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

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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,

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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

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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

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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

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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.

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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

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workflow. Differences in alpha diversity were determined using a *t*-test-based approach employing the non-parametric (Monte Carlo) method (999 permutations) implemented in the compare alpha diversity workflow. Bray-Curtis dissimilarity matrix were calculated and visualized via Principal Coordinate Analysis (PCoA) as previously described and ADONIS was used to evaluate group differences [31], [32]. Additionally, analysis and visualization of microbiome communities was conducted in R version 3.4.3. Plots were made using ggplot2 package version 2.2.1. Significant differences in the level of Tg between the groups were assessed using Welch's test. Correlation between the variables was computed by Spearman Rank correlation.

Differentially abundant taxa were determined by LEfSe analysis [33]. Only functional categories with log LDA scores of >2.0, and alpha values of < 0.05 for the factorial Kruskal-Wallis test among classes and pairwise Wilcoxon test between subclasses were considered as differential signatures discriminating between groups. A redundancy analysis (RDA) model was used to estimate the amount of variation among the most abundant mycobiome communities uniquely explained by dietary patterns after controlling for Tg status (Normal or Hypertriglyceridemia). The matrices were Hellinger-transformed using the "decostand" function followed by the "rda" function of the "vegan" package in R [34]. Significance levels determined by ANOVA and the R^2 values were generated by the "RsquareAdj" function in R [35], [36]. Correlation of anthropometric/body-composition data, fecal and plasma metabolome, and gut mycobiome associations were investigated by sparse Partial Least Squares (sPLS) performed using the R package mixOmics [37]. The Bonferroni or Benjamini-Hochberg approaches were used to adjust for multiple testing, where appropriate. For all statistical tests, unless stated otherwise, a p-value of $p < 0.05$ was considered as statistically significant.

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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

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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), $p = > 0.05$).

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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*,

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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*,

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Saccharomyces and *Cladosporium* are common gut commensal fungi, where the *Candida* genus predominantly forms the core mycobiome in the gut [15], [47], [48].

The causes of hypertriglyceridemia can be a result of interactions between genetic precursors [49], non-genetic factors such as unhealthy diet and lifestyle [50], diseases related to metabolic syndromes [51], and usage of some types of medicine [52]. A total of 25 of the included participants had Tg levels above the recommended level of 1.7 mmol/L [53]–[56]. We observed that the participants with high Tg levels were strongly associated with low in gut mycobiome community richness and diversity. Similarly, a similar pattern of good versus unhealthy VLDL cholesterol levels strongly linked to the mycobiome composition was observed. Hence, the increased trends in circulating cholesterol of Tg and VLDL in relation to specific gut mycobiome clusters could be used as potential indicators for describing the hypertriglyceridemia phenotype.

LEfSe analysis showed that an upsurge in *Penicillium* genus could be associated with hypertriglyceridemia. However, the utility of *Penicillium* as a biomarker in predicting the progression of atherosclerosis among older adults is unclear, and therefore, this association warrants further investigation. Another interesting observation was the positive association between the relative abundance of the genus *Mucor* and the subjects with normal Tg levels. This is in line with previous studies showing that *Mucor* is abundant in the gut of non-obese subjects [13], and confer protection from the risk of CVD [14]. In the present study, subjects stratified into NG and HG groups also differed in BMI levels (NG = 25.4±3.5; TG = 26.9±3.4 $kg \cdot m^{-2}$; $p = 0.003$), but no clustering between the gut mycobiome and BMI was observed.

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Interestingly, strong correlations between dietary data and gut mycobiome members and hypertriglyceridemia indicate a role of factors in the disease. Particularly, in the case of *Penicillium*, positive correlations with a diet rich in saturated fatty acids and other lipids are common indicators for higher Tg and VLDL cholesterol in circulating serum of hosts, which have been reported to be associated with signatures in coronary atherosclerotic plaques [57], aneurysms of the carotid artery [58], and negatively correlated with HDL-cholesterol [13]. Hence, we speculate that these dietary intakes such as fermented dairy products such as cheese[59] might contribute to increased Tg and VLDL cholesterol levels among the older adult subjects enrolled in this study.

