The gut microbiome in intestinal diseases

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CHAPTER 6

Proton pump inhibitors affect the gut microbiota

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Abstract

Background & aims

Proton pump inhibitors (PPI) are among the top ten most widely used drugs in the world. PPI use has been associated with an increased risk of enteric infections, most notably *Clostridium difficile*. The gut microbiota plays an important role in enteric infections, by resisting or promoting colonization by pathogens. In this study, we investigated the influence of PPI use on the gut microbiota.

Methods

The gut microbiota composition of 1815 individuals, spanning three cohorts, was assessed by tag-sequencing of the 16S rRNA gene. The difference in microbiota composition in PPI users vs. non-users was analysed separately in each cohort, followed by a meta-analysis.

Results

211 of the participants were using PPI at the moment of stool sampling. PPI use is associated with a significant decrease in Shannon's diversity and with changes in 20% of the bacterial taxa (FDR<0.05). Multiple oral bacteria were overrepresented in the faecal microbiota of PPI-users, including the genus *Rothia* (P=9.8x10^{-38}). In PPI users we observed a significant increase in bacteria: genera *Enterococcus*, *Streptococcus*, *Staphylococcus* and the potentially pathogenic species *Escherichia coli*.

Conclusions

The differences between PPI users and non-users observed in this study are consistently associated with changes towards a less healthy gut microbiota. These differences are in line with known changes that predispose to *C. difficile* infections and can potentially explain the increased risk of enteric infections in PPI users. On a population level, the effects of PPI are more prominent than the effects of antibiotics or other commonly used drugs.

Summary box

What is already known about this subject:

- PPI use is associated with increased risk of enteric infections, in particular with a 65% increase in incidence of Clostridium difficile infection.
- PPI is one of the most commonly used drugs.
- Changes in the gut microbiota can resist or promote the colonization of enteric infections.

What are the new findings:

- PPI use is associated with decreased bacterial richness and profound changes in the gut microbiota: 20% of the identified bacteria in this study showed significant deviation.
- Oral bacteria and potential pathogenic bacteria are increased in the gut microbiota of PPI users.
- On the population level we see more microbial alterations in the gut associated with PPI use than with antibiotics or other drug use.

How might it impact on clinical practice in the foreseeable future?

- Given the widespread use of PPI, the morbidity and mortality associated with enteric infections, and the increasing number of studies investigating the microbiota, both healthcare practitioners and researchers should take into consideration the influence of PPI on the gut microbiota.

Background & aims

Proton pump inhibitors (PPI) are among the top ten most widely used drugs in the world. In 2013, 7% of the population of the Netherlands used omeprazole. In the same year, esomeprazole was the second largest drug in terms of revenue in the United States.[1,2] PPI are used to treat gastro-oesophageal reflux disorder (GORD) and to prevent gastric and duodenal ulcers.[3,4] Of the general population, 25% report having heartburn at least once a month, explaining the large demand for PPI.[4] Nevertheless, PPI are frequently prescribed or taken for long periods without evidence-based indication.[5,6]

PPI use has been associated with increased risk of enteric infections.[5,7–9] A meta-analysis of 23 studies, comprising almost 300,000 patients, showed a 65% increase in the incidence of *Clostridium difficile*-associated diarrhoea among patients who used PPI.[9] In healthcare-related settings, PPI use also increases the risk of recurrent *C.*

difficile infections.[5] Another meta-analysis of 11,280 patients, from six studies evaluating *Salmonella*, *Campylobacter* and other enteric infections, also found an increased risk due to acid suppression, with a greater association with PPI than with H_2-receptor antagonists.[8] Recently, the Dutch National Institute for Public Health and the Environment (*RIVM*) noticed a marked increase in the occurrence of campylobacteriosis associated with increased PPI use in the Netherlands.[7]

The gut microbiota plays an important role in these enteric infections.[10–13] Gut microbiota can resist or promote the microbial colonization of the gut by *C. difficile* and other enteric infections through several mechanisms that either directly inhibit bacterial growth or enhance the immune system.[10,11] Moreover, substituting the gut microbiota of diarrhoea patients with *C. difficile* with a healthy microbiota through faecal transplantation has been proven to cure *C. difficile* infection.[14] The increased incidence of enteric infections in PPI users and the importance of the gut microbiota composition in the development of these infections led us to investigate the influence of PPI use on the gut microbiota.

Methods

Cohorts

We studied the effect of PPI use on the gut microbial composition in three independent cohorts from the Netherlands. These cohorts together comprise 1815 adult individuals, including both healthy subjects and patients with gastrointestinal diseases. Cohort 1 consists of 1174 individuals who participate in the general population study LifeLines-DEEP in the northern provinces of the Netherlands.[15] Cohort 2 consists of 300 Inflammatory Bowel Disease (IBD) patients from the department of Gastroenterology and Hepatology University Medical Center Groningen (UMCG), the Netherlands. Cohort 3 consists of 189 Irritable Bowel Syndrome (IBS) patients and 152 matched controls from Maastricht University Medical Center+ (MUMC+), the Netherlands. This study was approved by the institutional review boards of the UMCG and the MUMC+ (MUMC+ http://www.clinicaltrials.gov, NCT00775060). All participants signed an informed consent form.

