) performed using the R package mixOmics [37]. The Bonferroni
197 or Benjamini-Hochberg approaches were used to adjust for multiple testing, where
198 appropriate. For all statistical tests, unless stated otherwise, a p-value of $p < 0.05$ was
199 considered as statistically significant.

Data availability

The raw sequence data of this study were uploaded to EBI's ENA under accession codes PRJEB34758 and PRJEB34758.

RESULTS

Clinical Characteristics

In this study, a total of 100 home-dwelling rather sedentary elderly Danes above the age of 65 years without any known diseases were enrolled in the CALM study [22]. Blood parameters and anthropometric measurements were determined. Generally, all the participants had no systemic disease, did not receive any treatment with drugs that affected glucose and lipid metabolisms, nor did they take antibiotics. In this study we stratified the participants according to a newly proposed cut-off of fasting Tg levels; Tg > 1.70 mmol/l among the elderly [21], [38] defining a group of hypertriglyceridemia (HG, N=25) and normotriglyceridemia (NG, N=75). The HG group displayed the typical features of this phenotype in comparison with NG group, such as higher BMI ($p = 0.003$), higher blood pressure; diastolic ($p = 0.05$), higher lipid profiles; total cholesterol ($p = 0.001$), HDL ($p = <0.001$), and LDL ($p = 0.02$), and glucose metabolism; fasting OGTT ($p = 0.009$), Hemoglobin A1c ($p = 0.021$), Proinsulin C-peptide ($p = <0.001$) when compared by Welch t-test (Table 1). Nevertheless, age and fasting glucose did not present significant differences between the HG and NG groups.

Fungal Diversity and Composition in HG and NG

For the entire cohort, the average number of observed fungal species was 12 (min = 1, max = 86), but with large deviations between individuals (standard deviation = 14) (Supplementary Figure 1). The gut mycobiome of the investigated

older adults consist of a total of 4 phyla, 15 classes, 91 families and 128 different fungal genera. The elderly gut is home to three main phyla, namely Ascomycota, Basidiomycota and Zygomycota. The most prevalent genera among the elderly Danes were *Penicillium*, followed by *Candida*, and *Aspergillus* (Supplementary Table 1), as previously described in preliminary studies using similar cohort [39]–[41].

Associations with Serum Lipid Profiles for HG Phenotype

In order to determine whether the mycobiome was associated with host hypertriglyceridemia phenotypes, we utilized clinical metadata collected from CALM study participants focusing on biomarkers related to serum lipids and glucose metabolism. Alpha and beta diversity analyses showed clustering of samples according to Tg and VLDL cholesterol covariates. For both Tg and VLDL covariates, species richness and phylogenetic diversity (assessed using three different indexes, namely observed species, PD whole tree, and chao1) were significantly decreased in HG as compared with NG group samples (Figure 1(i to iii), and Figure 2(i to iii); $p < 0.05$).

Based on Tg levels, Bray-Curtis dissimilarity analysis confirmed that gut mycobiome composition was significantly associated with NG and HG status (Figure 1 (iv), $p = 0.001$, R = 0.06). Likewise, a significant association was observed between mycobiome and VLDL cholesterol status, based on Bray-Curtis dissimilarity analysis ($p = 0.002$, R = 0.06) as shown in Figure 2 (iv).

Importantly, analysis of previously published 16S rRNA gene amplicon data [24], showed that the prokaryote community does not cluster in relation to blood triglyceride, nor VLDL cholesterol levels (Figure 1 (v) and 2 (v), $p = > 0.05$).

Genus *Penicillium* associated with the HG

Interestingly, the genus *Penicillium* was prevalent in every individual classified with HG (Figure 3 (i)). To further investigate the relationship between the fungal taxa and Tg levels, Pearson's correlation tests were conducted to evaluate top most abundant taxa. Genus *Penicillium* showed strong correlation with increased levels of Tg (R=0.311, $p = 0.006$) while other abundant genera, namely *Candida*, *Aspergillus*, and Unclassified Saccharomycetales did not show any significant correlation with Tg levels (Figure 3 (ii)).

The most relevant taxa responsible for the differences between NG and HG were identified by LEfSe analysis. Healthy individuals had a significantly higher relative abundance of autochthonous mycobiome taxa, when compared with hypertriglyceridemia elderly from HG. The genus *Aspergillus*, as well as members of family Saccharomycetales, Saccharomycodaceae, Mucoraceae, Saccharomycetaceae and order Capnodiales were significantly more abundant in NG individuals, whereas genus *Penicillium* and the order Eurotiales were strongly associated with HG as shown in Figure 3 (iii).

Effect of Diet on the Mycobiome among NG and HG

Notably, RDA analysis showed significant clustering of NG and HG groups and dietary patterns, which again was reflected in the gut mycobiome. Among the HG population, the dietary elements related to saturated fatty acids ($p = 0.004$) and fats ($p< 0.05$) were associated with higher relative abundance of *Penicillium* and Rhodotorula species (Figure 4). Dietary elements related to vegetable oils, fibres, and legumes were shown to be modestly associated with lower Tg levels, no significant associations appeared with mycobiome profiles like *Aspergillus*, *Candida*, *Mucor*,

unclassified Saccharomycetales, unclassified Capnodiales and others (ANOVA with Bonferroni correction, $p > 0.05$).

SCFAs and Untargeted Serum and Fecal Metabolites Correlate with Gut Mycobiome of the Elderly

sPLS analyses were performed to determine possible correlations between the dominant fungal genera and untargeted plasma and fecal metabolites. *Aspergillus* and *Guehomyces* were positively correlated with levels of the stool metabolites butyrate, butanoic acid, and valeric acid. *Cyberlindnera* and an unclassified *Pleosporales* member were positively correlated with plasma metabolites such as ribitol and 1-piperidineacetonitrile (Figure 5).

DISCUSSION

Previous studies have characterized human gut fungal communities from diverse age groups [13], [15], [42], but information describing the gut mycobiome of older adults is sparse. Several studies suggest that prokaryote communities are hallmarks for atherosclerosis pathogenesis [43]–[46]. Here, we present data showing an association between gut mycobiome dysbiosis and hypertriglyceridemia in a homogeneous and well-characterized healthy cohort of older Danish adults.

Collectively, we found that the richness of the gut mycobiome among the studied population was low within individuals. Likewise, a previous study also showed lower alpha diversity of eukaryote community as compared to the gut bacterial community [15], which is furthermore decreasing throughout the course of life due to ageing [42]. In the present study, *Penicillium* was predominant in many of the subjects. In contrast, previous studies have indicated that *Candida*,

298 *Saccharomyces* and *Cladosporium* are common gut commensal fungi, where the
299 *Candida* genus predominantly forms the core mycobiome in the gut [15], [47], [48].
300 The causes of hypertriglyceridemia can be a result of interactions between
301 genetic precursors [49], non-genetic factors such as unhealthy diet and lifestyle [50],
302 diseases related to metabolic syndromes [51], and usage of some types of medicine
303 [52]. A total of 25 of the included participants had Tg levels above the recommended
304 level of 1.7 mmol/L [53]–[56]. We observed that the participants with high Tg levels
305 were strongly associated with low in gut mycobiome community richness and
306 diversity. Similarly, a similar pattern of good versus unhealthy VLDL cholesterol
307 levels strongly linked to the mycobiome composition was observed. Hence, the
308 increased trends in circulating cholesterol of Tg and VLDL in relation to specific gut
309 mycobiome clusters could be used as potential indicators for describing the
310 hypertriglyceridemia phenotype.
311 LEfSe analysis showed that an upsurge in *Penicillium* genus could be
312 associated with hypertriglyceridemia. However, the utility of *Penicillium* as a
313 biomarker in predicting the progression of atherosclerosis among older adults is
314 unclear, and therefore, this association warrants further investigation. Another
315 interesting observation was the positive association between the relative abundance of
316 the genus *Mucor* and the subjects with normal Tg levels. This is in line with previous
317 studies showing that *Mucor* is abundant in the gut of non-obese subjects [13], and
318 confer protection from the risk of CVD [14]. In the present study, subjects stratified
319 into NG and HG groups also differed in BMI levels (NG = 25.4±3.5; TG = 26.9±3.4
320 $kg \cdot m^{-2}$; $p = 0.003$), but no clustering between the gut mycobiome and BMI was
321 observed.