Finally, we investigated the relationship of the stool and plasma metabolomes and the gut mycobiome by performing regression-based modelling on 329 metabolites and 107 OTUs that were assigned to at least the genus level. We observed that *Aspergillus* together with *Guehomyces* was positively associated with faecal SCFA and specifically valeric, butyric and butanoic acids. Inversely, ribitol – the sugar alcohol from fruit fermentation by reduction of ribose [60], was positively correlated with *Cyberlindnera* and unclassified *Pleosporales*. Previously, *Aspergillus* was found to negatively correlate with SCFAs in subjects on a carbohydrate-rich diet [61]. However, a recent study showed that *Aspergillus* species are capable of producing SCFAs metabolites from fibre rich diet substances [62]. No significant correlations between *Penicillium* abundance and any of the metabolites were identified.

Most fungal species detected in gut mycobiome studies are considered transient components of the community, and putatively of environmental origin, where the composition in particular is influenced by food-borne fungi and life-style [63], [64], together with other factors such as age, gender and geographical setting

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[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.

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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

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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 (%).

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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.

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

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TABLE

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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,

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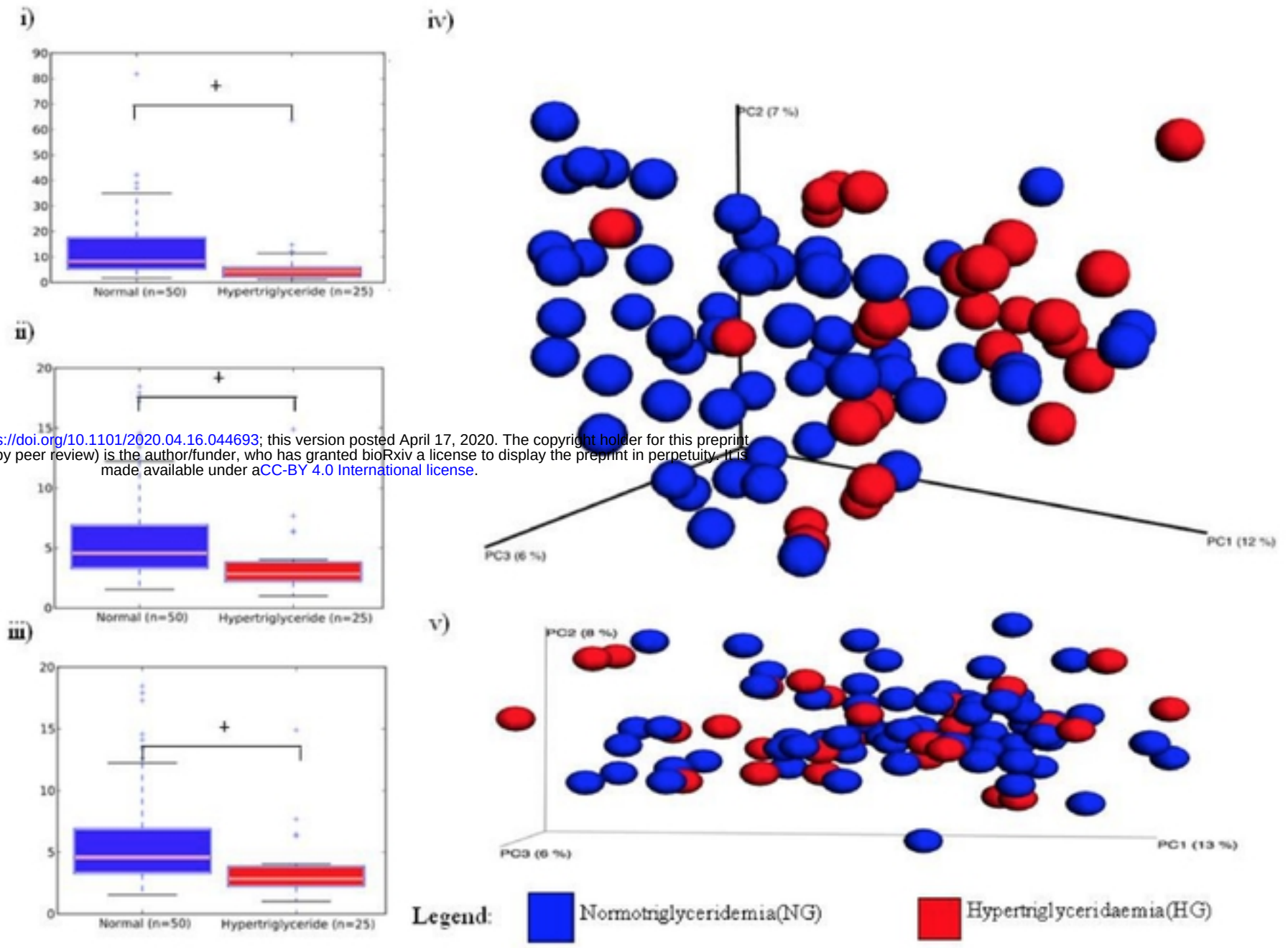