Medication use

Current medication use at the time of stool collection of Cohort 1 participants was extracted from a standardized questionnaire.[16] Two medical doctors reviewed all the medication for 1174 participants. PPI use was scored if participants used omeprazole, esomeprazole, pantoprazole, lansoprazole, dexlansoprazole or rabeprazole. To exclude other possible drug effects on the gut microbiota, medication use was scored in eight categories, allowing for

later correction of parameters or exclusion of certain participants. These categories were medication that: (1) changes bowel movement or stool frequency, (2) lowers triglyceride levels, (3) lowers cholesterol levels, (4) anti-diabetic medication (both oral and insulin), (5) systemic anti-inflammatory medication (excluding NSAIDs), (6) topical anti-inflammatory medication, (7) systemic antibiotics, including antifungal and antimalarial medication, and (8) antidepressants including serotonin-specific reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), mirtazapine, and tricyclic antidepressants (TCAs). The definitions of these categories are described in the Supplementary Appendix. Analysis of drugs used in Cohort 2 was based on the IBD-specific electronic patient record in the UMCG. Current PPI use, as well as current IBD medication (mesalazines, thiopurines, methotrexate, steroids, TNF-alpha inhibitors and other biologicals) were scored at the time of sampling by the gastroenterologist treating the IBD patient. Current PPI consumption in the IBS case-control Cohort 3 was based on self-reported questionnaires. Pseudonymized data for all three cohorts was provided to the researchers.

Gut complaints and other clinical characteristics

Information on age, gender and BMI was available for all three cohorts. In Cohort 1, gut complaints were investigated using an extensive questionnaire that included defecation frequency and the Bristol Stool Scale. Possible IBS and functional diarrhoea or constipation were determined using self-reported ROME III criteria. The IBD patients in Cohort 2 were diagnosed based on accepted radiological, endoscopic, and histopathological evaluation. All the IBD cases included in our study fulfilled the clinical criteria for IBD. IBS in Cohort 3 was diagnosed by a gastroenterologist according to the ROME III criteria.

Stool and oral cavity mucus sample collection

A total of 1815 stool samples and 116 oral cavity mucus samples were collected. Cohorts 1 and 2 used identical protocols to collect the stool samples. Participants of cohort 1 and 2 were asked to collect one stool sample at home. Stool samples were frozen within 15 minutes after stool production in the participants' home freezer and remained frozen until DNA isolation. A research nurse visited all participants to collect the stool samples shortly after production and they were transported and stored at –80°C. Participants of cohort 3 were asked to bring a stool sample to the research facility within 24 hours after stool production. These samples were immediately frozen upon arrival at –80°C. Oral cavity mucus samples were collected from 116 additional healthy volunteers using buccal swab.

DNA isolation and analysis of microbiota composition

Microbial DNA from stool samples was isolated with the Qiagen AllPrep DNA/RNA Mini Kit cat. # 80204. DNA isolation from oral cavity swabs was performed using the UltraClean microbial DNA isolation kit (cat.# 12224) from MoBio Laboratories (Carlsbad, CA, USA). To determine the bacterial composition of the stool and oral cavity mucus samples, sequencing of the variable region V4 of the 16S rRNA gene was performed using Illumina MiSeq. DNA isolation is described in the Methods section of the **Supplementary appendix**.

Taxonomy determination

Bacterial taxonomy was determined by clustering the sequence reads with UCLUST (version 1.2.22q) with a distance threshold of 97%, using Greengenes (version 13.8) as the taxonomy reference database. Sequencing and the determination of taxonomy are described in the Methods section of the **Supplementary appendix**.

Statistical analysis

In each cohort, differentially abundant taxa in the gut microbiota between PPI users and non-PPI users were analysed using the multivariate statistical framework MaAsLin.[17] MaAsLin performs boosted, additive, general linear models between meta-data and microbial abundance data. After running the association studies in the individual cohorts, we performed a meta-analysis of the three cohorts, using the weighted Z-score method. The Cochran's Q test was used to check for heterogeneity. The significance cut-off for the Cochran's Q test was determined by Bonferroni correction for the 92 significant results: $P<5.43x10^{-4}$. Differences in richness (the number of species within a sample), principal coordinate analyses (PCoA), and Shannon diversity analysis were determined using the QIIME microbiome analysis software.[18] The Wilcoxon test and Spearman correlations were used to identify differences in Shannon's diversity and relations between the PCoA scores of PPI users and non-PPI users, while the Chi-square test, Fisher's exact test, Spearman correlation and Wilcoxon-Mann-Whitney test (WMW test) were used to determine differences in age, gender, BMI, antibiotics use, and gut complaints between PPI users and non-users. In all the microbiome analyses, multiple test corrections were based on the false discovery rate (FDR). An FDR-value of 0.05 was used as a significance cut-off.

In addition to the PPI effect, we also tested the influence of other commonly used drugs in Cohort 1. Using MaAsLin with similar settings to those described above, we tested the microbial changes associated with the use of other drugs, with and without correction for PPI, and the changes when including these common drugs as a correcting factor in the PPI versus non-PPI analysis. Significant results were graphically represented in cladograms using GraPhlAn.[19] More details on the statistical analysis can be found in the Methods section **(Supplementary appendix)**.

Correction for factors influencing the gut microbiota

Differentially abundant taxa were corrected for several parameters, which were identified by statistical analysis of cohort phenotypes or univariate MaAsLin runs and subsequently added as co-factors to the additive linear model. Analyses in the general population Cohort 1 were corrected for age, gender, BMI, antibiotics use, sequence read depth, and ROME III diagnosis (IBS-Constipation (IBS-C), IBS-Diarrhoea (IBS-D), IBS-Mixed (IBS-M), IBS-Undetermined (IBS-U), functional bloating, functional constipation, functional diarrhoea, or none). The analysis of IBD patients in Cohort 2 was corrected for age, sex, BMI, antibiotics use, sequence read depth, diagnosis (Crohn's disease or ulcerative colitis) combined with disease location (colon, ileum or both) and IBD medication (use of mesalazines, steroids, thiopurines, methotrexate or anti-TNF antibodies). The analysis of the IBS case-control Cohort 3 was corrected for age, gender, BMI, sequence read depth, and IBS status according to the ROME III criteria. In the meta-analysis, all microbiome data were corrected for age, gender, BMI, antibiotics use, and sequence read depth.

