Supplementary Appendix

Proton Pump Inhibitors Affect the Gut Microbiome

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Supplementary Methods

Cohorts

Cohort 1: General Population; LifeLines-DEEP

LifeLines-DEEP is a sub-cohort of LifeLines: a multi-disciplinary prospective population-based study that examines health and health-related behaviors of 167,729 participants in the North of The Netherlands.[1] A subset of approximately 1,500 LifeLines participants also takes part in LifeLines-DEEP.[2] These participants are examined more thoroughly, specifically with respect to molecular data. This allows for more in-depth investigation of the genetic and phenotypic variation. Additional biological materials as well as additional information on environmental factors are collected. For a subset of 116 LifeLines participants, oral microbiome data is available as well. Consumption of PPI was obtained from questionnaire data. Analysis of gut complaints and presence of Irritable Bowel Syndrome (IBS) in LifeLines-DEEP cohort were defined according to the Rome III criteria.[3] The 116 LifeLines-DEEP participants from which oral microbiome samples were collected, were used to compare the gut microbiome compositions to oral composition

Cohort 2: IBD patients; Gastroenterology UMCG

The Groningen IBD Microbiome Cohort consists of more than 309 IBD patients from the Gastroenterology and Hepatology department of the University Medical Center Groningen. Stool samples are collected from these patients.

Cohort 3: IBS Case-Control

In Cohort 3, 193 IBS patients, between 18 and 75 years of age, were diagnosed by their general practitioner using the ROME III criteria.[3] Patients were recruited via the outpatient gastroenterology clinic of the MUMC+ (Maastricht University Medical Center, a secondary and tertiary referral centre) and via general practitioners in the area of Maastricht. Medical history was taken and GI endoscopy with biopsies, abdominal imaging and/or blood, breath and fecal analyses were performed to exclude organic disease, if indicated. Patients with a history of abdominal surgery, except appendectomy, laparoscopic cholecystectomy and hysterectomy, were excluded. Patients were assigned to IBS subtypes based on predominant bowel habits according to the ROME III criteria: diarrhea (IBS-D), constipation (IBS-C), mixed stool pattern (IBS-M) and unspecified subtype (IBS-U). Age and sex matched healthy controls (HC, n=152) were enrolled via public advertising. A brief medical history was taken to exclude the presence of previous or current GI disorders or complaints. All study participants gave written informed consent prior to inclusion, and completed questionnaires regarding demographic characteristics and lifestyle factors. A 14-day end-of-day GI symptom diary was completed, addressing symptoms GI complaints and medication information.

PPI definition

The PPI users were defined as users of: Omeprazol (Lozec), Esomeprazol (Nexium), Pantoprazol (Pantozol). In cohort 1 (LifeLines-Deep) the information of drugs usage was extracted from standardized questionnaire. In cohort 2 (IBD) drug analysis was performed

based on electronic patient's record. PPI consumption in IBS case-control cohort was performed based on self-reported questionnaires.

Definition of other drug groups (% of users in cohort 1)

(1) medication that changes bowel movement or stool frequency (2.8%)

a. Opiates (can cause severe constipation)
b. Laxatives

(2) lowers triglycerides levels (0.2%)

a. Fibrates

(3) lowers cholesterol levels (3.6%)

a. Statins

(4) anti-diabetic medication (both oral and insulin) (1.2%)

a. Insulin
b. Biguanides
c. Sulfonylureas
d. α-glucosidase Inhibitors
e. Dipeptidyl Peptidase-IV Inhibitors
f. Glinides
g. Thiazolidinediones

(5) systemic anti-inflammatory medication (does not include NSAIDs)(0.6%)

a. Thiopurines
b. Methotrexate
c. TNF-alpha inhibitors

d. Steroids

(6) topical anti-inflammatory medication (4.1%)

a. steroid cream, steroid nose drops or steroid nose spray

(7) systemic antibiotics, including antifungal and antimalarial medication (1.1%)

(8) antidepressants (3.2%)

a. SSRIs

b. SNRIs

c. Mirtazapine

d. Tricyclic antidepressants

Fecal sample collection

Fecal samples in all three cohorts were collected at home and immediately stored at -20˚C. After transport on dry ice, all samples were stored at -80˚C. Aliquots were made and DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. # 80204). Isolated DNA was sequenced at the Broad institute as described below.

Oral sample collection

Oral cavity samples were collected using sterilized cotton swabs. The cotton swabs were placed directly in tubes containing 300 µl MicroBead solution and stored at −80 °C before DNA extraction. DNA isolation from oral swabs was performed using the UltraClean microbial DNA isolation kit (cat.# 12224) from MoBio Laboratories, Carlsbad, CA. Isolated DNA was stored at −20 °C and sequenced at the Broad institute as described below.

Sequencing

Illumina MiSeq paired end sequencing was used to determine the bacterial composition of the fecal and oral samples. Hyper-variable region V4 was selected using forward primer 515F [GTGCCAGCMGCCGCGGTAA] and reverse primer 806R [GGACTACHVGGGTWTCTAAT]. We used custom scripts to remove the primer sequences and align the paired end reads. Details on the process can be found in Gevers et al[4].