322 Interestingly, strong correlations between dietary data and gut mycobiome
323 members and hypertriglyceridemia indicate a role of factors in the disease.
324 Particularly, in the case of *Penicillium*, positive correlations with a diet rich in
325 saturated fatty acids and other lipids are common indicators for higher Tg and VLDL
326 cholesterol in circulating serum of hosts, which have been reported to be associated
327 with signatures in coronary atherosclerotic plaques [57], aneurysms of the carotid
328 artery [58], and negatively correlated with HDL-cholesterol [13]. Hence, we speculate
329 that these dietary intakes such as fermented dairy products such as cheese[59] might
330 contribute to increased Tg and VLDL cholesterol levels among the older adult
331 subjects enrolled in this study.
332 Finally, we investigated the relationship of the stool and plasma metabolomes
333 and the gut mycobiome by performing regression-based modelling on 329 metabolites
334 and 107 OTUs that were assigned to at least the genus level. We observed that
335 *Aspergillus* together with *Guehomyces* was positively associated with faecal SCFA
336 and specifically valeric, butyric and butanoic acids. Inversely, ribitol – the sugar
337 alcohol from fruit fermentation by reduction of ribose [60], was positively correlated
338 with *Cyberlindnera* and unclassified *Pleosporales*. Previously, *Aspergillus* was found
339 to negatively correlate with SCFAs in subjects on a carbohydrate-rich diet [61].
340 However, a recent study showed that *Aspergillus* species are capable of producing
341 SCFAs metabolites from fibre rich diet substances [62]. No significant correlations
342 between *Penicillium* abundance and any of the metabolites were identified.
343 Most fungal species detected in gut mycobiome studies are considered
344 transient components of the community, and putatively of environmental origin,
345 where the composition in particular is influenced by food-borne fungi and life-style
346 [63], [64], together with other factors such as age, gender and geographical setting

[7], [42], [65]. However, due to the dearth of information related to gut mycobiome studies, little is known about its relationship with fecal metabolome and other factors such as environmental effects, diet and life style [66] that may lead to hypertriglyceridemia.

CONCLUSION

To the best of our knowledge, this is the first study to demonstrate that hypertriglyceridemia among elderly is associated with gut mycobiome dysbiosis characterized by overall reduction of the microbial richness and diversity as well as dysbiosis pattern of the gut mycobiome structure compared to those senior citizens with normal levels of circulating plasma triglycerides. These findings also highlight that the everyday diet shapes the gut mycobiome and host metabolome components among the older citizens. However, it remains unknown whether the microbial markers and patterns identified here are also adaptable to changes in life styles and applicable to other cultures in the world.

ACKNOWLEDGEMENTS

This project was supported by the University of Copenhagen-funded project "Counteracting Age-related Loss of Skeletal Muscle (CALM)", the Danish Dairy Research Foundation, Arla Foods Ingredients P/S, stipends from Universiti Malaysia Pahang, Malaysia, and Ministry of Education, Malaysia.

AUTHORS CONTRIBUTION

HFA performed laboratory procedures; DSN, LH, SBE, SR, JLC, HFA designed the study; RLB, SR, GWH, LH collected and provided samples as well as analyzed

clinical data; BK carried out metabolome analysis; WK carried out sequencing of libraries, HFA, JLC, ŁK, KF, DSN coupled and analyzed the different datasets of the study; HFA and DSN drafted the manuscript. All authors commented on, added paragraphs and approved the last version of this manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

This manuscript has not been published elsewhere and has not been submitted simultaneously for publication elsewhere. The authors declare no conflict of interest.

SUPPLEMENTARY INFORMATION

Supplementary Figure 1 : Alpha diversity. All the matrices showed that every individual contains low alpha diversity of fungal community at rarefaction of 1427 reads per sequence.

Supplementary Table 1 : Taxonomic composition of all fungi sequences identified at genera level among the healthy elderly Danes (%).

REFERENCES

[1] P. Alonso-Fernández and M. Fuente, “Role of the immune system in aging and longevity,” *Curr Aging Sci*, vol. 4, 2011.

[2] S. Rampelli *et al.*, “Functional metagenomic profiling of intestinal microbiome in extreme ageing,” vol. 5, no. 12, pp. 902–912, 2013.

[3] M. J. Claesson *et al.*, “Gut microbiota composition correlates with diet and health in the elderly.,” *Nature*, vol. 488, no. 7410, pp. 178–84, Aug. 2012.

[4] S. Saraswati and R. Sitaraman, “Aging and the human gut microbiota—

397 from correlation to causality ," *Frontiers in Microbiology* , vol. 5. p. 764,
398 2015.
399 [5] N. Thevaranjan *et al.*, "Age-Associated Microbial Dysbiosis Promotes
400 Intestinal Permeability, Systemic Inflammation, and Macrophage
401 Dysfunction," *Cell Host Microbe*, vol. 21, no. 4, pp. 455-466.e4, Apr. 2017.
402 [6] S. Z. N. Effa, S. J. Phang, and H. F. Ahmad, "Autoimmune Diseases and Gut
403 Symbionts : The Unpopular Liaison," *Malaysian J. Med. Heal. Sci.*, vol. 15, no.
404 13, pp. 165–172, 2019.
405 [7] E. Biagi *et al.*, "Gut Microbiota and Extreme Longevity," *Curr. Biol.*, vol. 26,
406 no. 11, pp. 1480–1485, Aug. 2016.
407 [8] M. A. Jackson *et al.*, "Signatures of early frailty in the gut microbiota,"
408 *Genome Med.*, vol. 8, no. 1, pp. 1–11, 2016.
409 [9] P. W. O'Toole and I. B. Jeffery, "Gut microbiota and aging," *Science (80-.).*,
410 vol. 350, no. 6265, pp. 1214–1215, Dec. 2015.
411 [10] S.-H. Park, K.-A. Kim, Y.-T. Ahn, J.-J. Jeong, C.-S. Huh, and D.-H. Kim,
412 "Comparative analysis of gut microbiota in elderly people of urbanized
413 towns and longevity villages," *BMC Microbiol.*, vol. 15, no. 1, pp. 1–9, 2015.
414 [11] C. A. Kumamoto, "The Fungal Mycobiota: Small Numbers, Large Impacts,"
415 *Cell Host Microbe*, vol. 19, no. 6, pp. 750–751, Jun. 2016.
416 [12] H. Sokol *et al.*, "Fungal microbiota dysbiosis in IBD," *Gut* , Feb. 2016.
417 [13] M. Mar Rodríguez *et al.*, "Obesity changes the human gut mycobiome," *Sci.*
418 *Rep.*, vol. 5, p. 14600, 2015.
419 [14] M. R. Chacón *et al.*, "The gut mycobiome composition is linked to carotid
420 atherosclerosis," *Benef. Microbes*, vol. 9, no. 2, pp. 185–198, Nov. 2017.
421 [15] A. K. Nash *et al.*, "The gut mycobiome of the Human Microbiome Project

422 healthy cohort," *Microbiome*, vol. 5, no. 1, p. 153, Nov. 2017.