Results

PPI use is associated to older age and higher BMI

PPI were used by 211 (11.6%) of the 1815 participants: 8.4% of the general population (Cohort 1), 20.0% of the IBD patients (Cohort 2) and 15.2% of the participants of case-control Cohort 3. Women use PPI more often than men: 9.2% versus 7.4%, albeit this was not significant (P = 0.61, Chi-square test). PPI users were generally older: 51.6 (SD 13.4) versus 44.4 (SD 14.7) years of age (P = 2.50 x 10^{-11}, WMW test) and have a higher BMI of 26.9 (SD 5.0) versus 24.9 (SD 4.2) for non-users (P = 1.89 x 10^{-8}, WMW test).

Table 1. Characteristics of the three independent cohorts in this study

	Cohort 1: LifeLines-DEEP (general population)		Cohort 2: IBD patients UMCG		Cohort 3: IBS case-control MUMC	
	PPI users (n=99)	Non-PPI users (n=1075)	PPI users (n=60)	Non-PPI users (n=240)	PPI users (n=52)	Non-PPI users (n=289)
	Average (SD)*	Average (SD)*	Average (SD)*	Average (SD)*	Average (SD)*	Average (SD)*
Age	51.94 (13.59)	44.79 (13.58)	50.87 (14.49)	42.45 (14.57)	51.94 (14.27)	44.57 (18.24)
BMI	27.73 (5.10)	25.05 (4.03)	26.14 (5.53)	25.58 (4.72)	26.24 (4.10)	24.16 (4.11)
Gender (% Male)	36.36%	42.05%	61.67%	39.17%	30.77%	33.56%
Reads per sample	48879 (43001)	55884 (40057)	51081 (43990)	52970 (37787)	43807 (28604)	65842 (119296)
Antibiotics (%)	2.02%	1.02%	31.67%	16.67%	0.00%	1.73%
IBD (%)	0.00%	0.00%	100.00%	100.00%	0.00%	0.00%
IBS (%)	34.34%	25.77%	0.00%	0.00%	90.38%	49.48%
Diarrhoea (%) (IBS-D and functional diarrhoea together)	7.07%	4.47%	-	-	28.4%	17.3%
Average bowel movements per day	1.36 (0.53)	1.38 (0.61)	-	-	1.60 (0.81)	1.92 (1.11)
AntiTNF (%)	-	-	38.33%	28.75%	-	-
Mesalazines (%)	-	-	26.67%	39.58%	-	-
Methotrexate (%)	-	-	16.67%	5.42%	-	-
Steroids (%)	-	-	30.00%	20.42%	-	-
Thiopurines (%)	-	-	21.67%	37.08%	-	-

* unless otherwise stated, BMI = body mass index, IBD = inflammatory bowel disease, IBS = irritable bowel syndrome, PPI = Proton Pump Inhibitor, SD = standard deviation, TNF = tumour necrosis factor UMCG = University Medical Center Groningen, MUMC = Maastricht University Medical Center

Antibiotics were concomitantly used by 2% of the 99 PPI users of Cohort 1 and 33% of the 60 PPI users of Cohort 2. There was no overlap between PPI users and antibiotics users in Cohort 3. Based on our data, we included age, gender, BMI and antibiotics as co-factors in the microbiome analyses. **Table 1** provides an overview of the characteristics per cohort and the use of PPI.

Composition of the gut microbiota

The predominant phylum in each cohort was Firmicutes with abundances of 76.7%, 73.8% and 77.4% in Cohorts 1, 2 and 3, respectively. Information on the composition of the gut microbiota for all three cohorts and on all taxonomic levels is provided in **Supplementary figures S1, S2 and Supplementary table S1**. Independent of PPI use, the overall high-level bacterial composition of the gut was homogeneous in all three cohorts (by phylum, class, and order level, Spearman correlations: rho>0.94; $P<1.6x10^{-13}$).

Reduced diversity of the gut microbiome associated with PPI use

In all three cohorts we identified a lower species richness and lower Shannon diversity, although not significant (Cohort 1, $P=0.85$; Cohort 2, $P=0.16$; Cohort 3, $P=0.53$), however in combined analysis of all three datasets we identified moderate but significant decrease in gut alpha diversity of PPI users was observed in the meta-analysis of all 1815 gut microbiota samples: Shannon index ($P=0.01$) and species richness ($P=0.02$)**(Supplementary figures S3 and S4)**.

Meta-analysis: differences in gut microbiome associated to PPI use

The meta-analysis across all three cohorts showed statistically significant alterations in 92 of the 460 bacterial taxa abundance (FDR<0.05). These changes are depicted in a cladogram in **Figure 1** and in a heatmap in **Figure 2**, and in **Supplementary figure S5**. Details of each taxon, including the individual direction, coefficient, P-value and FDR for each cohort, as well as the meta-analysis, are provided in **Supplementary tables S2 and S3**. Cochran's Q test was used to check for heterogeneity. None of the 92 reported associations were significantly heterogeneous at the Bonferroni corrected P-value cut off ($P<5.43x10^{-4}$) **(Supplementary table S2)**.