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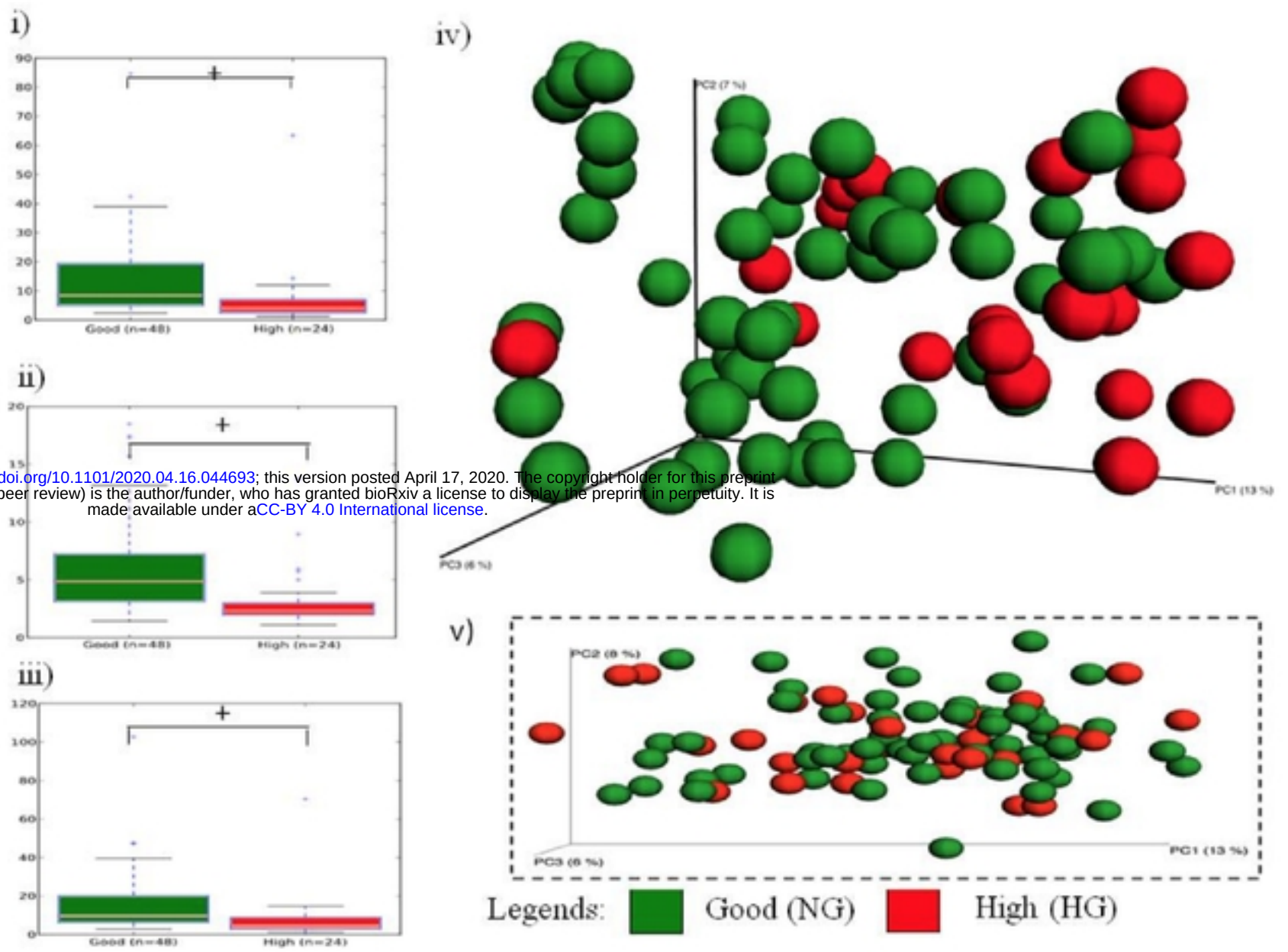

