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BUGS AND DRUGS IN FUNCTIONAL DYSPEPSIA

THE DUODENAL MICRO-ENVIRONMENT IN HEALTH AND FUNCTIONAL GASTROINTESTINAL DISORDERS

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List of abbreviations

Abbreviation	Full name
AE	Adverse Event
AIC	Akaike Information Criterion
BABD	Brisbane Aseptic Biopsy Device
BMI	Body Mass Index
BS	Bile Salt
BT	Breath Test
C	Cholic acid
CAR	Cortisol Awakening Response
CDC	Chenodeoxycholic acid
CFU	Colony-Forming Unit
CI	Confidence Interval
CLDN	Claudin
CLE	Confocal Laser Endomicroscopy
CLR	Centered log-ratio
CRH	Corticotrophin-Releasing Hormone
dbRDA	Distance-Based Redundancy Analysis
DC	Deoxycholic acid
EoE	Eosinophilic Esophagitis
EPS	Epigastric Pain Syndrome
FD	Functional Dyspepsia
Fd4	Fluorescein Isothiocyanate-labelled 4kda Dextran
FDR	False Discovery Rate
FODMAP	Fermentable Oligo-, Di-, Mono-Saccharides And Polyols
G	Glycine
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GERD	Gastro-Esophageal Reflux Disease
GI	Gastrointestinal
H2RA	Histamine-2 Receptor Antagonists
H&E	Hematoxylin and Eosin
H. pylori	*Helicobacter pylori*
HPA	Hypothalamic-Pituitary-Adrenal
hsCRP	High-Sensitivity C-Reactive Protein
IBS	Irritable Bowel Syndrome
IEL	Intraepithelial Lymphocyte
IF	Immuno-Fluorescence
IQR	Interquartile Range
ITT	Intention-To-Treat
LBP	Lipopolysaccharide-Binding Protein

LC	Lithocholic acid
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LPDS	Leuven Postprandial Distress Scale
MAM	Mucosa-Associated Microbiota
MBP	Major Basic Protein
NC	Negative Control
NFW	Nuclease Free Water
NNT	Number Needed To Treat
NSAID	Nonsteroidal Anti-Inflammatory Drugs
OCLN	Occludin
OLE	Open-Label Extension
OTU	Operational Taxonomic Unit
PAGI-SYM	Patient Assessment of Upper Gastrointestinal Symptom Severity Index
PAGI-QOL	Patient Assessment of Upper Gastrointestinal Disorders Quality Of Life
PBMC	Peripheral Blood Mononuclear Cells
PC	Positive Control
PCoA	Principal Coordinates Analysis
PDS	Postprandial Distress Syndrome
PERMANOVA	Permutational Multivariate Analysis Of Variance (MANOVA)
pi	Postinfectious
PP	Per-Protocol
PPI	Proton Pump Inhibitor
PSS	Perceived Stress Scale
QoL	Quality Of Life
qPCR	Quantitative Polymerase Chain Reaction (PCR)
RCT	Randomized Controlled Trial
RR	Relative Risk
rRNA	Ribosomal Ribonucleic Acid (RNA)
SD	Standard Deviation
SE	Standard Error
SIBO	Small Intestinal Bacterial Overgrowth
T	Taurine
TCA	Tricyclic Antidepressants
TEER	Transepithelial Electrical Resistance
Th	T helper
TLESR	Transient Lower-Esophageal Sphincter Relaxation
Treg	T regulatory
UDC	Ursodeoxycholic acid
US	Universal Standard
ZO	Zonula Occludens

CHAPTER 1
INTRODUCTION

This chapter was based on the following publications:

Wauters L, Dickman R, Drug V, Mulak A, Serra J, Enck P, Tack J, Consensus group. United European Gastroenterology (UEG) and European Society for Neurogastroenterology and Motility (ESNM) consensus on Functional Dyspepsia. *United European Gastroenterology Journal* 2021;9(3):307-331.

Wauters L, Ceulemans M, Walker MM, Vanuytsel T, Keely S, Talley NJ. Duodenal Inflammation: An Emerging Target for Functional Dyspepsia? *Expert Opinion on Therapeutic Targets* 2020;24:511-523.

Wauters L, Talley N, Walker MM, Tack J, Vanuytsel T. Novel concepts in the pathophysiology and treatment of functional dyspepsia. *Gut* 2020;69(3):591-600.

1 INTRODUCTION

1.1 Functional dyspepsia

1.1.1 Definition and diagnosis

Dyspepsia refers to chronic upper gastrointestinal (GI) symptoms originating from the gastroduodenal region with a significant impact on patients' lives.[1] According to the Rome IV criteria, functional dyspepsia (FD) is characterized by bothersome epigastric symptoms, which are unexplained after routine investigation, including upper GI endoscopy.[2] In contrast, organic dyspepsia may result from erosive esophagitis, peptic ulcer disease or cancer in a minority of patients.

Two subgroups of FD were proposed by the Rome III consensus and reiterated in the Rome IV version: postprandial distress syndrome (PDS) with postprandial fullness or early satiation, and epigastric pain syndrome (EPS) with epigastric pain or burning (**Table 1.1**).[2] Symptoms must be severe enough to impact on usual activities with a minimal frequency of 1 (EPS) or 3 (PDS) days per week, and be present for the past 3 months with symptom onset at least 6 months before diagnosis.[2]

Table 1.1: Rome IV criteria for Functional Dyspepsia.

	Rome IV criteria	**Frequency, duration & onset**	**Remarks and other symptoms**
FD	Bothersome postprandial fullness, early satiation, epigastric pain or burning AND no evidence of structural disease likely to explain symptoms.	≥1 symptom for the past 3 months with onset ≥6 months before diagnosis.	Vomiting suggests another disorder. Symptoms should not be relieved by evacuation of feces or gas. Symptoms of GERD and IBS may coexist with FD.
PDS	Bothersome postprandial fullness and/or early satiation, severe enough to interfere with daily activities or to prevent finishing a meal.	≥3 days/week for the past 3 months with onset ≥6 months before diagnosis.	Postprandial epigastric pain or burning, epigastric bloating, excessive belching, nausea and heartburn can be present.
EPS	Bothersome epigastric pain and/or epigastric burning severe enough to interfere with daily activities.	≥1 day/week for the past 3 months with onset ≥6 months before diagnosis.	Postprandial epigastric bloating, belching and nausea can be present. Pain may be induced or relieved by ingestion of a meal or occur while fasting, and does not fulfill biliary pain criteria.

EPS, epigastric pain syndrome; FD, functional dyspepsia; GERD, gastro-esophageal reflux disease; IBS, irritable bowel syndrome; PDS, postprandial distress syndrome.

Whereas PDS is characterized by meal-related symptoms, epigastric pain and burning in EPS can be unrelated to meals, although patients often underreport meal-related pain if it is delayed after eating.[3] Patients with symptoms of both syndromes are categorized as overlap syndrome and recognizing postprandial symptoms (including epigastric pain or burning) as part of the PDS subgroup substantially reduced overlap in Rome IV compared to Rome III definitions.[4,5]

Besides the cardinal FD symptoms, upper abdominal bloating, belching and nausea can be present, although functional bloating or nausea and vomiting are categorized separately.[6] Moreover, bloating or visible distention in the upper abdomen was not regarded as a dyspeptic symptom in a recent European consensus, with high agreement on the cardinal FD symptoms (**Figure 1.1**).[7]

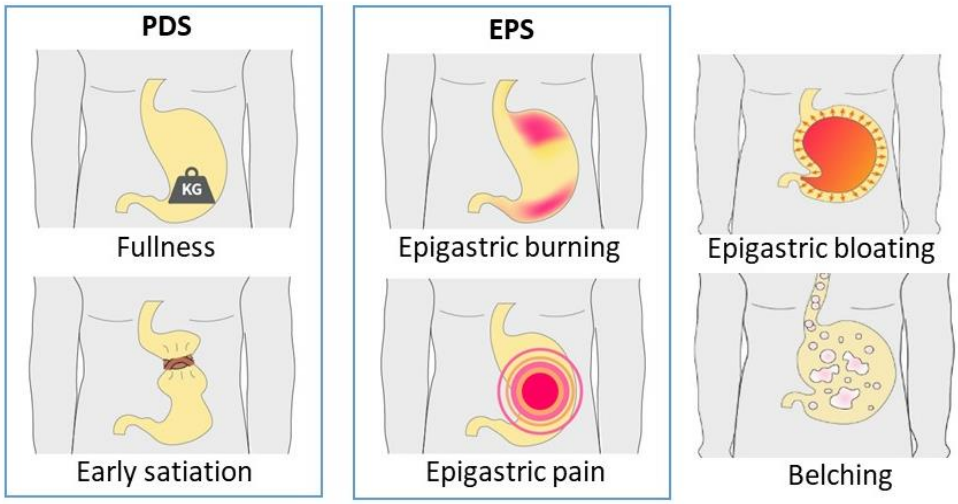

Figure 1.1: Cardinal and accessory symptoms in Functional Dyspepsia. EPS, epigastric pain syndrome; PDS, postprandial distress syndrome.