The overall difference of the gut microbiome associated to PPI use was also observed in the PCoA of all the datasets together **(Figure 3 and Supplementary figure S6)**. The same PCoA with separate colours for each cohort has been added in **Supplementary figure S7**. Notably, we observed statistically significant differences between PPI users and non-users in two principal coordinates (PCoA1: $P=1.39x10^{-20}$, PCoA3: $P=0.0004$, Wilcoxon test).

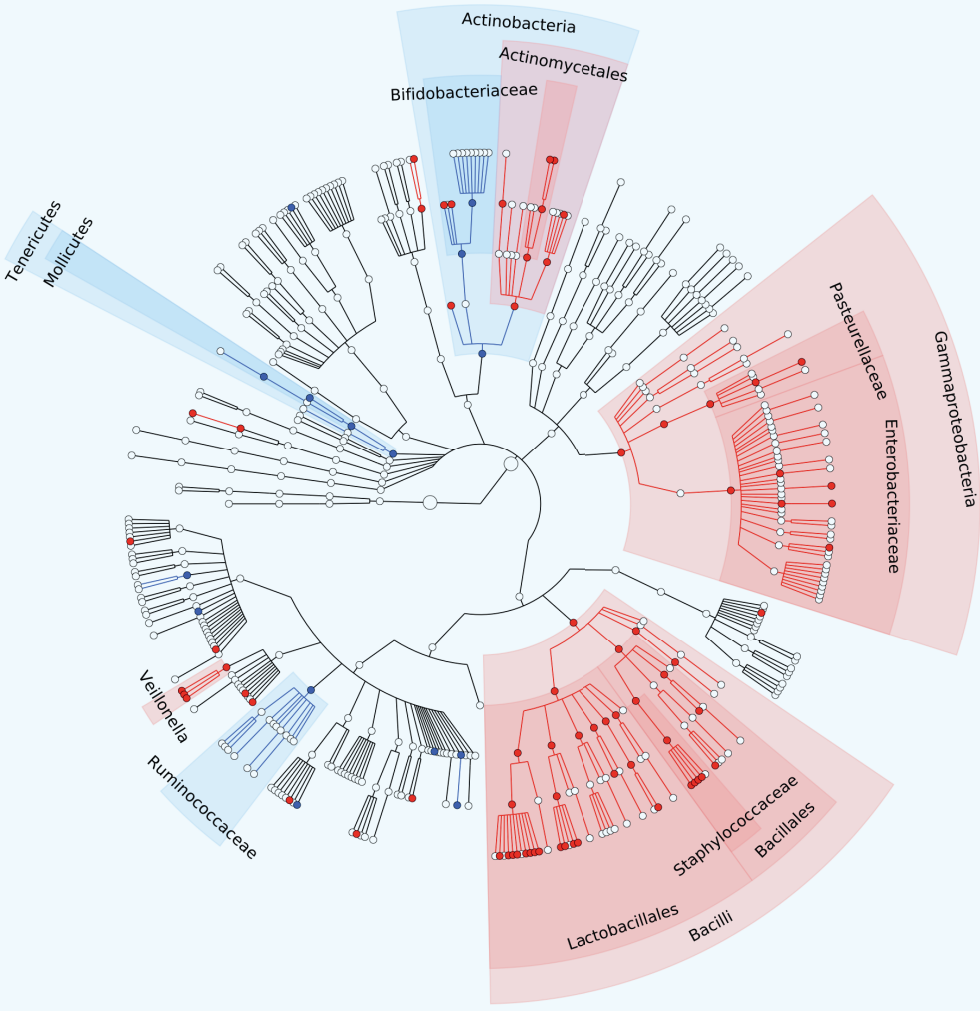

Figure 1. PPI-associated statistically significant differences in the gut microbiome

Meta-analysis of three independent cohorts comprising 1815 faecal samples, showing a cladogram (circular hierarchical tree) of 92 significantly increased or decreased bacterial taxa in the gut microbiome of PPI users compared to non-users (FDR<0.05). Each dot represents a bacterial taxon. The two most inner dots represent the highest level of taxonomy in our data: the kingdoms Archea and Bacteria (prokaryotes), followed outwards by the lower levels: phylum, class, order, family, genus and species. Red dots represent significantly increased taxa. Blue dots represent significantly decreased taxa.

Figure 2. Significantly altered families in PPI users consistent in three cohorts

Meta-analysis of three independent cohorts comprising 1815 faecal samples. The heatmap shows 19 families significantly increased or decreased associated with PPI use in the gut microbiome for each cohort and for the meta-analysis (meta-analysis FDR<0.05).

Similar changes in three independent cohorts were associated to PPI use

The order Actinomycetales, families *Streptococcoceae*, *Micrococcoceae*, genus *Rothia*, and species *Lactobacillus salivarius* were increased in participants using PPI in each cohort. None of the individual cohorts contained any significantly decreased taxa (FDR<0.05). In the general population (Cohort 1), 41 of the 829 bacterial taxa were significantly increased, including the class Gammaproteobacteria, the family *Enterococcoceae,* and the genera *Streptococcus*, *Veillonella* and *Enterococcus* (FDR<0.05) **(Supplementary Table S4)**. No effects due to PPI dosage were observed in the associated bacteria. In IBD patients (Cohort 2), PPI use was associated with an increase of 12 of the 667 bacterial taxa, including the family *Lactobacillaceae* as well as the genera *Streptococcus* and *Lactobacillus* (FDR<0.05) **(Supplementary Table S5)**. In IBS case-control Cohort 3, 18 of the 624 taxa were significantly increased, including the order Lactobacillales (FDR<0.05) **(Supplementary table S6)**.