423 [16] C. Andersson and R. S. Vasan, "Epidemiology of cardiovascular disease in
424 young individuals," *Nat. Rev. Cardiol.*, vol. 15, p. 230, Oct. 2017.

425 [17] J. Peng, F. Luo, G. Ruan, R. Peng, and X. Li, "Hypertriglyceridemia and
426 atherosclerosis," *Lipids Health Dis.*, vol. 16, p. 233, Dec. 2017.

427 [18] N. Sarwar *et al.*, "Triglycerides and the Risk of Coronary Heart Disease,"
428 *Circulation*, vol. 115, no. 4, pp. 450 LP – 458, Jan. 2007.

429 [19] N. BG, M. Benn, P. Schnohr, and A. Tybjærg-Hansen, "Nonfasting
430 triglycerides and risk of myocardial infarction, ischemic heart disease, and
431 death in men and women," *JAMA*, vol. 298, no. 3, pp. 299–308, Jul. 2007.

432 [20] Z. Liu *et al.*, "Associations of triglyceride levels with longevity and frailty: A
433 Mendelian randomization analysis," *Sci. Rep.*, vol. 7, p. 41579, Jan. 2017.

434 [21] Ž. Reiner, "Hypertriglyceridaemia and risk of coronary artery disease,"
435 *Nat. Rev. Cardiol.*, vol. 14, p. 401, Mar. 2017.

436 [22] R. Bechshøft *et al.*, "Counteracting Age-related Loss of Skeletal Muscle
437 Mass: a clinical and ethnological trial on the role of protein
438 supplementation and training load (CALM Intervention Study): study
439 protocol for a randomized controlled trial," *Trials*, vol. 17, no. 1, pp. 1–17,
440 2016.

441 [23] J. White, T.J., Bruns, T., Lee, S., & Taylor, "Amplification and direct
442 sequencing of fungal ribosomal RNA genes for phylogenetics.," in *PCR*
443 *Protocols: A guide to Methods and Applications.*, New York, USA: Academic
444 Press, Inc, 1990, pp. 315–322.

445 [24] J. L. Castro-Mejía *et al.*, "Physical fitness in community-dwelling older
446 adults is linked to dietary intake, gut microbiota, and metabolomic

447 signatures," *Aging Cell*, vol. n/a, no. n/a, p. e13105, Jan. 2020.

448 [25] R. C. Edgar, "Search and clustering orders of magnitude faster than

449 BLAST," *Bioinformatics*, vol. 26, 2010.

450 [26] R. C. Edgar, "UPARSE: Highly accurate OTU sequences from microbial

451 amplicon reads," *Nat. Methods*, vol. 10, no. 10, pp. 996–998, Oct. 2013.

452 [27] K. Urmas *et al.*, "Towards a unified paradigm for sequence-based

453 identification of fungi," *Mol. Ecol.*, vol. 22, no. 21, pp. 5271–5277, Aug.

454 2013.

455 [28] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, "Basic local

456 alignment search tool," *J. Mol. Biol.*, vol. 215, no. 3, pp. 403–410, 1990.

457 [29] D. S. Hibbett, "A phylogenetic overview of the Agaricomycotina,"

458 *Mycologia*, vol. 98, no. 6, pp. 917–925, Nov. 2006.

459 [30] J. G. Caporaso *et al.*, "QIIME allows analysis of high-throughput community

460 sequencing data," *Nat. Methods*, vol. 7, no. 5, pp. 335–336, 2010.

461 [31] A. R. Williams *et al.*, "Dietary cinnamaldehyde enhances acquisition of

462 specific antibodies following helminth infection in pigs," *Vet. Immunol.*

463 *Immunopathol.*, vol. 189, 2017.

464 [32] M. J. Anderson, "A new method for non-parametric multivariate analysis of

465 variance," *Austral Ecol.*, vol. 26, no. 1, pp. 32–46, Feb. 2001.

466 [33] N. Segata *et al.*, "Metagenomic biomarker discovery and explanation,"

467 *Genome Biol.*, vol. 12, no. 6, pp. R60–R60, Jun. 2011.

468 [34] P. Legendre and E. D. Gallagher, "Ecologically meaningful transformations

469 for ordination of species data," *Oecologia*, vol. 129, no. 2, pp. 271–280,

470 2001.

471 [35] C.-Y. Lay *et al.*, "Canola Root–Associated Microbiomes in the Canadian

Prairies ," *Frontiers in Microbiology* , vol. 9. p. 1188, 2018.

[36] G. Dubois, C. Girard, F.-J. Lapointe, and B. J. Shapiro, "The Inuit gut microbiome is dynamic over time and shaped by traditional foods," *Microbiome*, vol. 5, no. 1, p. 151, Nov. 2017.

[37] F. Rohart, B. Gautier, A. Singh, and K.-A. Lê Cao, "mixOmics: An R package for 'omics feature selection and multiple data integration," *PLOS Comput. Biol.*, vol. 13, no. 11, p. e1005752, Nov. 2017.

[38] A. F. Members: *et al.*, "'European Guidelines on cardiovascular disease prevention in clinical practice (version 2012)' The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by r," *Eur. Heart J.*, vol. 33, no. 17, p. 2126, Sep. 2012.

[39] H. F. Bin Ahmad *et al.*, "The gut mycobiome of elderly danes," in *Proceedings of the Danish Microbiological Society Annual Congress 2016*, 2016.

[40] H. F. Bin Ahmad *et al.*, "Deciphering the association networks of mycobiome communities among the elderly Danes," in *Cell Symposium: Exercise Metabolism*, 2017.

[41] R. Vemuri, M. E. Shankar, M. Chieppa, R. Eri, and K. Kavanagh, "Beyond Just Bacteria: Functional Biomes in the Gut Ecosystem Including Virome, Mycobiome, Archaeome and Helminths," *Microorganisms* , vol. 8, no. 4. 2020.

[42] F. Strati *et al.*, "Age and Gender Affect the Composition of Fungal Population of the Human Gastrointestinal Tract," *Front. Microbiol.*, vol. 7, p. 1227, 2016.

[43] J. M. Brown and S. L. Hazen, "The Gut Microbial Endocrine Organ: Bacterially-Derived Signals Driving Cardiometabolic Diseases," *Annu. Rev. Med.*, vol. 66, pp. 343–359, 2015.

[44] B. B. Lanter, K. Sauer, and D. G. Davies, "Bacteria Present in Carotid Arterial Plaques Are Found as Biofilm Deposits Which May Contribute to Enhanced Risk of Plaque Rupture," *mBio* , vol. 5, no. 3, Jul. 2014.

[45] D. A. Chistiakov, Y. V Bobryshev, E. Kozarov, I. A. Sobenin, and A. N. Orekhov, "Role of gut microbiota in the modulation of atherosclerosis-associated immune response," *Front. Microbiol.*, vol. 6, p. 671, Jun. 2015.

[46] D. Y. Li and W. H. W. Tang, "Gut Microbiota and Atherosclerosis," *Curr. Atheroscler. Rep.*, vol. 19, no. 10, p. 39, 2017.

[47] P. D. Scanlan and J. R. Marchesi, "Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces," *ISME J*, vol. 2, no. 12, pp. 1183–1193, Jul. 2008.

[48] D. M. Underhill and I. D. Iliev, "The mycobiota: interactions between commensal fungi and the host immune system," *Nat Rev Immunol*, vol. 14, no. 6, pp. 405–416, Jun. 2014.