OTU-picking

The operational taxonomic unit (OTU) formation was performed using the QIIME reference optimal picking, which uses UCLUST[5] (version 1.2.22q) to perform the clustering at 97% similarity. As a reference database we used a primer-specific version of the full Greengenes 13.8[6]. Using TaxMan[7], we created the primer-specific reference database containing only reference entries that matched the selected primers. During this process we restricted probe-reference mismatches to a maximum of 25%. The 16S regions that were captured by our primers, including the primer sequences, were extracted from the full 16S sequences. For each of the unique 16S sequences present in the reference database after primer selection, we determined the shared taxonomic label of the original sequences. This overlapping part of the taxonomy was used as the taxonomic label of the sequence. This process is based on work described in Bonder et al.[8] and Brandt et al.[7].

Statistical analysis

QIIME[9] was used for exploratory analysis on the microbiome datasets, gathering basic statistics, Shannon index, richness calculation and PCoA creation. PCoA metrics were

calculated using the Bray-Curtis dissimilarity statistic implemented in QIIME. Both alpha and beta diversity were calculated in a single rarefaction of the abundance table, setting the read depth at 10,000 reads. Differences in diversity, PCoA scores and other factors associated to PPI differences were performed using the Wilcoxon test, the Spearman correlation, Fischer exact test and the chi-squared test as implemented in R[10].

In the PCoA analysis, we compared the PPI group to the non-PPI group in terms of their scores on the first three components using a Wilcoxon test. After the test we compared the group averages to determine the direction of the shift.

To identify differentially abundant taxa between the PPI users and the non-PPI users we used MaAsLin[11]. In the analysis performed in MaAsLin we did not test individual OTUs, instead we focused on the most detailed taxonomic label each OTU represented. Using QIIMETOMAASLIN[12] tool we aggregated the OTUs if the taxonomic label was identical, moreover if multiple OTUs represented a higher order taxa we added this higher order taxa to the analysis. In this process we compress 60.000 OTUs to 1450 separate taxonomic units. We removed taxa from the tests if they were not present in at least 1% of our samples. During the conversion from OTU to taxonomies, QIIMETOMAASLIN transformed the microbiome data to percentages after which these percentages were arcsin square root transformed. This transforms the percentages into a normal distribution.

MaAsLin uses a boosted, additive general linear model to discriminate between groups of data. We used two different MaAsLin runs, correcting for different factors. We forced the cofactors: age, gender, BMI, read-depth and antibiotic usage and turned off the automatic filtering of input taxonomies. We did not remove any taxonomic inputs during the analysis to enable the meta-analysis.

GraPhlAn was used to represent the significant results from the MaAsLin analysis in cladograms.

Meta-analysis

The individual analyses were combined in a meta-analysis. For the meta-analysis a weighted Z-score approach was used. The individual P-values derived from the tests were transformed to Z-scores and the direction was taken from the coefficient of the test. The Z-scores were weighted according to the sample size and combined in a meta Z-score, this was subsequently transformed in a meta P-value.

Multiple testing correction

For correction for multiple testing in individual analyses and in meta-analysis we used the Q-value calculated using the R[10] Q-value package[13]. The Q-value is the minimal false discovery rate at which a test may be called significant. A Q-value of 0.05 was used as the as the cut-off point for significance.

References

1. Scholtens S, Smidt N, Swertz M a., et al. Cohort Profile: LifeLines, a three-generation cohort study and biobank. Int J Epidemiol 2014;1–9.

2. Tigchelaar EF, Zhernakova A, Dekens JAM, et al. An introduction to LifeLines DEEP: study design and baseline characteristics. 2014.

3. Drossman DA, Dumitrascu DL. Rome III: New standard for functional gastrointestinal disorders. J Gastrointestin Liver Dis 2006;15(3):237–41.

4. Gevers D, Kugathasan S, Denson LA, et al. The Treatment-Naive Microbiome in New-Onset Crohn's Disease. Cell Host Microbe. 2014 Mar 12;15(3):382-92

5. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1.

6. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 2006;72(7):5069–72.

7. Brandt BW, Bonder MJ, Huse SM, Zaura E. TaxMan: a server to trim rRNA reference databases and inspect taxonomic coverage. Nucleic Acids Res 2012;40:W82–7.

8. Bonder MJ, Abeln S, Zaura E, Brandt BW. Comparing clustering and pre-processing in taxonomy analysis. Bioinformatics 2012;28(22):2891–7.

9. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–6.

10. R Development Core Team RFFSC. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. 2008;1:2673. http://www.r-project.org

11. Tickle T, Waldron L, Yiren L, Huttenhower C. MaAsLin: Multivariate association of microbial communities with rich metadata in high-dimensional studies. https://bitbucket.org/biobakery/maaslin