Oral cavity bacteria are more abundant in the gut microbiota of PPI users

We hypothesized that the changes in the gut microbiota associated with PPI use are caused by reduced acidity of the stomach and the subsequent survival of more bacteria that are ingested with food and oral mucus. Indeed, some of the statistically significantly increased bacteria in PPI users (e.g. *Rothia dentocariosa*, *Rothia mucilaginosa*, the genera *Scardovia* and *Actinomyces* and the family *Micrococcaceae)* are typically found in the oral microbiota.[20] By analysing 116 oral microbiota samples from participants in Cohort 1, we could compare the overall composition of bacteria in the oral microbiota to the composition of the gut microbiota. We observed a statistically significant shift in Principal Coordinate 1 in the gut microbiome samples of the PPI users towards the oral samples, compared to non-PPI users (P=1.39x10^{-20}, Wilcoxon test) **(Figure 3)**. In **Supplementary figure S8**, the overrepresentation of oral cavity bacteria in the guts of PPI users is depicted in a cladogram.

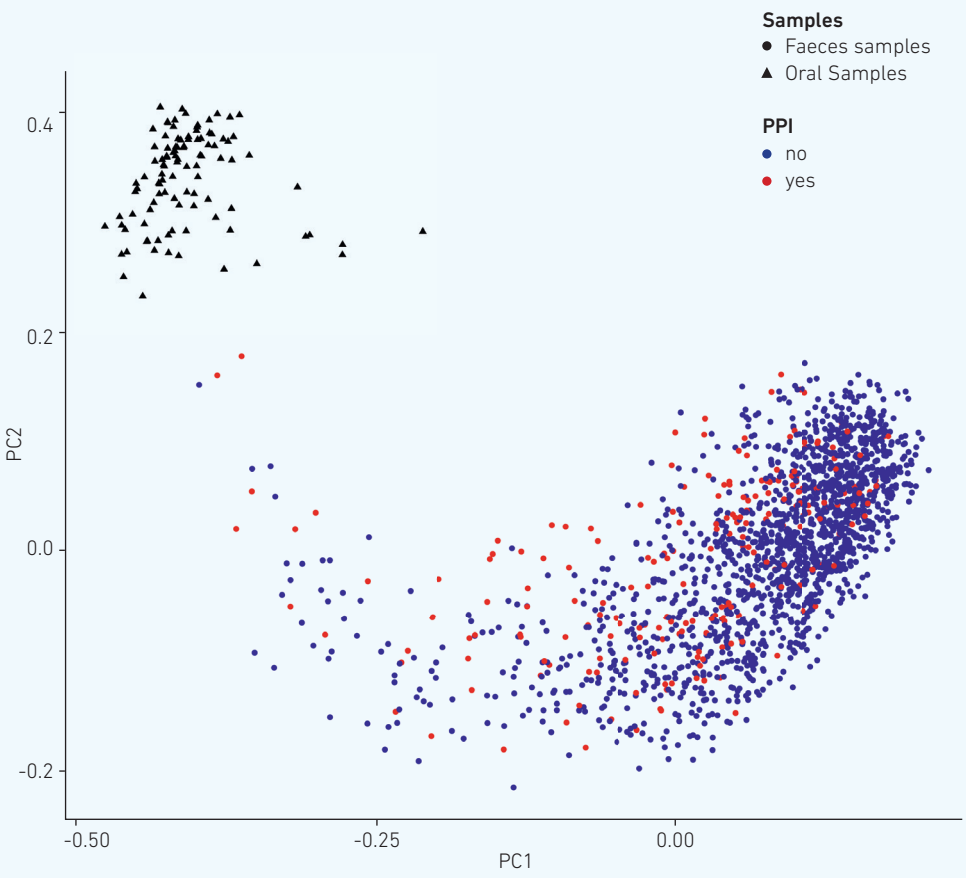

Figure 3. Principal Coordinate Analysis of 1815 gut microbiota samples and 116 oral microbiota samples.

Principal Coordinate Analysis: The gut microbiome of PPI users is significantly different to non-PPI users in the first Coordinate (PCoA1: P=1.39x10^{-20}, Wilcoxon test). For Principal Coordinate 1 there is a significant shift of the gut microbiome of PPI users towards the oral microbiome.

PPI use is independent of bowel movement frequency and stool consistency

Some of the significantly increased taxa were more abundant in the small intestine.[11] To ensure that the changes observed in microbiota composition were not due to diarrhoea and/or more frequent bowel movements, we checked in our general population whether clinical symptoms of diarrhoea were more often present in PPI users. Neither diarrhoeal complaints (IBS-D and functional diarrhoea, *P*=0.22, Fisher's exact test), stool consistency as defined by the Bristol Stool Scale (rho=0.027 *P*=0.36, Spearman correlation) nor the defecation frequency (rho=-0.001, *P*=0.98, Spearman correlation) of the participants in Cohort 1 were related to PPI use.

PPI, antibiotics and other commonly used drugs

In Cohort 1, sixteen taxa were associated to antibiotics and others commonly used drug categories besides PPI **(Supplementary table S7)**. After correction for PPI use, only six taxa remained associated to certain drugs: statins, fibrates and drugs that change bowel movements. All 92 alterations in bacterial taxa associated to PPI use remained statistically significant if we correct the microbiome analyses for antibiotics and other commonly used drugs.