[49] G. F. Watts, E. M. M. Ooi, and D. C. Chan, "Demystifying the management of hypertriglyceridaemia," *Nat. Rev. Cardiol.*, vol. 10, p. 648, Sep. 2013.

[50] P. M. Hunter and R. A. Hegele, "Functional foods and dietary supplements for the management of dyslipidaemia," *Nat. Rev. Endocrinol.*, vol. 13, p. 278, Jan. 2017.

[51] S. M. Grundy, "Hypertriglyceridemia, insulin resistance, and the metabolic syndrome," *Am. J. Cardiol.*, vol. 83, no. 9, Supplement 2, pp. 25–29, 1999.

[52] H. K. Singh, M. S. Prasad, A. K. Kandasamy, and K. Dharanipragada, "Tamoxifen-induced hypertriglyceridemia causing acute pancreatitis," *J. Pharmacol. Pharmacother.*, vol. 7, no. 1, pp. 38–40, Feb. 2016.

[53] A. C. Scott *et al.*, "Chemical Mediators of the Muscle Ergoreflex in Chronic Heart Failure," *Circulation*, vol. 106, no. 2, pp. 214 LP – 220, Jul. 2002.

[54] L. Berglund *et al.*, "Evaluation and Treatment of Hypertriglyceridemia: An Endocrine Society Clinical Practice Guideline," *J. Clin. Endocrinol. Metab.*, vol. 97, no. 9, pp. 2969–2989, Sep. 2012.

[55] T. J. Anderson *et al.*, "2012 Update of the Canadian Cardiovascular Society Guidelines for the Diagnosis and Treatment of Dyslipidemia for the Prevention of Cardiovascular Disease in the Adult," *Can. J. Cardiol.*, vol. 29, no. 2, pp. 151–167, Feb. 2013.

[56] T. Teramoto *et al.*, "Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan —2012 Version," *J. Atheroscler. Thromb.*, vol. 20, no. 6, pp. 517–523, 2013.

[57] S. J. Ott *et al.*, "Fungi and inflammatory bowel diseases: alterations of composition and diversity," *Scand J Gastroenterol*, vol. 43, 2008.

[58] A. Hot *et al.*, "Fungal Internal Carotid Artery Aneurysms: Successful Embolization of an Aspergillus-Associated Case and Review," *Clin. Infect. Dis.*, vol. 45, no. 12, pp. e156–e161, Dec. 2007.

[59] G. Gillot *et al.*, "Insights into Penicillium roqueforti Morphological and Genetic Diversity," *PLoS One*, vol. 10, no. 6, p. e0129849, Jun. 2015.

[60] R. L. P. Jump *et al.*, "Metabolomics analysis identifies intestinal microbiota-derived biomarkers of colonization resistance in clindamycin-treated

547 mice," *PLoS One*, vol. 9, no. 7, pp. e101267–e101267, Jul. 2014.

548 [61] C. Hoffmann *et al.*, "Archaea and Fungi of the Human Gut Microbiome:
549 Correlations with Diet and Bacterial Residents," *PLoS One*, vol. 8, no. 6, p.
550 e66019, Jun. 2013.

551 [62] E. Baltierra-Trejo, J. M. Sánchez-Yáñez, O. Buenrostro-Delgado, and L.
552 Márquez-Benavides, "Production of short-chain fatty acids from the
553 biodegradation of wheat straw lignin by Aspergillus fumigatus," *Bioresour.*
554 *Technol.*, vol. 196, pp. 418–425, 2015.

555 [63] A. M. Madsen *et al.*, "Generation and Characterization of Indoor Fungal
556 Aerosols for Inhalation Studies," *Appl. Environ. Microbiol.* , vol. 82, no. 8, pp.
557 2479–2493, Apr. 2016.

558 [64] H. E. Hallen-Adams and M. J. Suhr, "Fungi in the healthy human
559 gastrointestinal tract," *Virulence*, vol. 8, no. 3, pp. 352–358, Apr. 2017.

560 [65] T. Yatsunenko, F. E. Rey, M. J. Manary, I. Trehan, M. G. Dominguez-Bello,
561 and M. Contreras, "Human gut microbiome viewed across age and
562 geography," *Nature*, vol. 486, 2012.

563 [66] T. Jensen *et al.*, "Whey protein stories – An experiment in writing a
564 multidisciplinary biography," *Appetite*, vol. 107, 2016.

565

566

567

568

569

570

571

572 **Figure 1:** Gut mycobiome composition in association with Tg; Hypertriglyceridemia
573 (HG) is defined when Tg > 1.77 mmol/l. Normotriglyceridemia (NG) when Tg <
574 1.77 mmol/l.
575 i), ii) and iii) Alpha diversity measures. Differences in alpha diversity in gut
576 mycobiome between two groups according to triglycerides levels are shown by the
577 indices Observed species, PD whole tree and Chao1 *p<0.05.
578 iv) Gut Mycobiome composition is linked to Tg-levels. Principal Coordinates
579 Analysis (PCoA) plot based on Bray–Curtis dissimilarity matrix. Adonis analysis
580 showed significant separation between the groups (Bray-Curtis, R =0.06, adonis; p =
581 0.001).
582 v) Gut prokaryotic composition is not associated with Tg-levels. PCoA plot based on
583 Bray–Curtis dissimilarity matrix. Adonis-analysis showed no significant separation
584 between the groups.
585
586
587
588
589
590
591
592
593
594
595
596

Figure 2: Gut mycobiome composition in association with VLDL.

Hypertriglyceridaemia (HG) is defined when VLDL > 0.77 mmol/l.

i), ii) and iii) Alpha diversity measures. Differences in alpha diversity in gut mycobiome between two groups according to VLDL levels are shown by the indices Observed species, PD whole tree and Chao1 *p <0.05.

iv) Gut mycobiome composition is linked to VLDL-levels. Principal Coordinates Analysis (PCoA) plot based on Bray– Curtis dissimilarity matrix. Adonis analysis showed significant separation between the groups (Bray-Curtis, R =0.06, adonis; p = 0.002).

v) Gut prokaryotic composition is not associated with VLDL-levels. PCoA plot based on Bray–Curtis dissimilarity matrix. Adonis-analysis showed no significant separation between the groups.

622 **Figure 3**: Dysbiosis patterns of the gut mycobiome.

623 i) Gut mycobiome composition (relative abundance) of elderly Danes based as

624 determined by ITS2 high throughput amplicon sequencing.

625 ii) Correlation between the top most abundant taxa with Tg levels. The Spearman

626 Rank probability (P) and correlation (R) are shown in the graphs.

627 iii) LEfSe was conducted to explore potential mycobiome differences between NG

628 and HG groups. LDA Score was constructed, and the bar represents a log10

629 transformed LDA score. The red color represents taxa that corresponding to HG, and

630 the green color represents NG. All taxa presented are significant, $p < 0.05$ confirmed

631 by alpha value for the factorial Kruskal-Wallis test among classes, and the

632 discriminative threshold was set > 2.0.

633

634

635

636

637

638

639

640

641

642

643

644

645

646

647 **Figure 4:** RDA biplot at OTU level with Hellinger-transformed data. Red dots
648 represent individuals with high Tg levels (Hypertriglyceridemia, HG) and green dots,
649 individuals with normal Tg levels. Cut-off for plotted factors was ANOVA with
650 Bonferroni correction, $p < 0.05$.
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671

672 **Figure 5:** Sparse partial least squared correlations (sPLS) for mycobiome and
673 untargeted fecal metabolomes. sPLS in regression mode (predict Y from X) to model
674 a causal relationship between the most relevant of fungal genera and metabolites from
675 serum and stool. Heatmap displaying the relative accumulation patterns using color-
676 coding (green for negative correlation, and red for positive correlation) of 14
677 untargeted metabolites against 16 fungal communities.