12. Tickle T. QIIMEToMaAsLin. 2013; https://bitbucket.org/biobakery/qiimetomaaslin

13. Dabney A, Storey JD. qvalue: Q-value estimation for false discovery rate control. http://qvalue.princeton.edu/

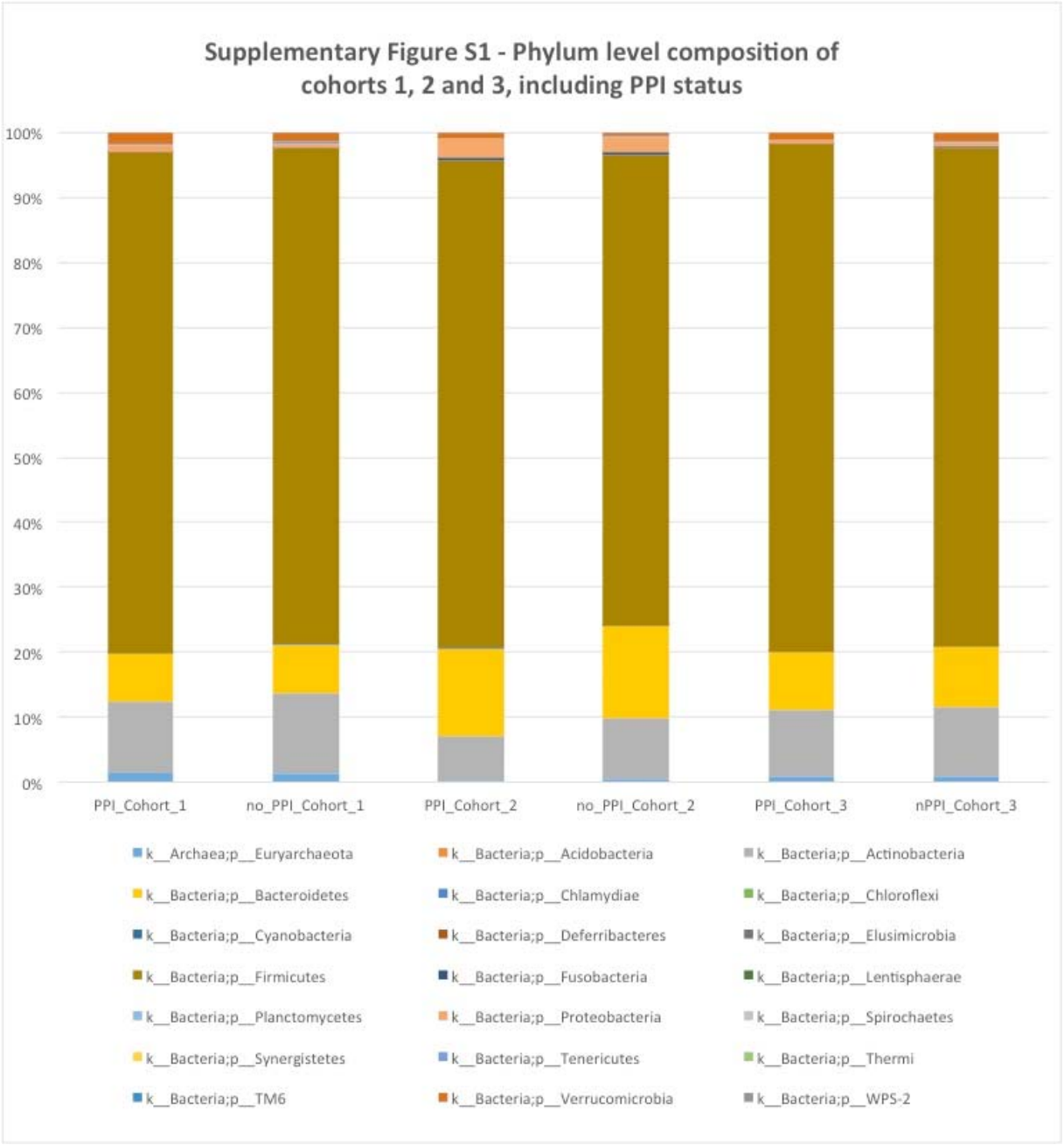
Supplementary Tables

1. Supplementary Table S1: Taxonomic comparison of cohort 1,2 and 3. Comparison of the taxonomic distributions in the datasets, including PPI status.
    a. File: SupplementaryTableS1_barplots_L2-L_IBD_LLD_MIBS_PPI.xlsx
2. Supplementary Table S2: Outcome meta-analysis: All bacterial taxa
    a. File: SupplementaryTableS2_MetaAnalysis.xlsx
3. Supplementary Table S3: Outcome meta-analysis: Annotation
    a. File: SupplementaryTableS6_MetaAnalysis_Sorted_Significantonly.xlsx
4. Supplementary Table S4: MaAsLin results: Cohort 1 LifeLines DEEP
    a. File: SupplementaryTableS4_Cohort1_MaAsLin_PPI.xlsx
5. Supplementary Table S5: MaAsLin results: Cohort 2 IBD UMCG
    a. File: SupplementaryTableS5_Cohort2_MaAsLin_PPI.xlsx
6. Supplementary Table S6: MaAsLin results: Cohort 3 IBS MUMC
    a. File: SupplementaryTableS6_Cohort3_MaAsLin_PPI.xlsx
7. Supplementary Table S7: Cohort 1 medication influencing the microbiome
    a. File: SupplementaryTableS7_Cohort1_FullMedicationCheck.xlsx