Conclusions

We show that PPI use is consistently associated with profound changes in the gut microbiota. In our study these changes were more prominent than changes associated with either antibiotics or other commonly used drugs. While PPI have proven to be useful in the prevention and treatment of ulcers and GERD, they have also been associated with an increased risk of *C. difficile*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and other enteric infections.[4,5,7–9] The increased risk of acquiring one of these enteric infections is likely due to changes in the PPI user's gut microbiota. Gut microbiota can resist or promote colonization of *C. difficile* and other enteric infections through mechanisms that either directly inhibit bacterial growth or enhance the immune system.[10–13] In the case of *C. difficile,* spores might be able to germinate more easily because of metabolites synthesized by certain gut bacteria.[12,13]
We hypothesized that PPI change the gut microbiota through their direct effect on stomach acid. This acidity forms one of the main defences against the bacterial influx that accompanies ingesting food and oral mucus. PPI reduce the acidity of the stomach, allowing more bacteria to survive this barrier. We have shown here that species in the oral microbiota are more abundant in the gut microbiota of PPI users. Moreover, a study looking into the effect of PPI on the oesophageal and gastric microbiota in oesophagitis and Barret's oesophagus showed similar bacterial taxa associated with PPI use, including increased levels of Enterobacteriaceae, Micrococcaceae, Actinomycetaceae and Erysipelotrichaceae.[21] Gastric bypass surgery compromises the stomach acid barrier and leads to gut microbiota changes similar to the PPI-associated alterations in this study, thereby supporting our hypothesis.[22]

We looked at the role of the gut microbiota in *C. difficile* infections, which cause 12.1% of all nosocomial infections and were responsible for half a million infections and associated with 29,000 deaths in the United States in 2011.[23,24] Virulent strains of *C. difficile* can only colonize a susceptible gut, after which toxins are produced and spores are shed. This leads to a wide spectrum of symptoms varying from mild diarrhoea to fulminant relapsing diarrhoea and pseudomembranous colitis.[25] Recent human, animal and in vitro studies show an overlap between the specific alterations in the gut microbiota associated with PPI use found in this study and bacterial changes that lead to increased susceptibility to *C. difficile*. The reduced alpha diversity in PPI-users is associated with increased susceptibility to *C. difficile* infection.[13,27,28] The PPI-associated decreases of the family Ruminococcoceae and the genus Bifidobacterium, as well as the PPI-associated increases of the class Gammaproteobacteria, the families Enterobacteriaceae, Enterococcoceae, Lactobacillaceae and the genera *Enterococcus* and *Veillonella,* have been consistently linked to increased susceptibility to *C. difficile* infection. **(Table 2)**[10,13,26–32]

The Ruminococcoceae family is significantly decreased in *C. difficile* patients and enriched in healthy controls.[28,29,31] Moreover, mice that have been treated with a mixture of antibiotics that do not become clinically ill after a challenge with *C. difficile* have higher levels of Ruminococcaceae.[26] Within the Ruminococcaceae family, the *Faecalibacterium* genus was significantly increased in patients who recovered from *C. difficile* illness, whereas it was severely decreased in *C. difficile* patients with active disease.[31] Last, a decreased *Ruminococcus torques* OTU was significantly associated with *C. difficile* infection in another study, although their OTU-picking was done using a different reference database and associations were performed using OTU-level, making direct comparisons with our study difficult.[13]

Species of the *Bifidobacterium* genus: *Bifidobacterium longum*, *Bifidobacterium lactis*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium breve*, *Bifidobacterium pseudolongum*, *Bifidobacterium adolescentis* and *Bifidobacterium animalis lactis* have been shown to inhibit or prevent *C. difficile* infection.[10] The administration of antibiotics that enhance the susceptibility to *C. difficile* in an in vitro model of the gut also significantly reduce the genus *Bifidobacterium*.[30] Moreover, active *C. difficile* diarrhoea is associated with decreased *Bifidobacteria* in elderly patients.[29]

The class Gammaproteobacteria and the family Enterobacteriaceae are both significantly increased in PPI users. Gammaproteobacteria are enriched in *C. difficile* patients compared to healthy controls.[28] Within the class Gammaproteobacteria, the family Enterobacteriaceae dominate the murine gut microbiota after administration of clindamycin. Those mice that became clinically ill after the administration of an antibiotic cocktail containing clindamycin and a *C. difficile* challenge, had profoundly increased levels of Enterobacteriaceae in their gut microbiota, while mice that did not become clinically ill had a gut microbiota that predominantly consisted of Firmicutes.[26] The family Enterobacteriaceae is also increased in hamsters that were treated with clindamycin and subsequently infected with *C. difficile*.[32]

The *Enterococcus* genus, which is also more abundant in PPI-users, is significantly enriched in *C. difficile*-infected patients compared to healthy controls.[28,31] An *Enterococcus faecalis* OTU and an *Enterococcus avium* OTU are both significantly associated with increased susceptibility to *C. difficile* infections in mice.[13] Moreover, an *Enterococcus avium* OTU is also significantly associated with *C. difficile* in humans.[13] The administration of the antibiotic ceftriaxone lead to an increase in the genus *Enterococcus* and enhanced the susceptibility to *C. difficile* in an in vitro model of the gut.[30]

The increased abundance of the family Lactobacillaceae in PPI users was associated with increased risk of *C. difficile* infection in several studies. Mice treated with a cocktail of antibiotics (consisting of kanamycin, gentamycin, colistin, metronidazole and vancomycin), cefoperazone or a combination of clindamycin and cefoperazone have higher levels of Lactobacillaceae in their gut.[26] Mice treated with cefoperazone and clindamycin that developed *C. difficile* infection after being challenged with the pathogen also had a higher

level of Lactobacillaceae.[26] Within the Lactobacillaceae family, the *Lactobacillus* genus is significantly enriched in *C. difficile* infection patients compared to healthy controls.[28] *Lactobacillus spp* in the gut microbiota are also associated with active *C. difficile* diarrhoea in patients. [29] In contrast to these studies, the *Lactobacillus* species *Lactobacillus delbrueckii*, *Lactobacillus plantarum* and a *Lactobacillus reuteri* OTU increased colonization resistance to *C. difficile*.[10,13] However, in concordance with increased risk, a *Lactobacillus johnsonii* OTU enhanced *C. difficile* infection.[13]