Although no evidence can be present for an underlying organic, metabolic or systemic disease, Rome IV criteria do not exclude the presence of microscopic pathology as discussed in **section 1.2**.[2]

1.1.2 Epidemiology and impact

Using a broad definition of (uninvestigated) dyspepsia, the global prevalence was 21%.[8] The prevalence varied between countries, even when using uniform symptom-based criteria in a recent systematic review and meta-analysis.[9] While 10% of adults fulfilled Rome IV criteria of 'functional' dyspepsia in a large internet-based cross-sectional health survey, organic pathology was not systematically ruled out by endoscopy.[10] As approximately 80% of symptomatic patients will have a normal endoscopy,[11] a prevalence of 16% in the general population was recently proposed but varied between countries.[12,13] Differences in the cultural, dietary, environmental, ethnic or genetic backgrounds are therefore important in FD, but the underlying mechanisms are unclear.

The prevalence of dyspepsia was higher in women, smokers, users of nonsteroidal anti-inflammatory drugs (NSAIDs) and subjects with *Helicobacter pylori* (*H. pylori*) infection.[8,14] Female predominance was confirmed in the Rome Global Epidemiology Study.[15] However, NSAIDs appeared only relevant for uninvestigated dyspepsia and not FD.[7] An association with antibiotics for non-enteric infections was reported,[16] but less likely than FD following acute gastroenteritis or post-infectious (pi) FD, which is also different from *H. pylori*-associated FD.[17] Finally, anxiety but not depression was associated with an increased risk of FD in a 10-year follow-up study,[18] although bidirectional interactions exist (see below).

Regarding subgroups, PDS is consistently more prevalent (61% vs. 18% for EPS and 21% for overlap) with a similar distribution across the USA, UK and Canada.[10] Results were similar for FD subgroups in secondary care from Belgium (57% PDS, 8% EPS and 35% overlap group).[5] In addition, FD often co-exists with gastroesophageal reflux disease (GERD) and/or irritable bowel syndrome (IBS). Symptoms of GERD were present in almost 1/3 of FD patients,[19] which may be due to pathological acid reflux or functional heartburn.[20,21] Both functional heartburn (12%) and IBS (32%) were independent factors associated with all FD subtypes, with the strongest association for the overlap subgroup.[10]

Health impairment and health-care visits were higher in dyspeptic patients, especially for the overlap subgroup,[10] with frequent absenteeism and impact on daily life in a Belgian study.[22] Quality of life (QoL) in FD is impaired in all main domains (physical, mental and social aspects).[23] Besides the increased health care costs and impaired work productivity,[24] the lack of cost-effective treatments (discussed in **section 1.3**) results

in a substantial economic burden.[24] Despite the high prevalence with significant impact on QoL and health care expenses, the pathophysiology remains unknown.

1.1.3 Gastric impairments

Abnormalities of gastric function including impaired accommodation, hypersensitivity to distention and delayed emptying have been reported in FD (**Figure 1.2**), but these changes correlate poorly or not at all with symptoms.[25] Moreover, the prevalence of gastric impairments was similar in the Rome III-defined PDS and EPS subgroups in a large tertiary-care study, suggesting only a limited contribution of altered gastric sensorimotor function to symptom generation.[25]

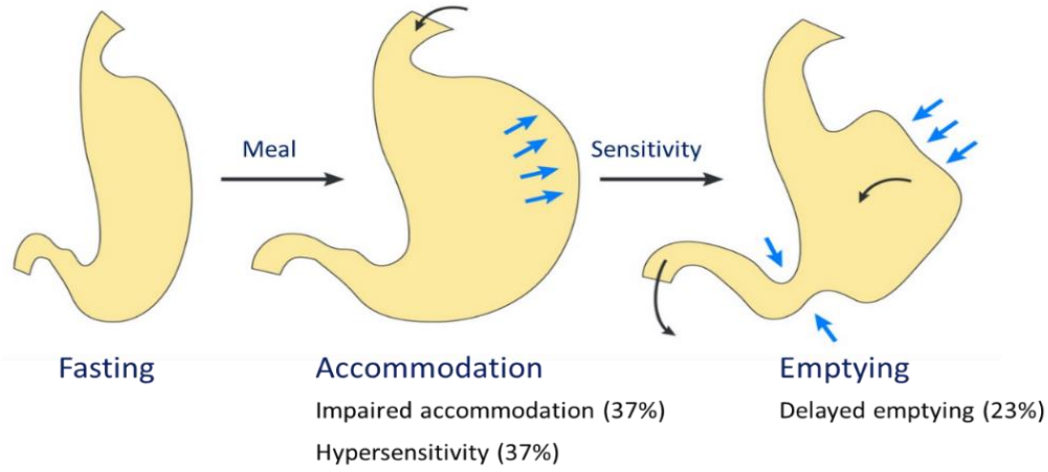

Figure 1.2: Gastric sensorimotor abnormalities in Functional Dyspepsia.

Although cardinal FD symptoms did not correlate with gastric emptying in other studies,[26,27] a recent systematic review and meta-analysis supported associations between upper GI symptoms and gastric emptying when optimal test methods were used.[28] However, the distinction with gastroparesis should be made as nausea may be present but vomiting is unusual in FD,[2] and both nausea and vomiting are significant symptoms in idiopathic and diabetic gastroparesis.[29,30] Impaired accommodation and hypersensitivity may overlap and an association was only found with severe PDS symptoms.[25,31] Finally, both gastric function and symptom severity are determined by psychosocial factors in FD.[31,32]

1.1.4 Gut-brain interactions

Functional GI disorders are classified by the Rome IV criteria as disorders of gut-brain interaction with contributions of both altered brain processing and luminal changes.[33,34] Indeed, up to 50% of subjects with uninvestigated dyspepsia in the general population identify stress as a trigger for their symptoms.[22] A systematic review of functional neuroimaging studies concluded that several brain regions including the frontal and somatosensory cortex showed anomalies in FD.[35] Moreover, symptom severity was correlated with the glyco-metabolism in several brain regions.[36]

Abnormal central modulation (brain to gut) and overactive visceral sensory signaling (gut to brain) may both be involved in the pathophysiology of FD.[35,37] Evidence for bidirectional interactions between central and peripheral manifestations illustrated that depression without GI symptoms at baseline predicted FD whereas anxiety and depression developed in FD patients without psychological co-morbidity at baseline after 1 and 12 years of follow-up.[38,39] On average, a period of more than 3 years was noted before the development of GI symptoms in patients with mood or anxiety disorders.[40]

As the majority of subjects had gut to brain abnormalities and gastric dysfunction fails to explain symptoms,[39] recent research has shifted to the duodenum with evidence of mucosal and luminal alterations (**Figure 1.3**).[41]

Interestingly, duodenal mucosal eosinophilia was linked not only to impaired gastric accommodation and reflux symptoms via transient lower esophageal sphincter relaxations (TLESR),[42,43] but also to anxiety.[44] Moreover, overlapping IBS symptoms in FD may also be explained by more extensive intestinal inflammation with alterations of neuronal signaling and visceral hypersensitivity.[45,46] Thus, the potential key role of the duodenum will be the focus of the next section.