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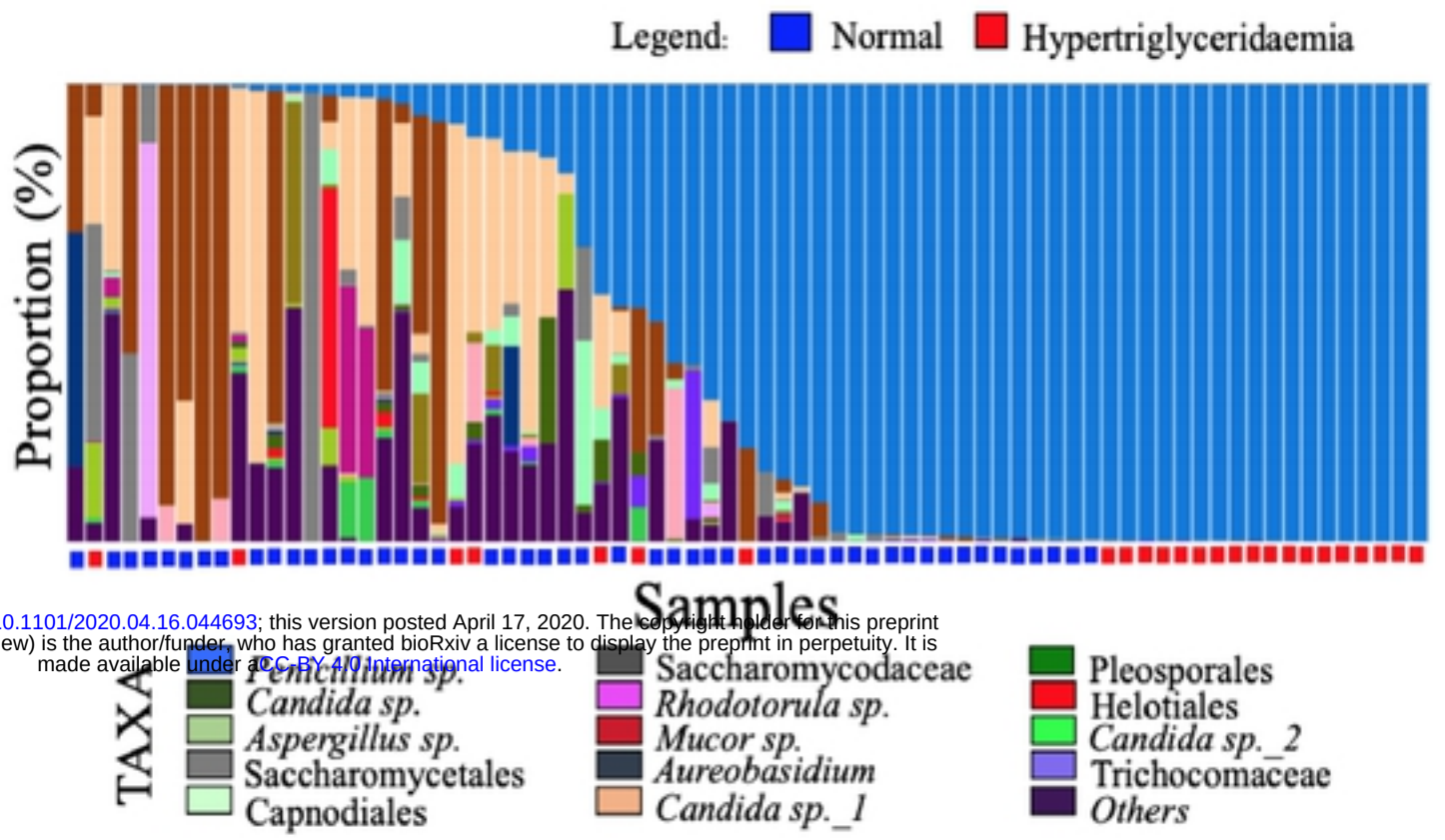
i)