Supplementary Figures

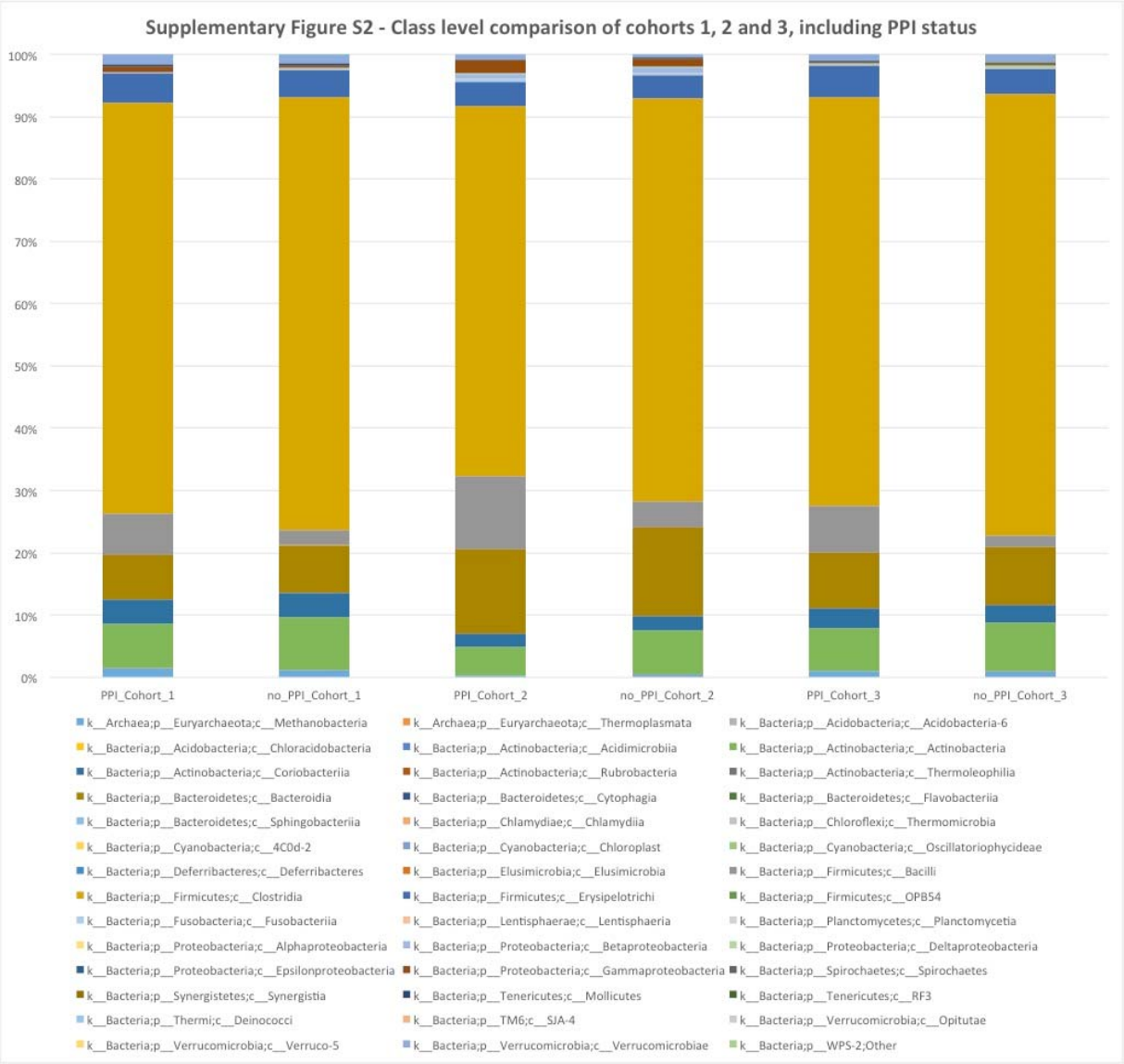
Supplementary Figure S1

Comparison of the three datasets on phylum level. Cohort 1: LifeLines-DEEP, Cohort 2: IBD UMCG, Cohort 3: IBS MUCM. PPI are the PPI users, nPP are the non-PPI users.