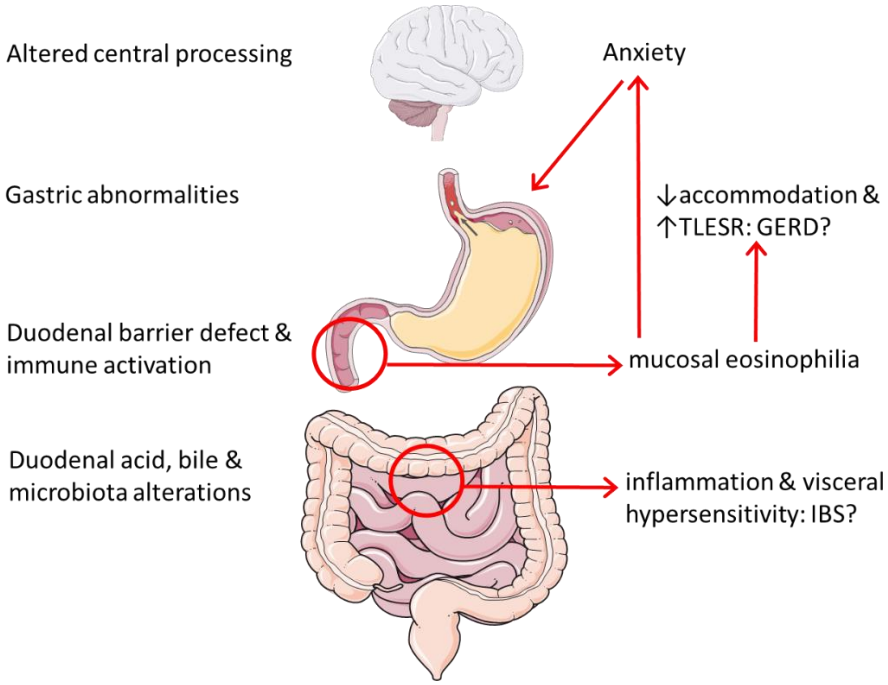

Figure 1.3: Gut-brain axis and mechanisms of overlap in Functional Dyspepsia. GERD, gastro-esophageal reflux disease; IBS, irritable bowel syndrome; TLESR, transient lower-esophageal sphincter relaxation.

1.2 Duodenal pathophysiology

1.2.1 Duodenal mucosal barrier defect

The duodenum has emerged as a key player in GI- and metabolic diseases as it regulates the passage of food as chyme from the stomach to the small intestine, where nutrients are absorbed.[47] Auto- and paracrine mechanisms in the duodenum are also involved in the mucosal defense to acid and luminal digestion of nutrients with secretion of bile and pancreatic juice.[48] Activation of duodeno-gastric feedback mechanisms by chemical or mechanical triggers influences gastric emptying, with an important role of intestinal or pancreatic hormones including incretins and orexigenic hormones, signaling from the stomach to the brain.[49] The role of luminal content is discussed further below.

Besides nutrient sensing, transmucosal passage of luminal content is possible in the duodenum due to regional variation in the mucosal barrier and crypt-villus axis, ranging from >20 angstrom (Å) at the crypt to 5 Å at the villus tip in the proximal small intestine, which is also the most permeable region with the largest intercellular pores of the GI tract.[50] Although the small bowel microenvironment with a loose glycocalyx (covering the brush border on the apical surface of the epithelial cells) allows for a closer interaction between the lumen and host cells in comparison to the colon, the defense against potential noxious luminal factors is reflected in the barrier function and distribution of immune cells.[51]

Ussing chamber experiments have shown increased duodenal mucosal permeability in FD patients with a lower transepithelial electrical resistance and higher paracellular passage of fluorescently-labelled dextran.[52] The expression of cell-to-cell adhesion proteins, including tight junction (zonula occludens-1 and occludin) or adherens junctions (β-catenin and E-Cadherin) and desmosomes were also decreased at the gene- and protein-level.[52] In contrast, claudin (CLDN) transmembrane proteins were unaltered at the gene- and/or

protein-level,[52–55] except for CLDN1 and CLDN3.[54,56] Recently, increased duodenal permeability was confirmed *in vivo* with mucosal impedance and confocal laser endomicroscopy (CLE).[53,54,57,58] However, only baseline impedance was consistently altered for different duodenal regions in FD patients.[58] In summary, multiple independent findings using different methods provided strong evidence for the occurrence of duodenal mucosal barrier dysfunction in FD patients (**Table 1.2**).

Table 1.2: Impaired duodenal mucosal permeability in Functional Dyspepsia.

Type	Findings	References
Ex vivo	↓TEER, ↑FD4-passage (Ussing chambers) ~symptoms	Vanheel *et al.* 2014, 2018[52,59]; Beeckmans *et al.* 2020[60]; Nojkov *et al.* 2020[54]
	↓bacterial passage (Ussing chambers)	Beeckmans *et al.* 2020[60]
In vivo	↓mucosal impedance	Ishigami *et al.* 2017[57]; Komori *et al.* 2019[53]; Nakagawa *et al.* 2020[58]
	↑epithelial gap density (CLE)	Nojkov *et al.* 2020[54]
In vitro	↓ZO-1 (qPCR, IF)	Vanheel *et al.* 2014[52]; Komori *et al.* 2019[53]
	↓OCLN (qPCR, WB), ↓p-OCLN (IF) ↓β-catenin (qPCR, IF), ↓E-Cadherin (IF) ↓DSC-2 (qPCR), ↓DSG-2 (RNA, protein)	Vanheel *et al.* 2014[52]
	↑DIS (TEM), ~symptoms	Tanaka *et al.* 2016[61]
	↓CLDN1 (qPCR, IHC), ↓CLDN3 (qPCR)	Du *et al.* 2018[56]; Taki *et al.* 2019[55]; Nojkov *et al.* 2020[54]
	↑pyroptosis (caspase-1 IHC)	Nojkov *et al.* 2020[54]

CLDN, claudin; CLE, confocal laser endomicroscopy; DIS, dilated intercellular spaces; DSC, desmocollin; DSG, desmoglein; FD4, fluorescein isothiocyanate-labelled 4kDa dextran; IF, immuno-fluorescence; IHC, immuno-histochemistry; OCLN, occludin; qPCR, quantitative PCR; TEER, transepithelial electrical resistance; TEM, transmission electron microscopy; WB, Western blot; ZO, zonula-occludens. ~ for correlation.

1.2.2 Duodenal and systemic immune activation

Eosinophils and mast cells are normally present in the GI-tract except for the esophagus, but increased numbers and activation (e.g. clustering and degranulation) have been described in FD (**Table 1.3**).[41,62,63] Duodenal eosinophil infiltration in FD was first described in pediatric patients from the USA[64] and a nested case-control study of adults from Sweden.[65] A predominance of eosinophils in PDS was confirmed in the UK[66] and Australia,[67] with a similar prevalence between PDS and EPS subtypes in other cohorts.[52,61,68–71] The finding of duodenal eosinophilia was accompanied by mast cell[52,59,69,70] infiltration in a subset of studies.

Regarding systemic immune activation, increased proportions of β7+ T cells were consistently reported in IBS patients,[72] but α4β7 co-expression or increased 'gut-homing' lymphocytes was only found in FD.[73] Indeed, a higher fraction of CD4+ α4β7+ CCR9+ T-cells in the peripheral blood was found with increased production of TNF-α, IL-1β and IL-10, correlating with gastric emptying and symptoms.[73] In addition, increased CD3+ CD45RA+ CD45RO+ lymphocytes with a shift to a T-helper(Th)-2 cytokine profile were found, including increased IL-5 and IL-13 and decreased IL-10 and IFN-γ production by stimulated lymphocytes of FD patients.[74] Although eosinophils are critical effector cells of Th2 and allergic-type inflammation,[62] no mucosal Th2-signal has yet convincingly been demonstrated.[75,76]

Immune activation in the pathogenesis of FD is evident in the post-infectious setting,[77] with persisting changes in duodenal mucosal immune cells after the initial event and systemic immune activation in acute compared to unspecified-onset FD, indicating the inability of the immune system to recover from the triggering infectious insult.[74,77] Infiltration of both eosinophils and CCR2-positive macrophages with increased counts surrounding the crypts and focal CD8+ T-cell aggregates were found in pi-FD, indicating a persisting cellular immune response.[77,78]

Table 1.3: Duodenal and systemic immune activation in Functional Dyspepsia.