678

679

680

681

682

683

684

685

686

687

688

689

690

691

692 **TABLE**

693 **Table 1.** Clinical and anthropometrical features of the study groups. Data are given as
694 mean ± standard deviation (SD). The Welch's t-test outcomes are presented and
695 significant *P*-values indicated by * are included, $p < 0.05$.
696
697
698
699
700
701

Table 1. Clinical and anthropometrical features of the study groups. Data are given as mean ± standard deviation (SD). The Welch's t-test outcomes are presented and significant *P*-values indicated by * are included, $p < 0.05$.

Features	Normotriglyceridemia Group (NG) Tg <1.70 mmol/l	Hypertriglyceridemia Group HG Tg >1.70 mmol/l	*P*-value
Total participants, N	70	30	
Age (years)	69.27±3.48	70.27±3.94	0.106
BMI (kg/cm^2)	24.81±3.29	26.87±3.43	*0.003
Blood pressure			
Systolic (mmHg)	142.86±21.37	144.57±15.54	0.347
Diastolic (mmHg)	83.79±10.03	87.67±11.97	*0.050
Lipid profile			
Total cholesterol (mmol/l)	5.54±0.89	6.14±0.91	*0.001
HDL-cholesterol (mmol/l)	1.92±0.46	1.50±0.43	*<0.001
LDL-cholesterol (mmol/l)	3.12±0.86	3.53±0.96	*0.020
VLDL-cholesterol (mmol/l)	0.51±0.14	1.04±0.24	*<0.001
Fasting triglycerides (mmol/l)	1.11±0.30	2.43±0.72	0.08
Glucose metabolism			
Fasting glucose (mmol/l)	5.37±0.43	5.51±0.59	0.115
OGTT 120 glucose (mmol/l)	6.50±1.60	7.35±1.57	*0.009
Haemoglobin A1c (mmol/mol)	35.19±3.21	36.57±2.81	*0.021
Proinsulin C-peptide (*p*mol/l)	623.27±213.56	916.46±314.86	*<0.001

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; OGTT 120, oral glucose tolerant test at 120 minutes; Haemoglobin A1c, glycated haemoglobin,

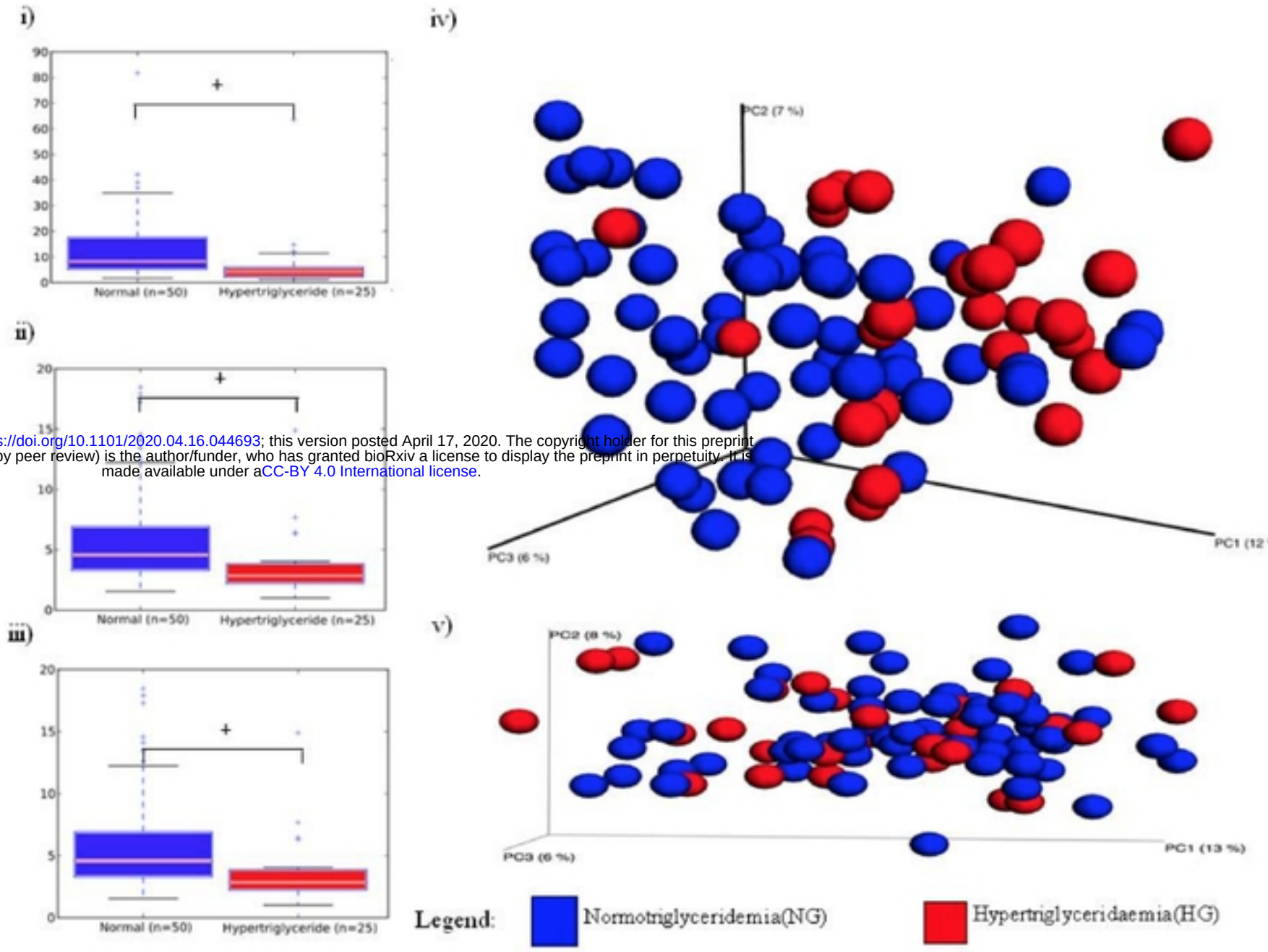


Figure 1: Gut mycobiome composition in association with Tg; Hypertriglyceridemia (HG) is defined when Tg > 1.77 mmol/l. Normotriglyceridemia (NG) when Tg < 1.77 mmol/l. i), ii) and iii) Alpha diversity measures. Differences in alpha diversity in gut mycobiome between two groups according to triglycerides levels are shown by the indices Observed species, PD whole tree and Chao1 $^{+}p<0.05$. iv) Gut Mycobiome composition is linked to Tg-levels. Principal Coordinates Analysis (PCoA) plot based on Bray–Curtis dissimilarity matrix. Adonis analysis showed significant separation between the groups (Bray-Curtis, R =0.06, adonis; $p = 0.001$). v) Gut prokaryotic composition is not associated with Tg-levels. PCoA plot based on Bray–Curtis dissimilarity matrix. Adonis-analysis showed no significant separation between the groups.