Last, the *Veillonella* genus that is increased in PPI users is significantly enriched in *C. difficile* patients compared to healthy controls.[28]

The prevention of healthcare-associated *C. difficile* infections is a priority in the United States and reduction targets for 2020 have been established.[5,33] A recent study looking into the effect of PPI on the risk of developing recurrent *C. difficile* infections found that of 191 PPI users admitted to a hospital, only 47.1% had an evidence-based indication for PPI use.[5] Moreover, PPI use was discontinued in only 0.6% of the cases.[5] The U.S. Food and Drug Administration already recommends limiting PPI use to a minimum dose and duration.[34] Despite these recommendations, PPI are still often over-prescribed.[5,6] The risk of unnecessary antibiotics use is already addressed.[35] However, limiting the unnecessary use of PPI should also be considered in preventing *C. difficile* and other enteric infections.

The microbiota is being intensively studied in various diseases and conditions including IBD, IBS, obesity, old age, non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD).[36] PPI users are overrepresented in these groups as they more likely to have gastrointestinal complaints or experience GERD, either due to their health condition or their associated lifestyle. Prominent microbiome studies looking into obesity, IBD and NAFLD include results that researchers have contributed to the condition under study, but we show they are also associated to PPI use.[17,37] It could well be that some of the observed effects should rather have been attributed to the use of PPI. Future microbiome studies in humans should therefore always take the effect of PPI on the gut microbiota into account.

This paper reports the largest study to date investigating the influence of Proton Pump Inhibitors on the gut microbiota. The profound alterations seen in the gut microbiota could be linked to the increased risk of *C. difficile* and other enteric infections. Given the widespread use of PPI, the morbidity and mortality associated with enteric infections, and the increasing number of studies investigating the microbiome, both healthcare practitioners and microbiome researchers should be fully aware of the influence of PPI on the gut microbiota.

Table 2. Taxa and microbiota aspects associated with both PPI use and increased risk of C. difficile infection

Taxa or microbiota aspect	Direction in PPI users that increases the risk of C. difficile infection	References of role on risk of C. difficile infection.
Alpha diversity	**Reduced**	[13] Buffie et al. Nature. 2015 [27] Chang et al. J. Infect. Dis. 2008 [28] Antharam et al. Journal of Clinical Microbiology. 2013
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales **f__Ruminococcaceae**	**Decreased**	[26] Reeves et al. Gut Microbes. 2011 [28] Antharam et al. Journal of Clinical Microbiology. 2013. [13] Buffie et al. Nature. 2015. Extended Figure 3d and 3e [31] Schubert et al. Mbio. 2014. [29] Rea et al. Journal of Clinical Microbiology. 2011.
k__Bacteria p__Actinobacteria c__Actinobacteria o__Bifidobacteriales f__Bifidobacteriaceae ***g__Bifidobacterium***	**Decreased**	[10] Buffie et al. Nature Reviews Immunology. 2013 [29] Rea et al. Journal of Clinical Microbiology. 2011 [30] Baines et al. Journal of Antimicrobial Chemotherapy. 2013
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales f__Enterococcaceae ***g__Enterococcus***	**Increased**	[28] Antharam et al. Journal of Clinical Microbiology. 2013 [31] Schubert et al. Mbio. 2014 [29] Rea et al. Journal of Clinical Microbiology. 2011 (Figure 4) [13] Buffie et al. Nature. 2015 (Extended figure 3d and 3e) [30] Baines et al. Journal of Antimicrobial Chemotherapy. 2013
k__Bacteria p__Firmicutes c__Bacilli o__Lactobacillales **f__*Lactobacillaceae*,** ***g__Lactobacillus*,** ***s__delbrueckii*,** ***s__plantarum*** ***and s__reuteri***	**Increased**	[26] Reeves et al. Gut Microbes. 2011 [28] Antharam et al. Journal of Clinical Microbiology. 2013 [29] Rea et al. Journal of Clinical Microbiology. 2011 [10] Buffie et al. Nature Reviews Immunology. 2013 [13] Buffie et al. Nature. 2015
k__Bacteria p__Firmicutes c__Clostridia o__Clostridiales f__Veillonellaceae ***g__Veillonella***	**Increased**	[28] Antharam et al. The Journal of Clinical Microbiology. 2013
k__Bacteria p__Proteobacteria **c__Gammaproteobacteria** o__Enterobacteriales **f__Enterobacteriaceae** ***g__Escherichia*** ***s__coli***	**Increased**	[28] Antharam et al. Journal of Clinical Microbiology. 2013 [26] Reeves et al. Gut Microbes. 2011 [31] Schubert et al. Mbio. 2014 [32] Peterfreund et al. PLOS ONE. 2012

PPI, proton pump inhibitor.
k__, kingdom; p__, phylum; c__, class; o__, order; f__, family; g__, genus; s__, species
associations are in bold

Declarations

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Supplementary documents

All supplementary materials are available online (open access):
https://gut.bmj.com/content/65/5/740