Type	Findings	References
Mucosal immune cells	Eosinophil infiltration and degranulation (H&E, IHC, TEM), ~symptoms	Talley et al. 2007[65]; Walker et al. 2009, 2010, 2014[66–68]; Vanheel et al. 2014, 2018[52,59]; Cirillo et al. 2015[69]; Wang et al. 2015[70]; Du et al. 2016[71]; Tanaka et al. 2016[61]; Lee et al. 2019[79]; Taki et al. 2019[55]; Järbrink-Sehgal et al. 2020[80]
	Mast cell infiltration and degranulation (H&E, IHC, TEM), ~symptoms	Vanheel et al. 2014, 2018[52,59]; Cirillo et al. 2015[69]; Wang et al. 2015[70]; Yuan et al. 2015[81,82]; Taki et al. 2019[55]; Giancola 2020[83]
Systemic immune cells	CD4+ α4β7 CCR9+ cells with ↑TNFα, IL-1β, IL-6, IL-10, ~symptoms	Liebregts et al. 2011[73]
	CD3+ CD45RA+ CD45RO+ cells with ↑IL-5, IL-13 and ↓IL-10, IFN-γ	Kindt et al. 2009[74]
Mucosal cytokines	↑IL-1β, IL-6 (qPCR)	Komori et al. 2019[53]; Nojkov et al. 2020[54]
Systemic cytokines	↓IL-5, MCP-1, BDNF and ↑TGF-β3	Cheung et al. 2018[84]
Post-infectious or acute onset FD		
Mucosal immune cells	Eosinophil infiltration and duodenitis (H&E), ~symptoms	Futagami et al. 2010[78]
	↑macrophage (CCR2+) (IHC)	Futagami et al. 2010[78]; Kindt et al. 2009[77]
	↑CD8+ aggregates, ↓CD4+ (IHC)	Kindt et al. 2009[77]
Systemic immune cells or cytokines	CD3+ CD45RA+ CD45RO+ cells with ↑TNF-α (stimulated) and ↑IL-10 (basal)	Kindt et al. 2009[74]

BDNF, brain-derived neurotrophic factor; H&E, hematoxylin and eosin; IHC, immuno-histochemistry; MCP, Monocyte Chemoattractant Protein; TEM, transmission electron microscopy. ~ for correlation

1.2.3 Duodenal luminal content

The causes of the mucosal barrier defect and immune activation in FD are unknown, but likely candidates include duodenal luminal components.[41] Interestingly, duodenal acid perfusion resulted in delayed gastric emptying, impaired accommodation and hypersensitivity to distension in healthy subjects, suggesting a role for duodenal acid in gastric sensorimotor dysfunction in FD.[85–87] Although these perfusion experiments do not reflect physiological changes, increased duodenal acid exposure has been reported in FD patients, possibly due to delayed acid clearance as gastric acid secretion was normal.[88,89] Also, duodenal acid perfusion resulted in mucosal hyperpermeability and mast cell activation, which may be linked to gastric relaxation by activation of duodeno-gastric reflexes.[90]

The role of food in triggering FD symptoms and its role in duodenal low grade inflammatory changes is also unclear. Duodenal hypersensitivity to lipids has been reported in FD,[91] with modulation of upper GI symptoms via cholecystokinin-signaling in response to fat.[92] Moreover, alterations in duodenal motor responses were only found after luminal acid and lipid but not dextrose infusion, implying chemospecific effects.[93] Wheat sensitivity has been proposed but a pilot randomized and placebo-controlled trial (RCT) did not identify specific gluten or fructans as triggers (see **section 1.3**).[94,95]

The release of bile salts (BS) in the duodenum has been implicated in the onset or worsening of dyspeptic symptoms after a meal.[96] A bi-directional relationship exists between BS and bacteria since specific BS have antimicrobial effects and bacteria are responsible for BS-transformation, mainly in the colon with reabsorption and reconjugation of primary and secondary BS in the liver before excretion in the duodenum (**Figure 1.4**).[97] Although bacterial deconjungation may also occur in the small bowel, deconjugated BS were only reported at or below the detection limit in nasoduodenal aspirates from FD patients with decreased fasted concentrations of primary BS.[98] While secondary BS where similar, a decreased ratio of primary to secondary BS may play a role in FD.[99]

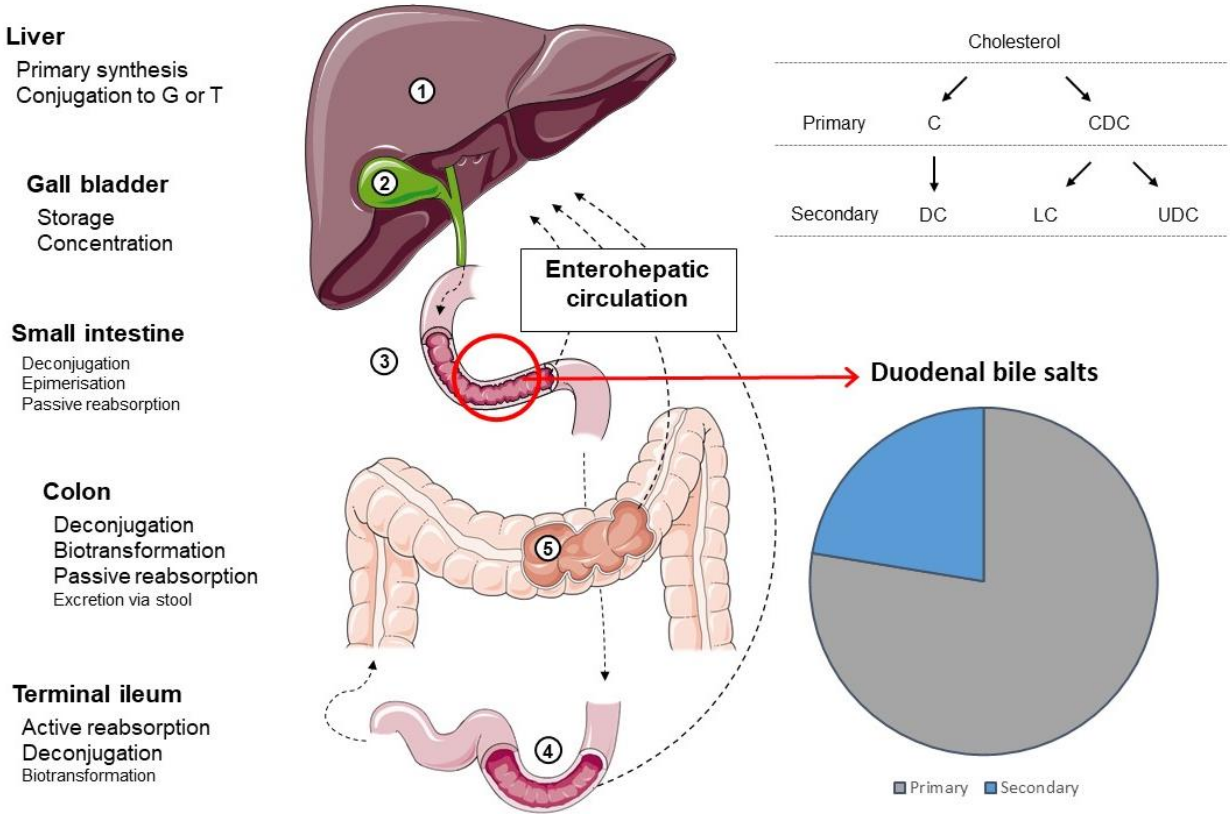

Figure 1.4: Enterohepatic circulation of bile salts, proportions according to Riethorst *et al.*[100] C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; G, glycine; LC, litocholic acid; T, taurine; UDC, ursodeoxycholic acid.

1.2.4 Duodenal microbial dysbiosis

The combination of gastric acid, bile, digestive enzymes and rapid transit time contribute to a hostile environment with predominantly gram-positive aerobes in the duodenum compared to obligate anaerobes in the colon.[101,102] Although more challenging to study, microbiota-immune interactions are probably more important in the small intestine.[51] The disruption in structural and/or functional microbial configuration, defined as "dysbiosis", has been studied in different GI disorders.[101,103] Culture-independent metagenomics and high-throughput 16S ribosomal RNA (16S rRNA) gene sequencing have revolutionized our understanding of the human gut microbiome or collective genome of micro-organisms inhabiting the GI-tract.[104] However,

the focus has mainly been on fecal microbiota, which do not reflect the mucosa-associated microbiota (MAM) or small intestinal microbiome.[105]

Data on the duodenal MAM in FD are currently limited to one pilot study involving 9 FD patients, with a different composition and correlations between total bacterial load and meal-related symptoms or QoL.[106] However, sample size was small with no information on medication, which is an important confounder, nor IBS-overlap, which has also been associated with fecal and duodenal dysbiosis.[107–112] Interestingly, a study using duodenal brushes reported different findings (**Table 1.4**).[113] Although the presence of intestinal-like bacteria in gastric fluid suggested small intestinal bacterial overgrowth (SIBO) in a subset of FD patients,[114,115] no standardized diagnosis of SIBO is available and the majority of studies have used non-invasive breath tests in FD with different substrates (glucose or lactulose), doses or type of exhaled gases (hydrogen and/or methane) and 'controls' (**Table 1.4**).[116]

Table 1.4: Small intestinal microbiota changes and overgrowth in Functional Dyspepsia.