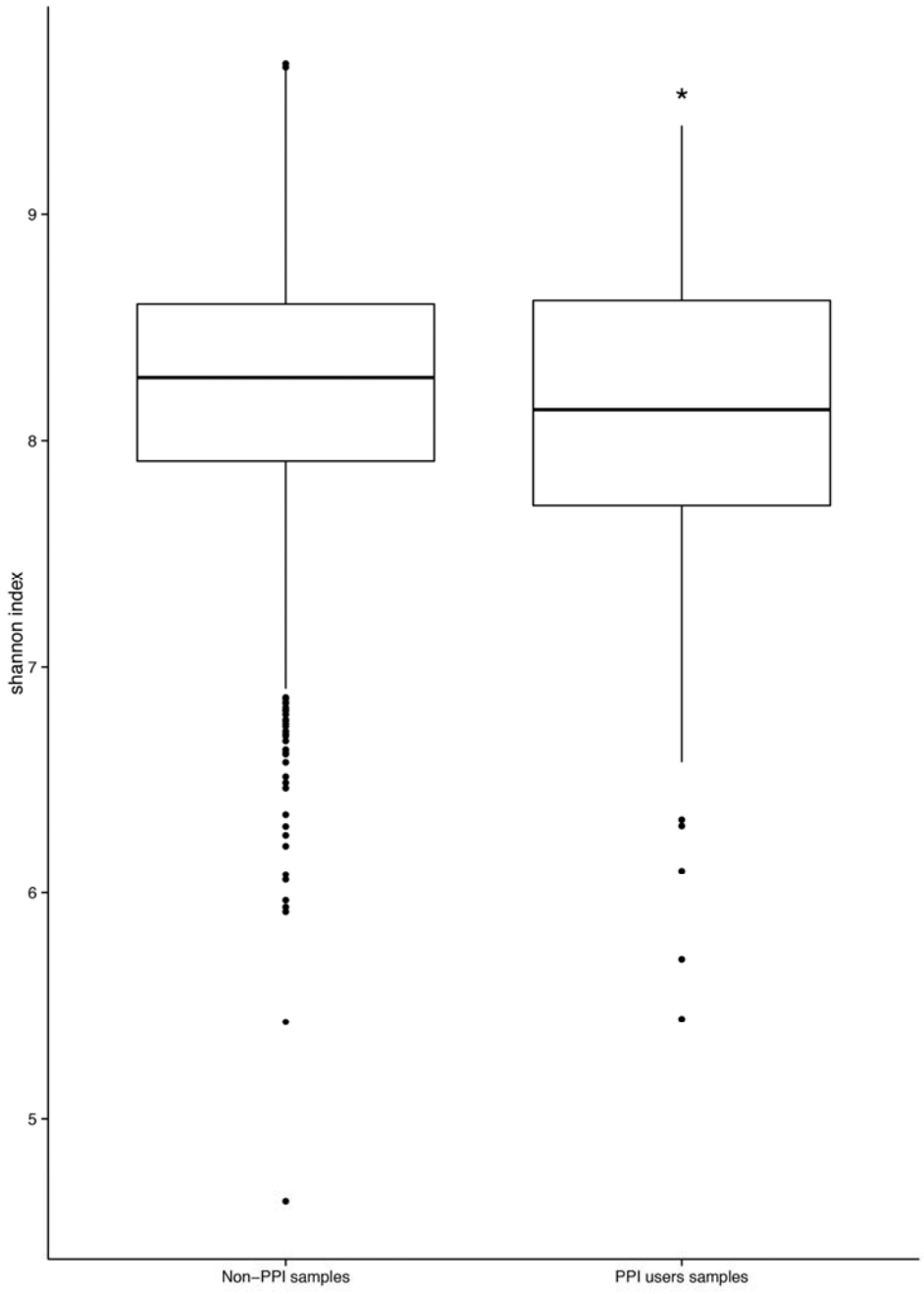
Supplementary Figure S2

Comparison of the three datasets on class level. Cohort 1: LifeLines-DEEP, Cohort 2: IBD UMCG, Cohort 3: IBS MUCM. PPI are the PPI users, nPP are the non-PPI users.



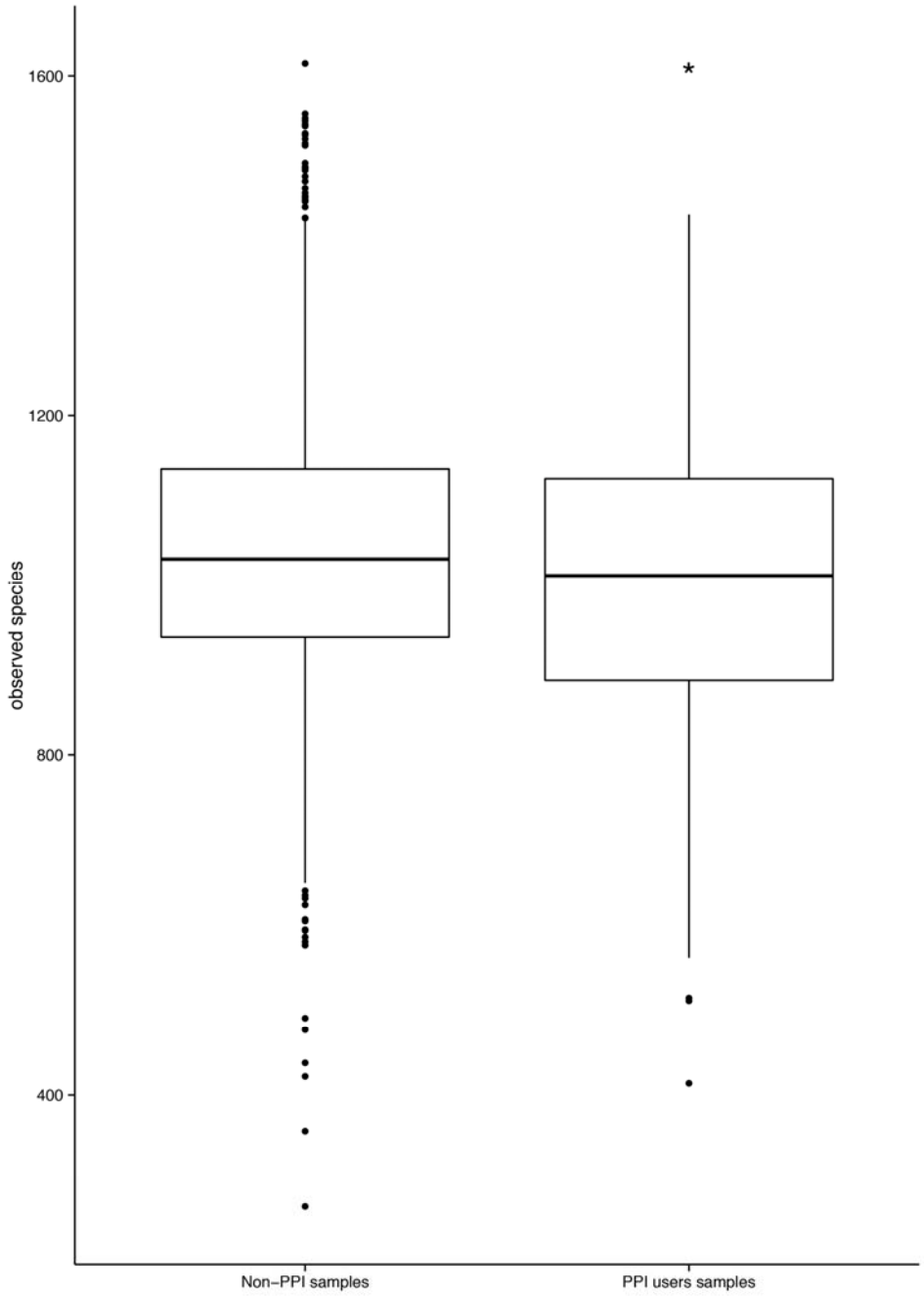
Supplementary Figure S3:

Shannon diversity: Gut microbiome diversity is significantly diminished in PPI users. (p = 0.01)



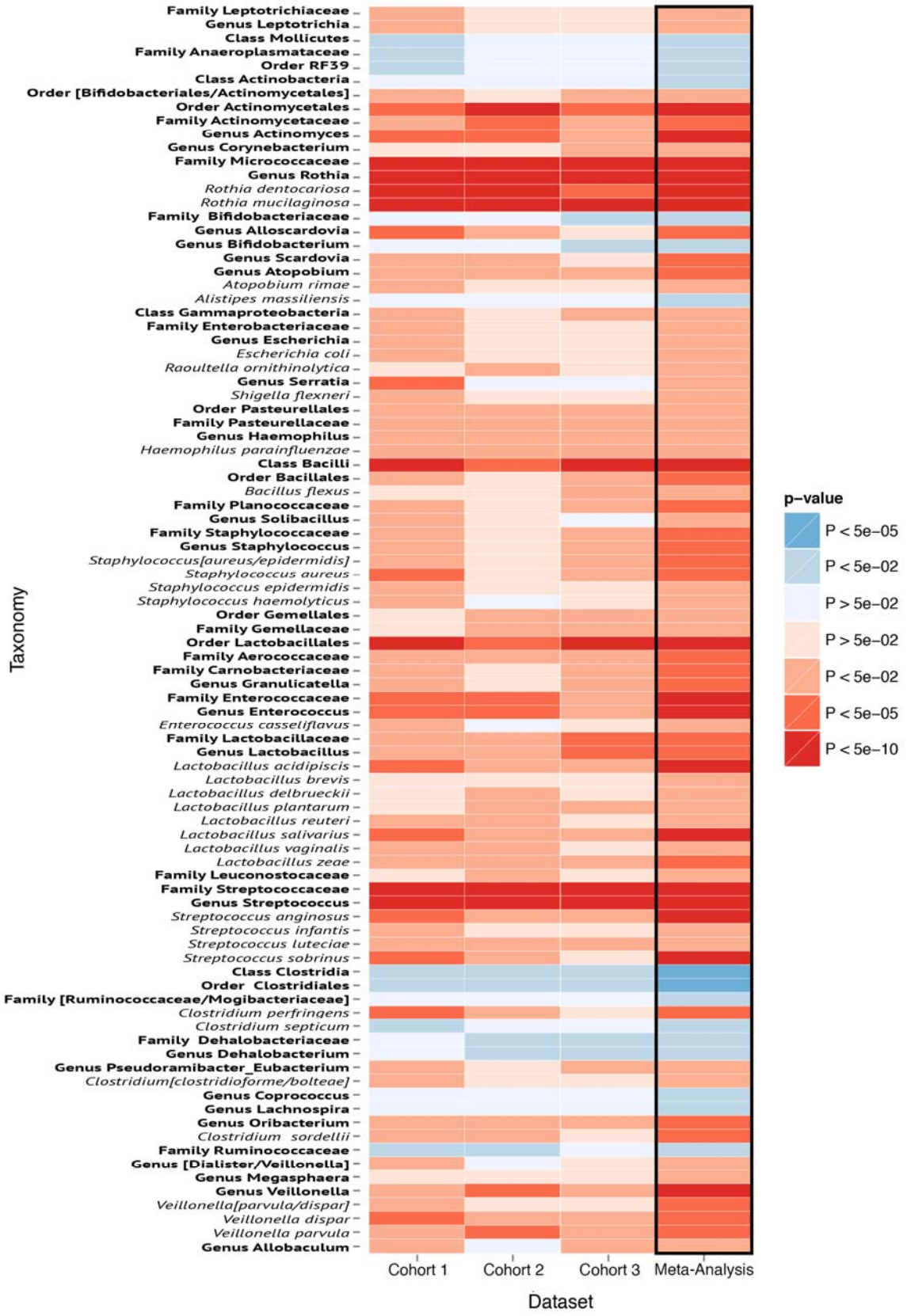
Supplementary Figure S4:

Richness: gut microbiome of PPI-users contains significantly less observed species. (p = 0.02)