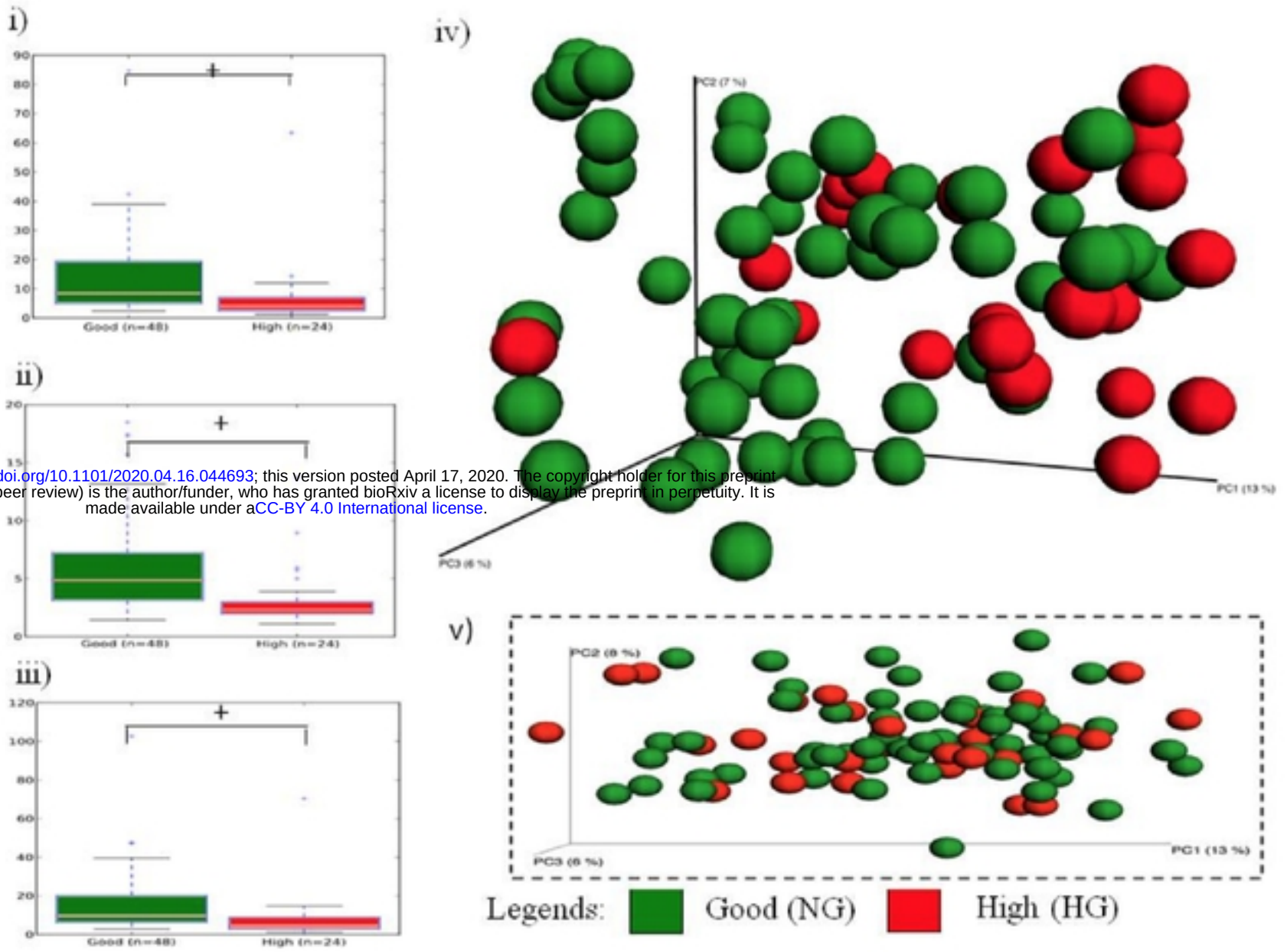
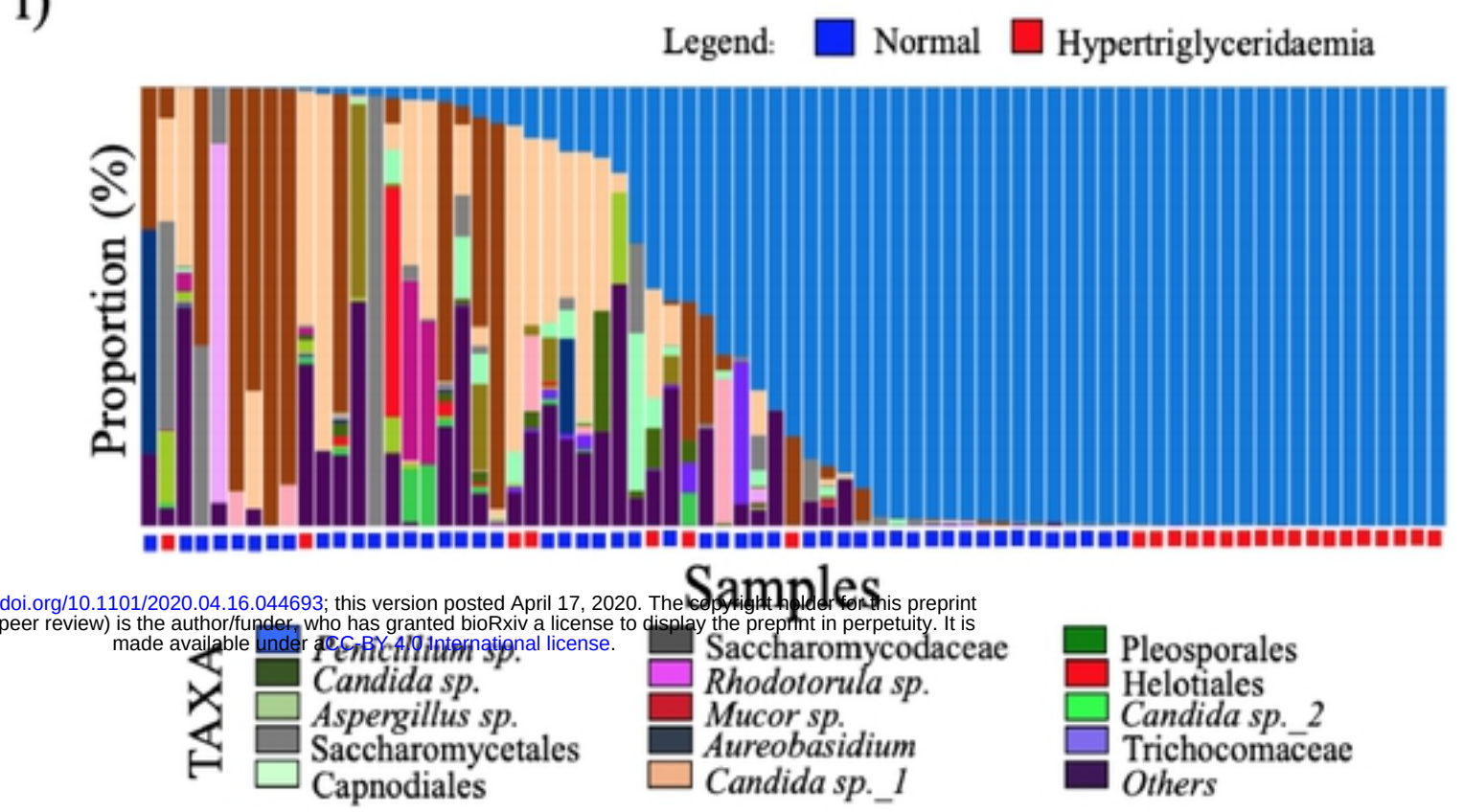