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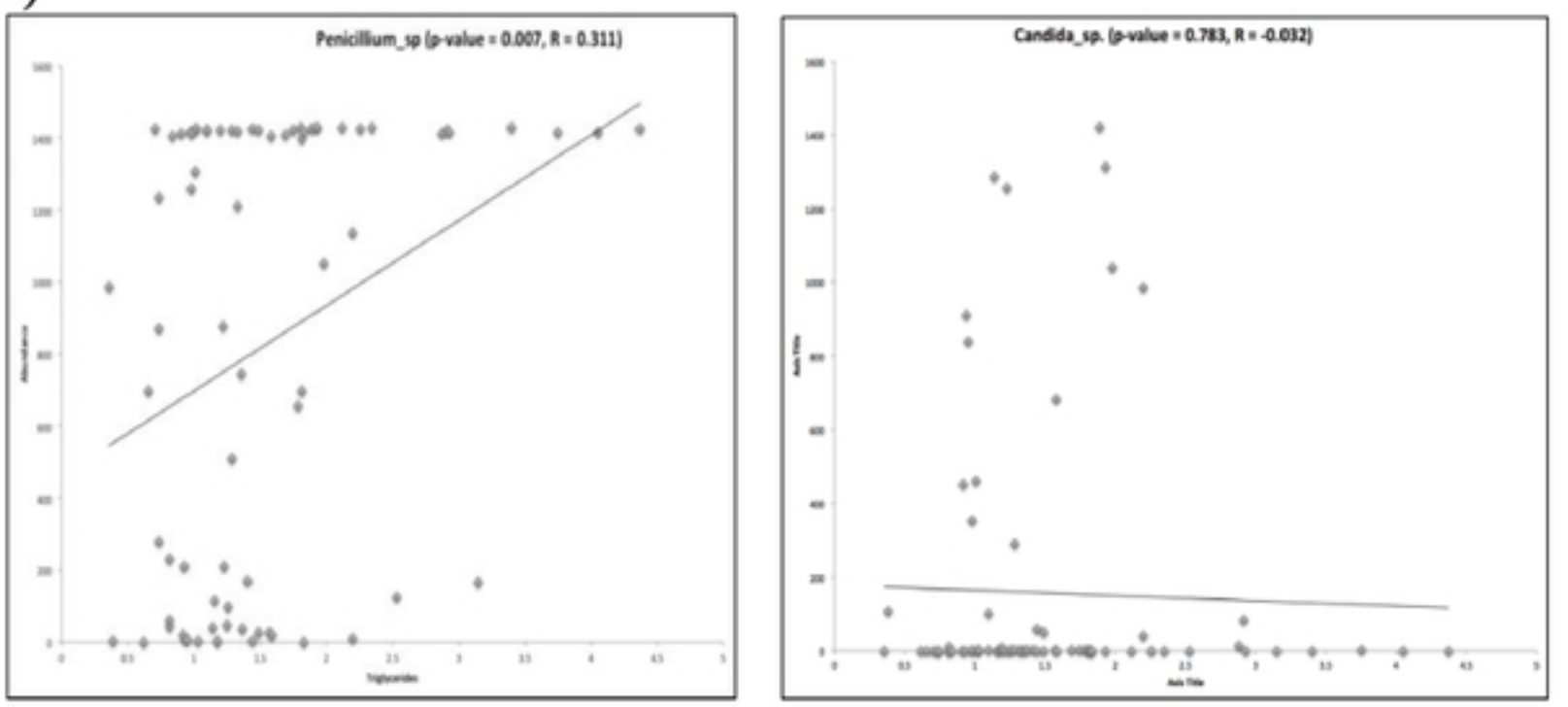
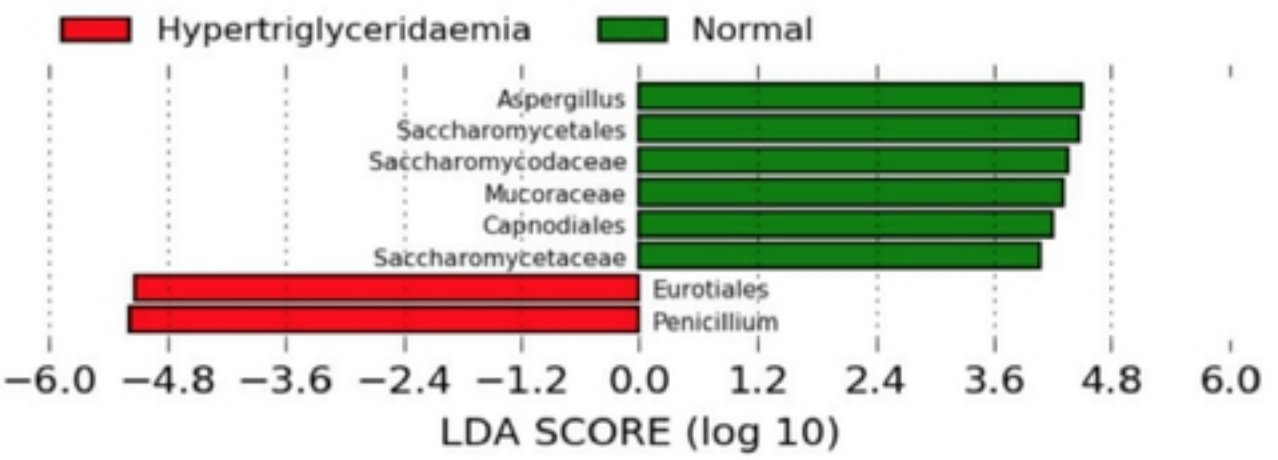
ii)