- **Supplementary appendix.** Online Methods
- **Supplementary table S1.** Taxonomic comparison of cohort 1,2 and 3
- **Supplementary table S2.** Outcome meta-analysis: All bacterial taxa
- **Supplementary table S3.** Outcome meta-analysis: Annotation
- **Supplementary table S4.** MaAsLin results: Cohort 1 LifeLines-DEEP
- **Supplementary table S5.** MaAsLin results: Cohort 2 IBD UMCG
- **Supplementary table S6.** MaAsLin results: Cohort 3 IBS MUMC
- **Supplementary table S7.** Cohort 1 medication influencing the microbiota
- **Supplementary figure S1**. Bar charts: Gut microbiota composition phylum level
- **Supplementary figure S2.** Bar charts: Gut microbiota composition class level
- **Supplementary figure S3.** Alpha diversity: Shannon index
- **Supplementary figure S4.** Alpha diversity: Richness
- **Supplementary figure S5.** Heatmap all significant associated taxa in all cohorts
- **Supplementary figure S6.** PCoA component 1 and component 3
- **Supplementary figure S7.** PCoA separate for individual cohorts.
- **Supplementary figure S8**. Cladogram: Oral cavity bacteria marked

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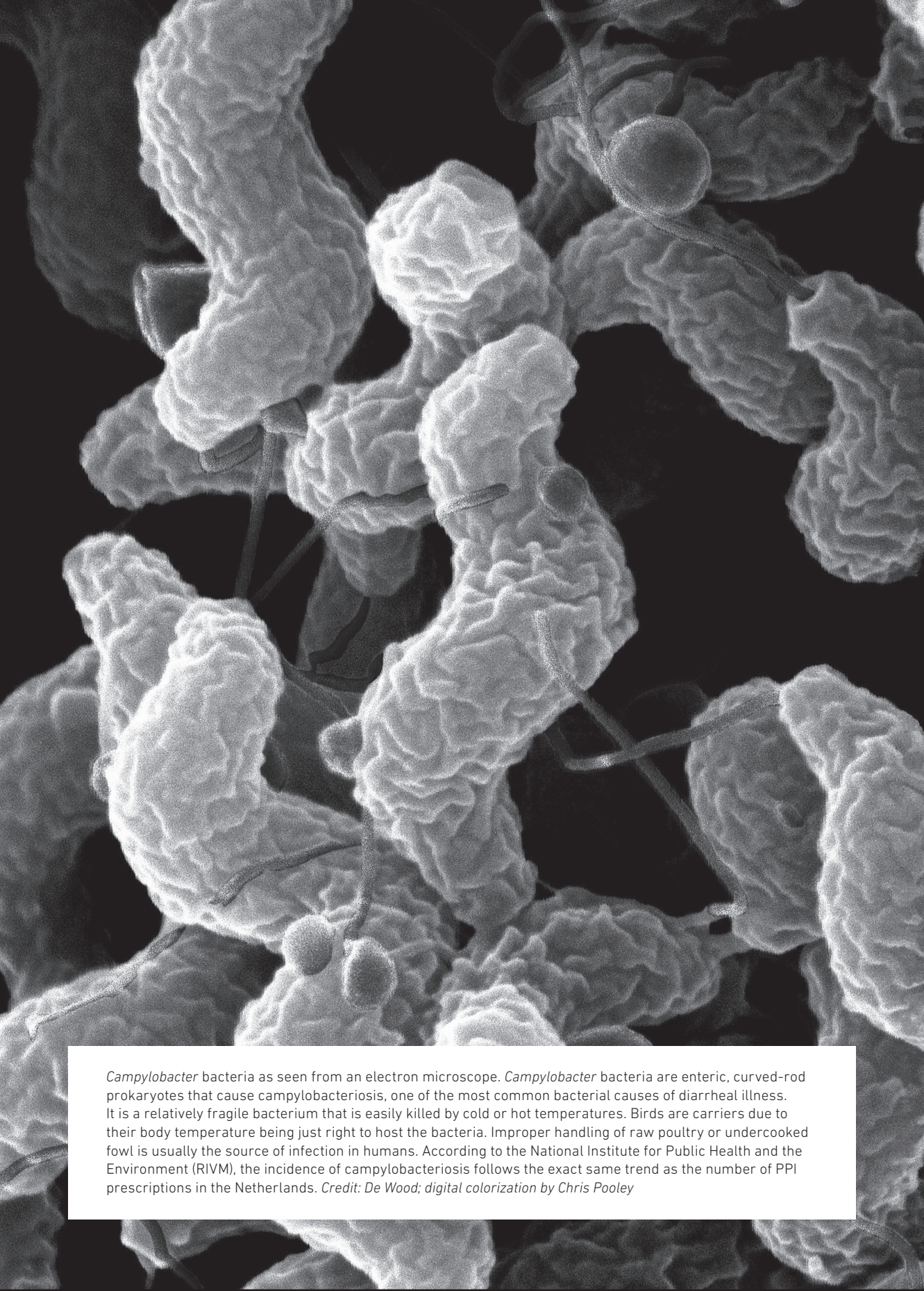

Campylobacter bacteria as seen from an electron microscope. *Campylobacter* bacteria are enteric, curved-rod prokaryotes that cause campylobacteriosis, one of the most common bacterial causes of diarrheal illness. It is a relatively fragile bacterium that is easily killed by cold or hot temperatures. Birds are carriers due to their body temperature being just right to host the bacteria. Improper handling of raw poultry or undercooked fowl is usually the source of infection in humans. According to the National Institute for Public Health and the Environment (RIVM), the incidence of campylobacteriosis follows the exact same trend as the number of PPI prescriptions in the Netherlands. *Credit: De Wood; digital colorization by Chris Pooley*