Type	Findings	References
Mucosal (biopsy)	↓*Actinomyces, Atopobium, Leptotrichia, Prevotella, Veillonella* vs. iron deficiency (no HV) (16S rRNA) Bacterial load (qPCR) ~symptoms/QoL	Zhong et al. 2017[106]
	↑load vs. iron deficiency/FOBT + (no HV) (qPCR)	Shah et al. 2020[117]
Luminal (aspirate or brush)	↑*Bifidobacterium* and ↓*Prevotella, Clostridia* clusters vs. HV (T-RFLP), ~symptoms	Nakae et al. 2016[114]
	Bacteroidetes > *Proteobacteria* vs. HV (16S rRNA)	Igarashi et al. 2017[115]
	↑*Streptococcus* vs. HV (16S rRNA), ~symptoms	Fukui et al. 2020[113]
Small intestinal bacterial overgrowth		
Culture (aspirate)	44/227 (19.4%) FD vs. 1/30 (3.3%) NERD (no HV)	Tziatzios et al. 2020[118]
Glucose (H_2-positive)	2/28 (7.1%) FD vs. 0/36 (0%) HV	Shimura et al. 2016[119]
	17/82 (20.7%) FD (no HV)	Petzold et al. 2019[120]
Glucose (H_2 and/or CH_4-positive)	2/10 (20%) FD vs. 8/44 (18.2%) iron deficiency/ FOBT + (no HV)	Shah et al. 2020[117]
Lactulose (H_2-positive)	13/23 (56.5%) FD vs. 0/11 (0%) HV	Costa et al. 2012[121]
	1/8 (12.5%) FD vs. 0/15 (0%) HV	Nakagawa et al. 2020[58]

CH_4, methane; FOBT, fecal occult blood test, H_2, hydrogen; HV, healthy volunteer; NERD, non-erosive reflux disease; QoL, quality of life; rRNA, ribosomal RNA; T-RFLP, terminal restriction fragment length polymorphism.

1.2.5 Changing the paradigm

The most attractive hypothesis for symptom generation is loss of mucosal integrity as a primary event, leading to immune activation through antigen presentation with eosinophil- and mast cell- activation, triggering visceral hypersensitivity and altered motor control (**Figure 1.5**).[41] Indeed, functional and structural submucosal neuronal changes have been reported in the duodenum of FD patients, correlating with the accumulation of eosinophils and mast cells in close proximity to the neurons.[69] This proximity to nerve cells is similar to findings of colonic mast cells in IBS patients,[122] and duodenal mast cells were also elevated in FD patients.[52,69,70] Thus, activation and degranulation of immune cells with normal numbers may also result in neuronal changes with dyspeptic symptoms.[59,64,71] In addition, changes in systemic immune activation,

including small bowel homing T cells, and correlations with symptoms also point to the importance of local duodenal changes in FD patients.[73]

The consistent finding of duodenal eosinophilia in FD may also help to explain the observed link with allergy and atopy.[66,123,124] Moreover, associations of dyspepsia in the general population with herbivore but not carnivore pets and antibiotics suggest the involvement of microbiota-related components.[125] As mentioned above, pi-FD results in important immune activation and, similar to IBS, higher titers of antibodies to Cytolethal distending toxin B (produced by Gram-negative bacteria causing infection) were found in FD vs. controls in an Australian population-based study, suggesting possible under-recognition of pi-FD.[126] Thus, duodenal dysbiosis or other luminal triggers (acid, bile, food or secreted mediators) may cause the mucosal barrier defect and immune activation in FD.[41] However, clinical case-control studies do not allow any inference of causality or directionality and the potential role of increased permeability and inflammation in FD remains elusive in the absence of specific therapies targeting the underlying pathophysiology, as discussed in the next section.

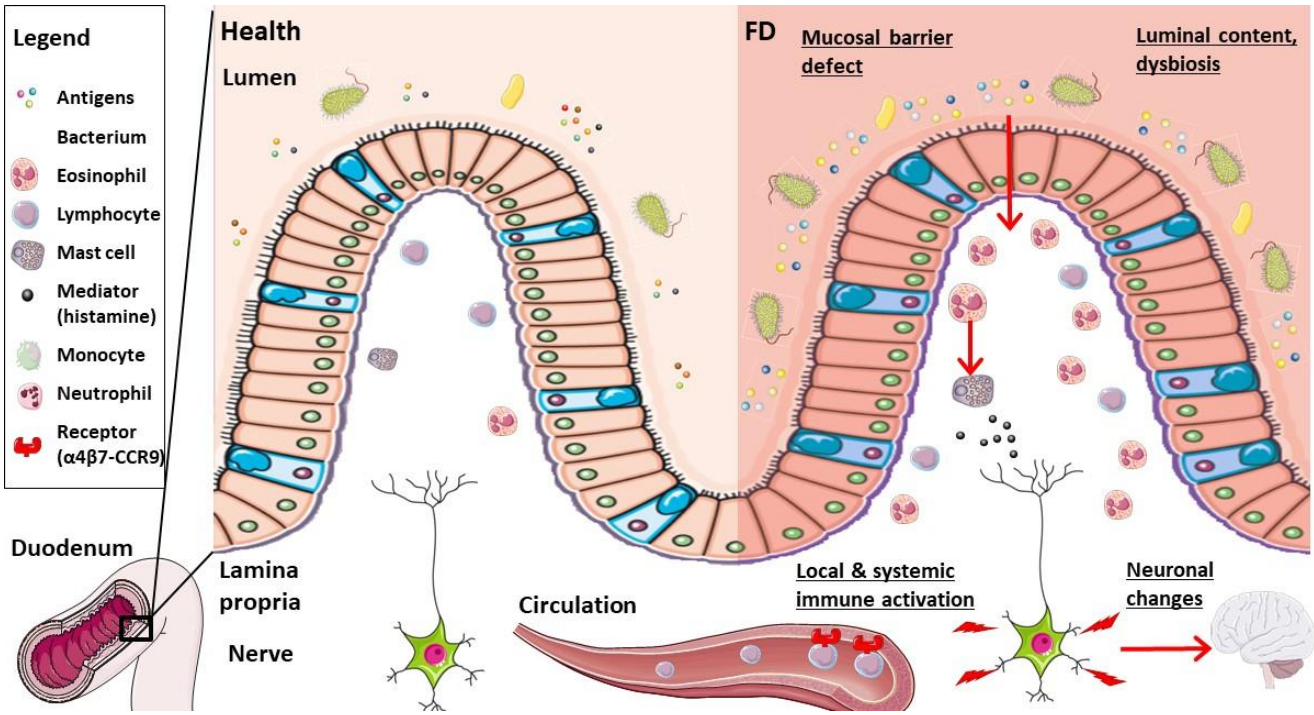

Figure 1.5: Pathways underlying duodenal pathology in Functional Dyspepsia compared to health. In health (left), the duodenal luminal content is separated from the mucosal lamina propria by an intact barrier. In case of duodenal barrier dysfunction such as observed in FD (right), altered luminal content and/or duodenal dysbiosis may trigger local (eosinophil-mast cell axis) and systemic (gut-homing lymphocytes) immune activation. This in turn may lead to neuronal changes with disturbed duodeno-gastric feedback signaling (causing delayed gastric emptying) and dyspeptic symptom generation by signaling to the brain. FD, functional dyspepsia.