Penicillium (p-value = 0.007, R = 0.311) *Candida* (p-value = 0.783, R = -0.032)

iii)



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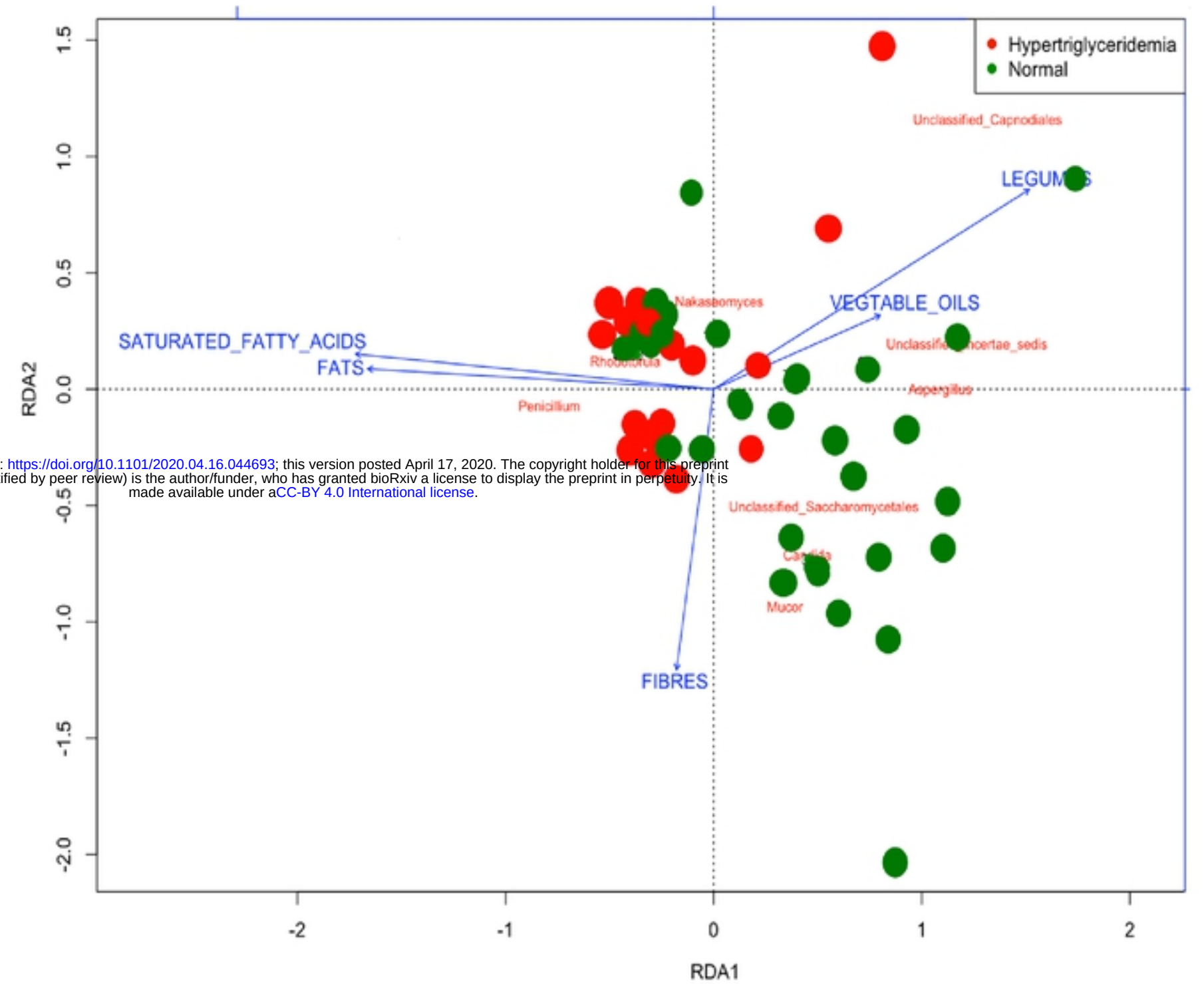