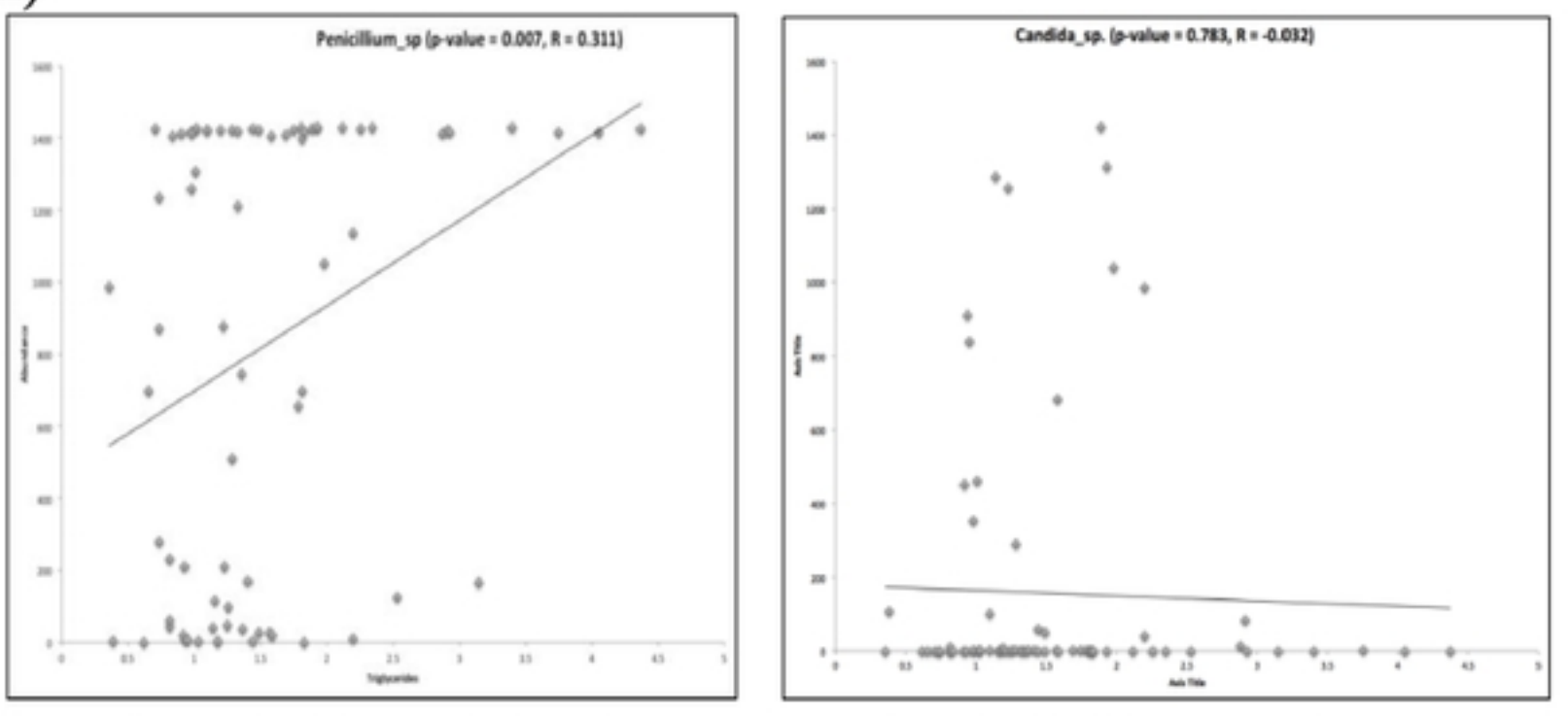
Figure 2: Gut mycobiome composition in association with VLDL.

Hypertriglyceridaemia (HG) is defined when VLDL > 0.77 mmol/l. i), ii) and iii) Alpha diversity measures. Differences in alpha diversity in gut mycobiome between two groups according to VLDL levels are shown by the indices Observed species, PD whole tree and Chao1 ^{+}p <0.05. iv) Gut mycobiome composition is linked to VLDL-levels. Principal Coordinates Analysis (PCoA) plot based on Bray– Curtis dissimilarity matrix. Adonis analysis showed significant separation between the groups (Bray-Curtis, R =0.06, adonis; p = 0.002). v) Gut prokaryotic composition is not associated with VLDL-levels. PCoA plot based on Bray–Curtis dissimilarity matrix. Adonis-analysis showed no significant separation between the groups.

i)



ii)



Penicillium (p-value = 0.007, R = 0.311)

Candida (p-value = 0.783, R = -0.032)

iii)

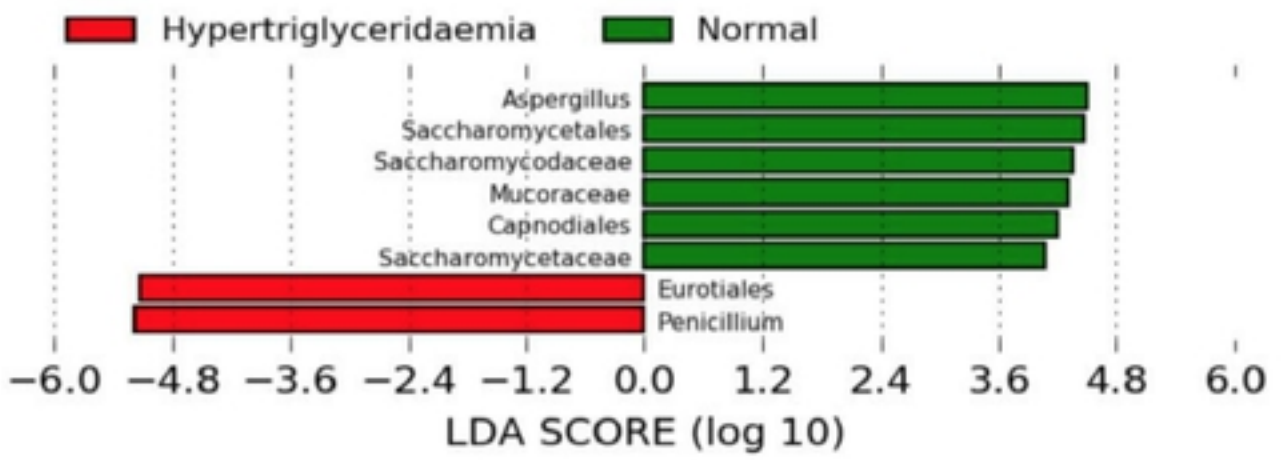


Figure 3: Dysbiosis patterns of the gut mycobiome. i) Gut mycobiome composition (relative abundance) of elderly Danes based as determined by ITS2 high throughput amplicon sequencing. ii) Correlation between the top most abundant taxa with Tg levels. The Spearman Rank probability (P) and correlation (R) are shown in the graphs. iii) LEfSe was conducted to explore potential mycobiome differences between NG and HG groups. LDA Score was constructed, and the bar represents a log10 transformed LDA score. The red color represents taxa that corresponding to HG, and the green color represents NG. All taxa presented are significant, $p < 0.05$ confirmed by alpha value for the factorial Kruskal-Wallis test among classes, and the discriminative threshold was set > 2.0.