1.3 Therapeutic options

1.3.1 General recommendations

Despite the recognition of meal-related symptoms, especially for PDS, no specific dietary factors have been convincingly linked with the pathophysiology of FD. Although patients often link certain food components with symptoms after ingestion, there is a lack of controlled dietary intervention studies. While fatty foods were more commonly associated with symptoms than isocaloric high-carbohydrate meals,[127] symptoms were related to the fat label (independent of content) indicating a significant nocebo effect.[128] Regarding sensitivity to wheat, only 1 small RCT has separated gluten and fructans (fermentable oligo-, di-, mono-saccharides and polyols or FODMAPs) and identified no specific triggers in FD.[95] In contrast, a gluten re-challenge was positive

in up to 1/5 of FD patients responsive to a gluten-free diet.[129] Also, a higher response to low-FODMAP vs. standard dietetic advice was found in an observational study,[130] and this should include frequent small size meals with avoidance of high-fat food items, alcohol or coffee and smoking cessation. More studies including the psychological or multimodal aspects of food and multidisciplinary care should be performed in FD.[131]

Regarding medical treatment, few options exist and no cost-effective therapy is available, except for antibiotic eradication with acid-suppression in case of *H. pylori* infection.[1] While this is also based on the reduced risk of peptic ulcer disease and gastric cancer rates in infected patients, clinical efficacy was significantly greater vs. placebo antibiotics (relative risk (RR) of dyspepsia remaining .91, 95% confidence interval (CI) [.88;.94]) with a number needed to treat (NNT) of 12.5 (95% CI [10;20]).[1] The separate entity of *H. pylori*-associated dyspepsia is distinguished from FD in case of sustained relief of symptoms after 6-12 months of eradication.[17] Interestingly, the effect of eradication was greater with metronidazole and in case of microscopic duodenitis,[132,133] suggesting an effect on the duodenal microbiome which is distinct from eradication of *H. pylori*.[41] Following exclusion and/or eradication of *H. pylori*, further therapy is advised as discussed in the next sections.[1,7]

1.3.2 Acid suppression

Acid-suppressive therapy with proton pump inhibitors (PPI) is recommended as first-line therapy by the North American and UK guidelines after eradication or in *H. pylori*-negative patients.[1,134] PPI-therapy was also recognized as an effective treatment for FD in the recent European consensus.[7] Indeed, a Cochrane meta-analysis including 6,172 patients confirmed a reduction of global dyspeptic symptoms (RR= .88 [.82;.94], NNT= 11) with similar QoL on PPI compared to placebo.[135] This was confirmed by another meta-analysis (NNT= 10), with no differences for different doses or types of PPI.[1,135] According to the Rome IV consensus, PPIs were considered ineffective for the PDS subgroup,[2] which was based on the results from an older meta-analysis, showing efficacy of PPIs in the epigastric pain but not dysmotility-like FD subgroups (Rome I and II criteria).[136] However, a tendency for higher efficacy of PPI in PDS (RR= .89 [.77;1.03]) vs. EPS (RR= .99 [.76;1.28]) was reported, although the number of included 'pure EPS' patients was low.[135] While the exact mechanism of action in FD is unknown, PPIs are beneficial for overlapping GERD symptoms and may also reduce duodenal acid exposure in FD patients.[91]

Although international guidelines advice against dose escalation in case of insufficient improvement with a once daily dose of PPI during 4-8 weeks, inappropriate and prolonged use of PPIs even in the absence of clinical benefit is frequently reported. Moreover, PPI have been associated with an increased risk of enteric infections (including *Clostridioides difficile*)[137,138] and overabundance of oral or potentially pathogenic flora in the gastric[139] and fecal microbiome.[140] Cessation of PPI or association of probiotics have been proposed (see below), although clinical and microbial evidence for both approaches is still lacking in FD. Acid suppression can also be achieved with histamine-2 receptor antagonists (H2RA), with no difference between both treatments (RR .88 [.74;1.04]).[135] However, due to the lack of high-quality comparative trials and the superior anti-secretory effect of PPIs, the North American but not UK guidelines still advise PPI over H2RA as first-line therapy.[1,134] Interestingly, the similar efficacy could also be explained by anti-histamine effects, which is the main mast cell-mediator and a potential target of the eosinophil-mast cell-axis (see below).

1.3.3 Prokinetics

Prokinetics enhance gastric emptying and although delayed gastric emptying and impaired gastric accommodation may be more common in PDS patients, this was not confirmed in a large study which showed a similar prevalence of gastric motor abnormalities in PDS and EPS.[25] Delayed gastric emptying may be present in about 23% of FD patients and prokinetics also affect gastric accommodation and sensitivity, which were disturbed in 37% of FD patients.[25] These effects may explain the significant benefit of prokinetics in

reducing ongoing dyspeptic symptoms (RR= .81 [.74;.89], NNT= 7) in a meta-analysis of 29 studies involving 10,044 FD patients.[141] However, significant heterogeneity and publication bias were noted and the overall effect was less convincing after removing cisapride from the meta-analysis (NNT= 12), which was withdrawn from the market due to cardiac adverse events.[141] However, QT-prolongation (domperidone) or extra-pyramidal side effects (metoclopramide) limit their chronic use and other prokinetics such as itopride or acotiamide are not widely available (**Figure 1.6**).

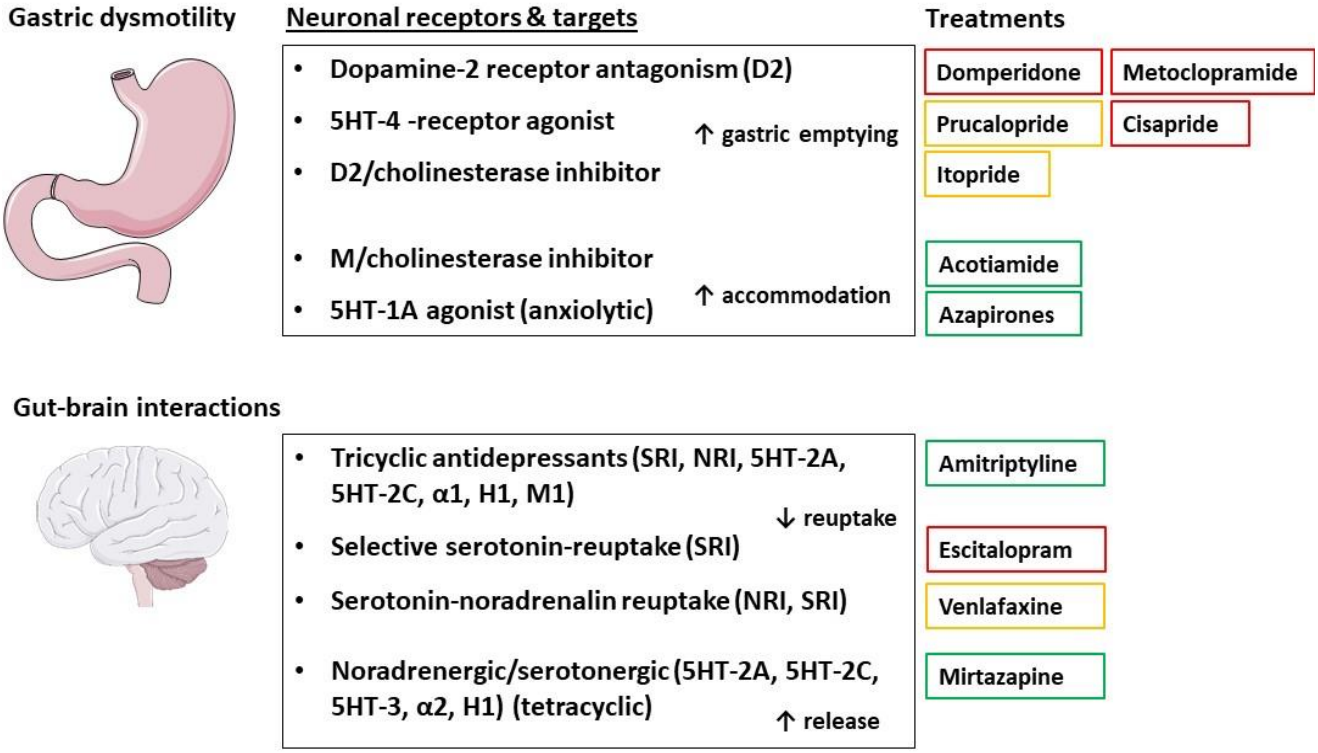

Figure 1.6: Neuronal targets for gastric dysmotility and gut-brain interactions in Functional Dyspepsia. Effective (green) or potentially effective (yellow) and harmful or withdrawn (red) treatments are depicted on the right. 5-HT, 5-hydroxytryptamine (serotonin); α, α-noradrenalin receptor; D, dopamine receptor; H, histamine receptor; M, muscarinic acetylcholine receptor; NRI, noradrenalin reuptake inhibition; SRI, serotonin reuptake inhibition.