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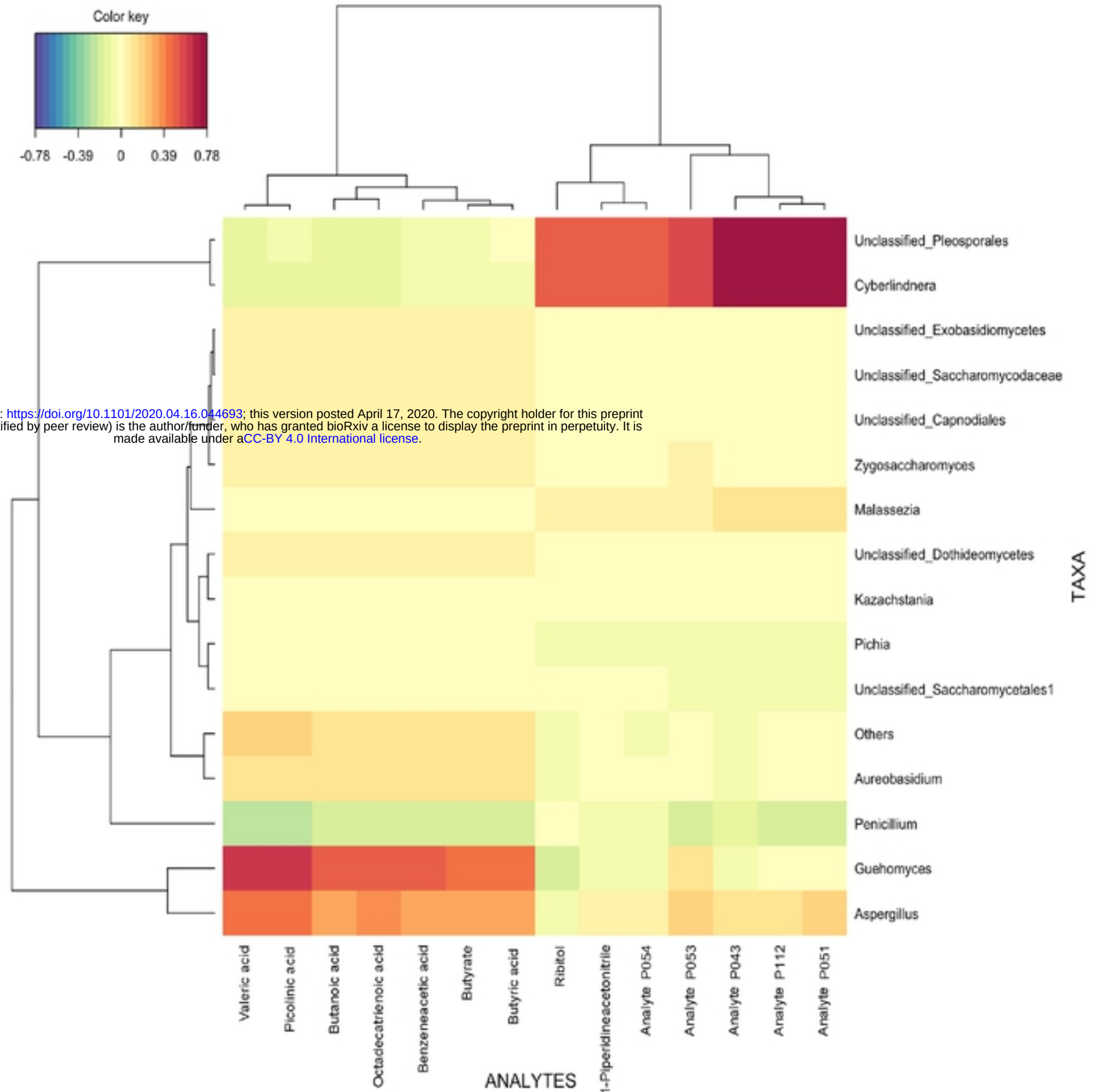

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