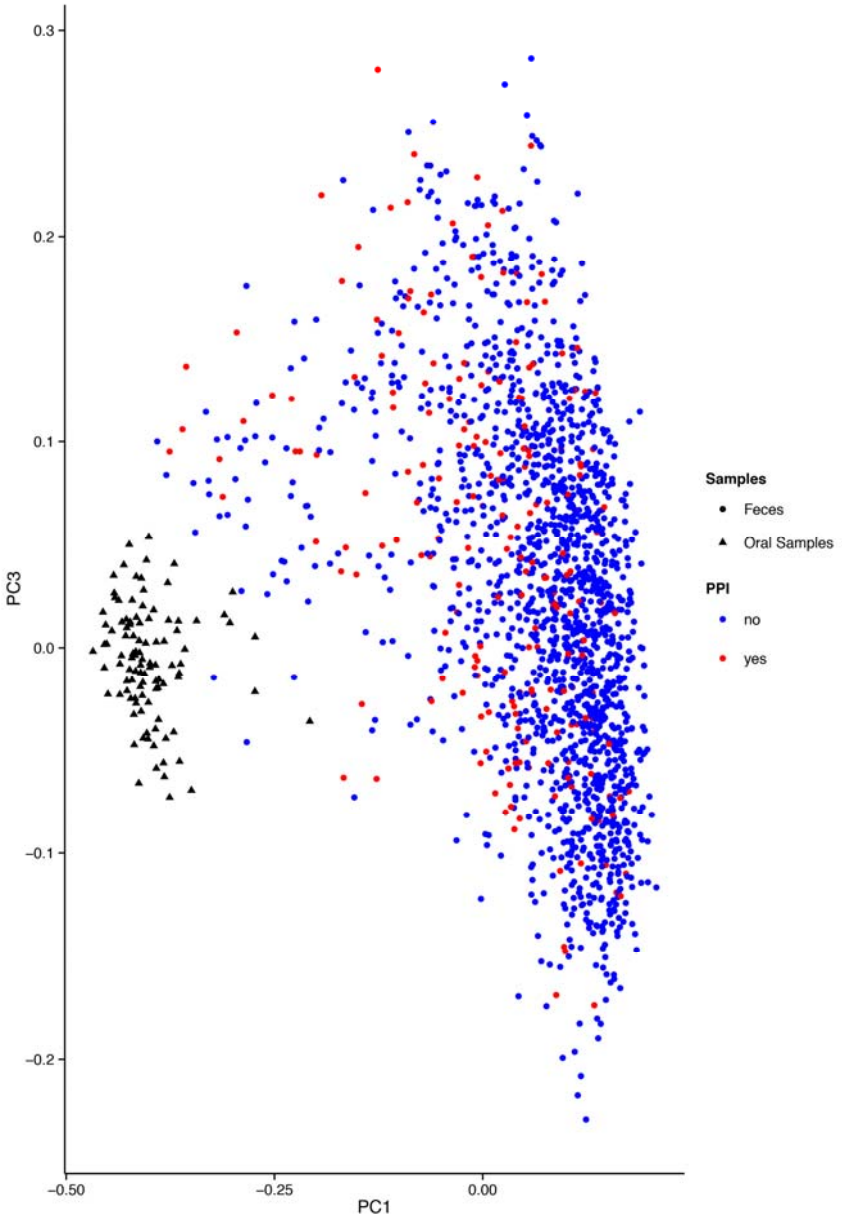
Supplementary Figure S5:

Significantly altered taxa in PPI users is consistent in three cohorts. Meta-analysis of three independent cohorts comprising 1815 fecal samples. The heatmap shows 92 significantly increased or decreased taxa associated with PPI use in the gut microbiome for each cohort and for the meta-analysis (meta-analysis FDR < 0.05).


Family Leptotrichiaceae
Genus Leptotrichia
Class Mollicutes
Family Anaeroplasmataceae
Order RF39
Class Actinobacteria
Order [Bifidobacteriales/Actinomycetales]
Order Actinomycetales
Family Actinomycetaceae
Genus Actinomyces
Genus Corynebacterium
Family Micrococcaceae
Genus Rothia
Rothia dentocariosa
Rothia mucilaginosa
Family Bifidobacteriaceae
Genus Alloscardovia
Genus Bifidobacterium
Genus Scardovia
Genus Atopobium
Atopobium rimae
Alistipes massiliensis
Class Gammaproteobacteria
Family Enterobacteriaceae
Genus Escherichia
Escherichia coli
Raoultella ornithinolytica
Genus Serratia
Shigella flexneri
Order Pasteurellales
Family Pasteurellaceae
Genus Haemophilus
Haemophilus parainfluenzae
Class Bacilli
Order Bacillales
Bacillus flexus
Family Planococcaceae
Genus Solibacillus
Family Staphylococcaceae
Genus Staphylococcus
Staphylococcus[aureus/epidermidis]
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus haemolyticus
Order Gemellales
Family Gemellaceae
Order Lactobacillales
Family Aerococcaceae
Family Carnobacteriaceae
Genus Granulicatella
Family Enterococcaceae
Genus Enterococcus
Enterococcus casseliflavus
Family Lactobacillaceae
Genus Lactobacillus
Lactobacillus acidipiscis
Lactobacillus brevis
Lactobacillus delbrueckii
Lactobacillus plantarum
Lactobacillus reuteri
Lactobacillus salivarius
Lactobacillus vaginalis
Lactobacillus zeae
Family Leuconostocaceae
Family Streptococcaceae
Genus Streptococcus
Streptococcus anginosus
Streptococcus infantis
Streptococcus luteciae
Streptococcus sobrinus
Class Clostridia
Order Clostridiales
Family [Ruminococcaceae/Mogibacteriaceae]
Clostridium perfringens
Clostridium septicum
Family Dehalobacteriaceae
Genus Dehalobacterium
Genus Pseudoramibacter_Eubacterium
Clostridium[clostridioforme/bolteae]
Genus Coprococcus
Genus Lachnospira
Genus Oribacterium
Clostridium sordellii
Family Ruminococcaceae
Genus [Dialister/Veillonella]
Genus Megasphaera
Genus Veillonella
Veillonella[parvula/dispar]
Veillonella dispar
Veillonella parvula
Genus Allobaculum
Taxonomy
Cohort 1
Cohort 2
Cohort 3
Meta-Analysis
Dataset
p−value
P < 5e−05
P < 5e−02
P > 5e−02
P > 5e−02
P < 5e−02
P < 5e−05
P < 5e−10