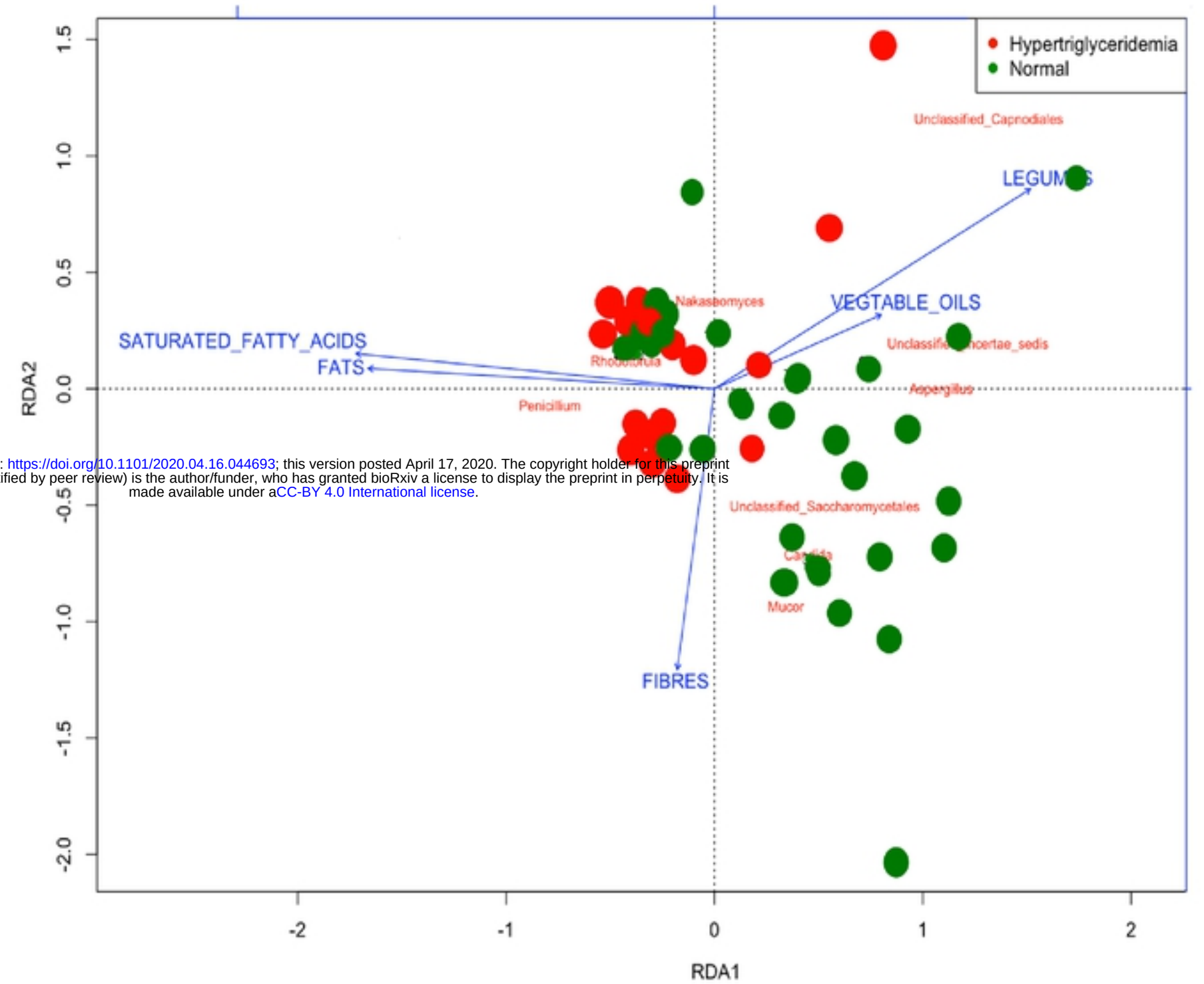


Figure 4: RDA biplot at OTU level with Hellinger-transformed data. Red dots represent individuals with high Tg levels (Hypertriglyceridemia, HG) and green dots, individuals with normal Tg levels. Cut-off for plotted factors was ANOVA with Bonferroni correction, $p < 0.05$.

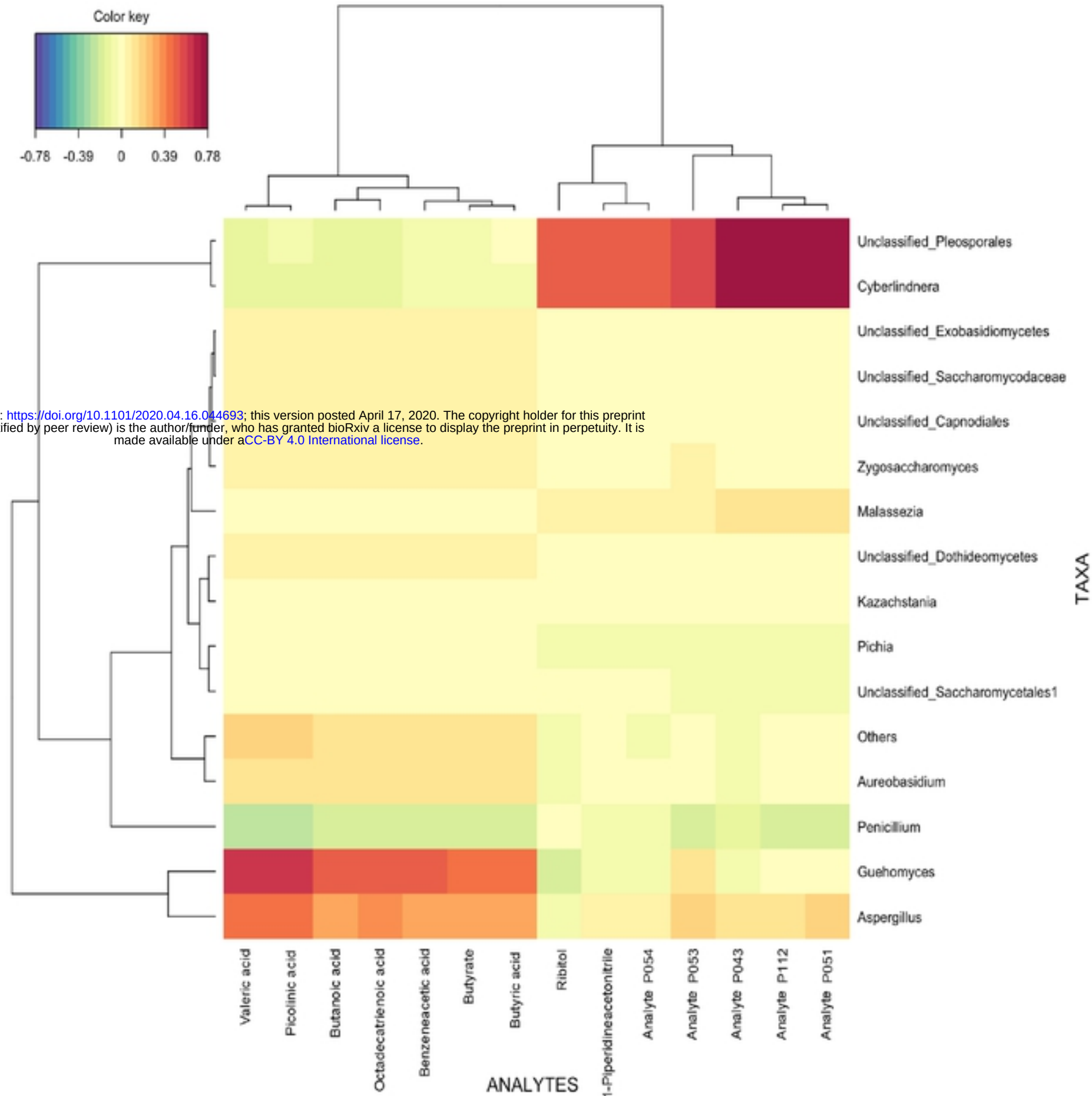


Figure 5: Sparse partial least squared correlations (sPLS) for mycobiome and untargeted faecal metabolomes. sPLS in regression mode (predict Y from X) to model a causal relationship between the most relevant of fungal genera and metabolites from serum and stool. Heatmap displaying the relative accumulation patterns using color-coding (green for negative correlation, and red for positive correlation) of 14 untargeted metabolites against 16 fungal communities.