1.3.4 Neuromodulators

Efficacy of neuromodulators (RR= .78 [.68;.91], NNT= 6) was limited to antipsychotics and tricyclic antidepressants (TCA) in a systematic review and meta-analysis of 13 studies with 1241 FD patients.[142] Amitriptyline (TCA) but not escitalopram (selective serotonin-reuptake inhibitor) was effective for the treatment of ulcer-like FD, with a 3-fold increased odds of reporting adequate relief of symptoms compared to placebo.[143] Although patients with delayed gastric emptying did not respond to amitriptyline, this was not related to a treatment-induced delay in gastric emptying, indicating an effect on visceral sensitivity and abdominal pain.[143,144] However, little is known on the longer-term efficacy of neuromodulators as the longest treatment duration was 12 weeks.[142] The tetracyclic antidepressant mirtazapine improved PDS-symptoms, QoL, nutrient tolerance and body weight in a RCT of 34 FD patients with significant weight loss (>10% of original body weight) and no depression or anxiety, which could be linked to the antagonism of histamine-1, adrenergic α2- and both serotonin-2A/C and -3 receptors.[145] In addition, treatment with buspirone, a serotonin-1A receptor agonist which relaxes the proximal stomach in healthy individuals, reduced both overall and PDS-type symptoms in FD and improved gastric accommodation.[146] Although the gastric emptying rate for solids was unchanged, a delay in gastric emptying for liquids was noted but the relevance for dyspeptic symptoms is probably limited.[146]

Based on the efficacy of TCA and safety concerns on prokinetics, North American guidelines advised the former before the latter in PPI-refractory FD patients.[1] A recent systematic review and network meta-analysis confirmed the superior efficacy of neuromodulators for symptom improvement, while symptom resolution was most common with standard-dose PPI.[147] Despite TCA being mainly tested in refractory FD patients, they were still among the most efficacious drugs, suggesting that earlier use may be beneficial in FD.[147] Although subgrouping FD patients based on GI symptoms and psychological comorbidity is possible using latent class analysis,[148] it is still unclear whether such subgroups could predict the disease course and therapeutic response. In the absence of biomarkers, the subdivision in PDS and EPS is common and agreement was only reached for PPI as an effective therapy and nutritional support in case of severe weight loss (**Figure 1.7**).[7]

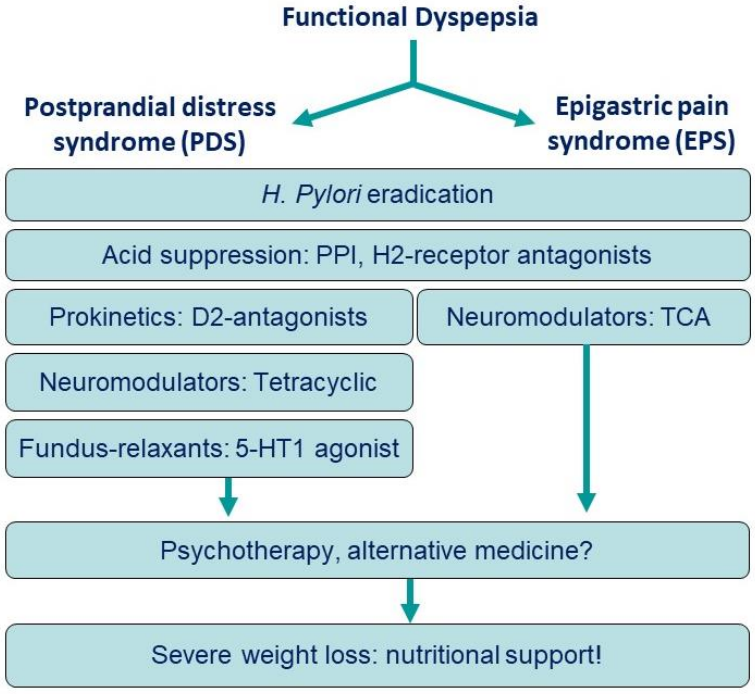

Figure 1.7: Proposed treatment algorithm for Functional Dyspepsia. 5-HT, 5-hydroxytryptamine (serotonin); D, dopamine receptor; EPS, epigastric pain syndrome; H, histamine receptor; PDS, postprandial distress syndrome; PPI, proton pump inhibitor; TCA, tricyclic antidepressant.

Psychological and alternative therapies have also been tested in FD and although Iberogast (STW5), composed of nine different extracts (e.g. Iberis, peppermint, chamomile), and peppermint oil (with or without caraway oil) were superior vs. placebo, the comparative efficacy of herbal medicine or phytotherapy with existing medical treatments remains to be established and none of these treatments reached agreement in the recent European consensus.[7,149]

1.3.5 Immune and microbial targets

Despite the growing recognition of duodenal immune activation, only few studies have investigated anti-inflammatory therapies in FD. Similar to IBS,[150] histamine-1 receptor antagonism may improve symptoms by blockade of histamine effects in patients with mast cell infiltration. Indeed, response rates of 79% were observed in an early and open-label study of refractory dyspeptic patients with increased antral mast cells.[151] As mentioned above, H2RA may have anti-inflammatory effects and combined H1- and H2-receptor blockade led to a 50% symptom improvement in dyspeptic children with duodenal eosinophilia.[152] A similar retrospective case series in adult FD showed a 71% symptom improvement with a trend for baseline duodenal eosinophilia in responders.[153] A single RCT also confirmed the efficacy of a leukotriene-1 receptor antagonist (montelukast) in pediatric FD.[154]

Following early reports of dysbiosis, probiotics have been tested for dyspeptic symptoms, even in the presence of *H. pylori*.[155] Despite the lack of effect on *H. pylori*, daily intake of *Lactobacillus gasseri* OLL2716 (LG21) but not placebo yoghurt decreased postprandial fullness scores after 12 weeks.[156] Although probiotic efficacy was more likely related to the reduction in side effects of PPI rather than direct effects on *H. pylori*,[157] similar eradication rates were reported for the combination of a *L. reuteri* strain with Pantoprazole 40mg/day for 8 weeks.[158] While beneficial clinical effects were described for different *Lactobacillus* strains, no single study performed and/or specified negative investigations (even *H. pylori*[159,160]) (**Table 1.5**). Due to the higher prevalence of *H. pylori*, this is even more critical for Asian studies.[161] In addition, randomization was only performed in one study, with a 'placebo' or fermented yoghurt without the additional probiotic under study, and all studies lacked a validated questionnaire as advised by FDA/EMA.

Table 1.5: Probiotics for uninvestigated dyspeptic symptoms (*H. pylori* negative or unknown).

Type	Findings	References
Lactobacillus	↓nausea, postprandial fullness, gastric distention, belching for *L. reuterii, L. rhamnosus + Saccharomyces boulardii* vs. antioxidants or no addition to olive oil with meals (each 1w)	Ianiro et al. 2013[162]
	↓PDS- & EPS-like symptoms with open-label *L. gasseri* (12w)	Igarashi et al. 2016[115]; Nakae et al. 2016[114]
	↓postprandial fullness and epigastric bloating for *L. gasseri* vs. placebo yoghurt (RCT, 12w)	Ohtsu et al. 2017[163]
	↓main and minor PDS- & EPS-symptoms with open-label combination *L. rhamnosus, L. pentosus, L. plantarum, L. delbrueckii* + N-ACC +/- PPI, prokinetics or antacids (30+15d)	Drago et al. 2021[164]
	↓abdominal pain and distention, and belching with open-label *L. paracasei* (28d)	Sun et al. 2021[159]
Bifido-bacterium	↓acid-related dyspepsia with open-label *B. bifidum* (4w)	Urita et al. 2015[160]

EPS, epigastric pain syndrome; N-ACC, N-acetylcysteine; PDS, postprandial distress syndrome; PPI, proton pump inhibitor; RCT, randomized-controlled trial.

As mentioned above, probiotics have been proposed in case of PPI-related side effects,[165] although this has not yet been studied in FD. Besides limited evidence suggesting that microbiota changes may cause persisting dyspeptic symptoms on-PPI,[166] others have questioned the long-term use of PPI in FD patients due to their limited efficacy.[1] Of note, a two-week treatment with rifaximin, a non-absorbable and selective antibiotic, was superior to placebo for the adequate relief of global dyspeptic symptoms at 8 weeks and post-prandial fullness, bloating and belching at 4 weeks in Asian and *H. pylori*-negative FD patients.[161] The effect on symptoms was more pronounced in women with a similar incidence of adverse effects in the active and control group.[161] Although the exact mechanism of action is unknown, the increased solubility of rifaximin with bile salts could explain the therapeutic antibiotic effect in FD patients with changes in the duodenal micro-environment.[41] Whether these selective antibiotics would also benefit FD patients with dysbiosis on long-term PPI has not yet been investigated (**Figure 1.8**).