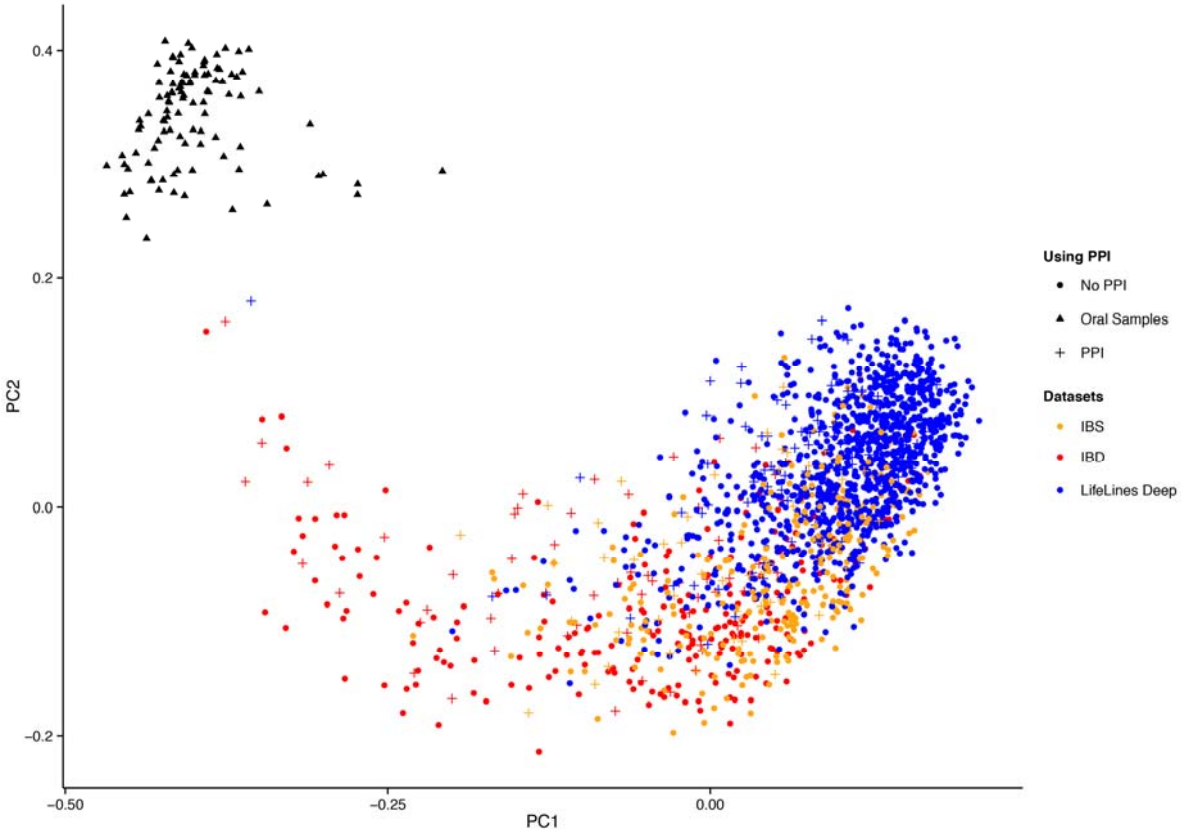
Supplementary Figure S6:

Principal Coordinate Analysis: The gut microbiome of PPI users (red dots) is significantly different to non-PPI users (blue dots) in the first coordinate (PCoA1: $P = 1.39 \times 10^{-20}$, Wilcoxon test) and third coordinate (PCoA3: P = 0.0004, Wilcoxon test).



Supplementary Figure S7:

PCoA with separate colors for the individual cohorts. The gut microbiome of PPI users was significantly closer to the oral microbiome.



Supplementary Figure S8:

Cladogram of PPI-associated changes in the gut microbiome. Red dots represent significantly increased bacterial taxa. Blue dots represent significantly decreased bacterial taxa ($q < 0.05$). Yellow areas represent bacterial taxa of the gut microbiome that are more abundantly present in the oral microbiome.