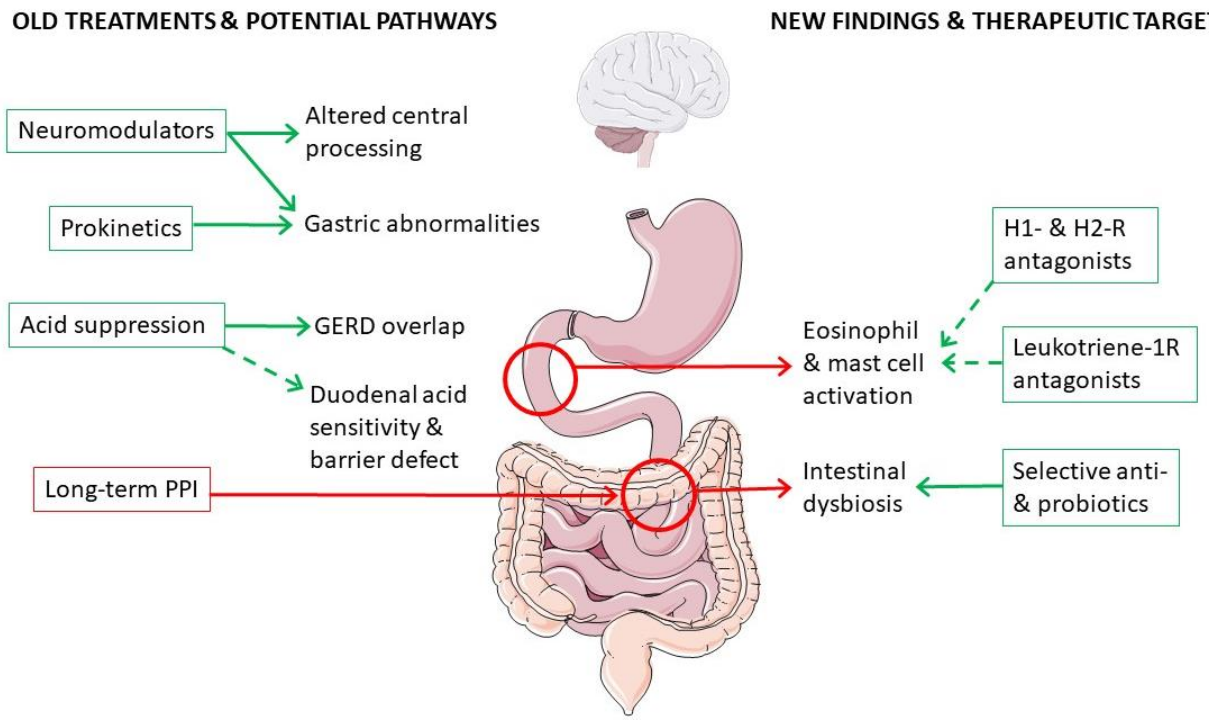

Figure 1.8: Old and new treatments based on alterations and therapeutic targets in functional dyspepsia. Effective treatments are indicated with green boxes and arrows, with solid lines for evidence from controlled trials and dashed lines for evidence from open-label trials. Red boxes indicate potentially harmful side-effects. GERD, gastro-esophageal reflux disease; H, histamine-receptor; PPI, proton pump inhibitor.

1.4 Introductory conclusions

Despite the occurrence of FD in up to 20% of the population with a considerable impact on QoL and health care expenses, the underlying pathophysiology remains unclear and treatment options are limited in efficacy and/or safety. Increasing data from our group and others have shifted the paradigm of FD as a pure 'functional' GI-disorder by observations of structural alterations in the duodenum. Although duodenal changes may also be linked to the traditional views of gastric sensorimotor and central dysfunction, no treatments targeting these duodenal alterations have been identified and hence the potential relevance to symptom generation is still unclear.

Moreover, little is known about the mechanism of action of PPI in FD, which are still the first-line therapy, including their potential effect on the duodenal microbiome. This is especially relevant considering the important microbiota-immune interactions and preliminary data on dysbiosis of the small intestine in dyspeptic patients. Besides the heterogeneous designs, populations and outcomes of probiotic trials for dyspeptic symptoms, the microbial and immune effects of probiotics have not been investigated in relation to PPI-use, which is relevant regarding the potential effect of both treatments on luminal as well as systemic changes including immune activation.

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CHAPTER 2
RESEARCH OBJECTIVES

2 RESEARCH OBJECTIVES

Despite the growing recognition of duodenal alterations in the pathophysiology of FD, the contribution to symptom generation and the effect and mechanism of PPI or first-line therapy remain unclear. Considering the potential role of both duodenal and systemic alterations and the fact that PPI may affect different factors, a comprehensive and prospective study in FD patients is needed. Moreover, long-term PPI-therapy has been scrutinized due to an increased risk of side effects including enteric infections, and changes in the duodenal microbiome have not been assessed in relation to PPI. Finally, clinical, immune and microbial effects of probiotics may also differ depending on the use of PPI.

We hypothesized that duodenal rather than systemic or central changes drive dyspeptic symptoms and that these are affected by treatment with PPI and/or probiotics (**Figure 2.1**).

The **first objective** was to study the effects of PPI-therapy on previously described duodenal and systemic alterations in FD patients compared to controls.

The **second objective** was to study the duodenal microbiome in relation to other duodenal luminal and mucosal factors and the effect of PPI in FD patients vs. controls.

The **third objective** was to study the efficacy and safety of spore-forming probiotics in FD patients with or without PPI-therapy, as changes in symptoms and systemic immune activation may differ depending on the cumulative effect on the microbiome.

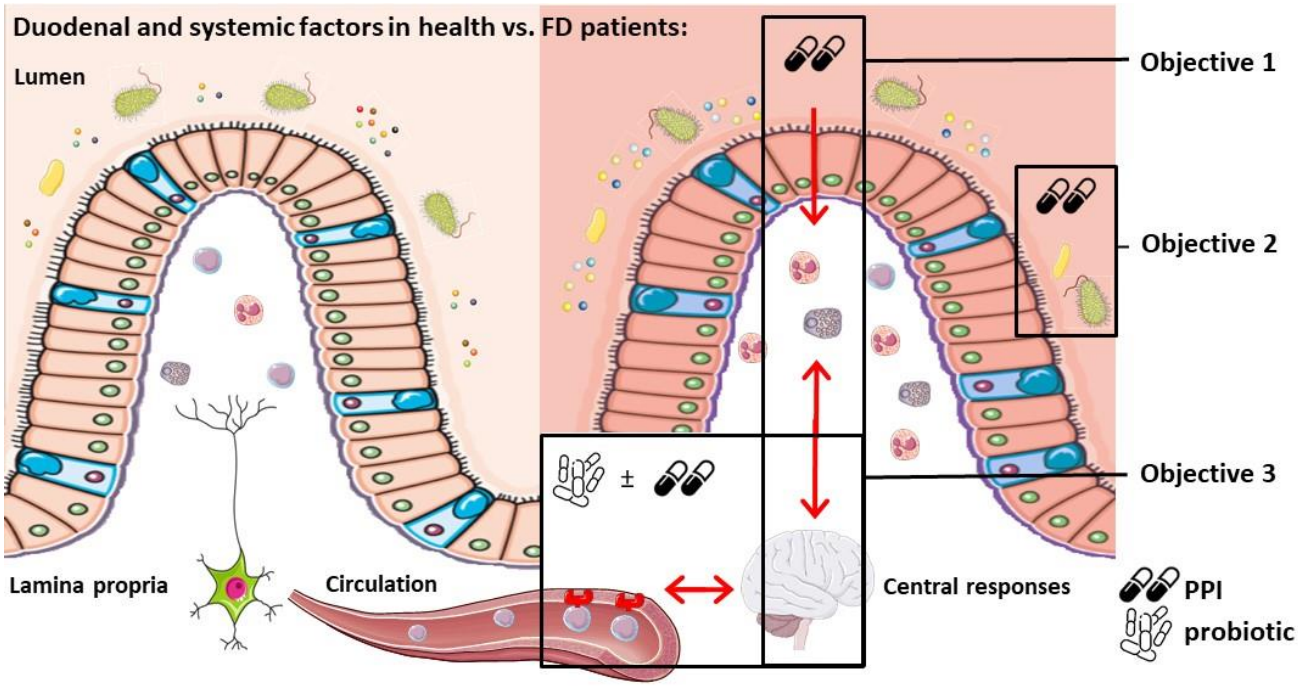


Figure 2.1: Objectives of the current doctoral thesis. FD, functional dyspepsia; PPI, proton pump inhibitor.

CHAPTER 3

PROTON PUMP INHIBITORS REDUCE DUODENAL EOSINOPHILIA, MAST CELLS AND PERMEABILITY IN PATIENTS WITH FUNCTIONAL DYSPEPSIA

This chapter has been adapted from: **Wauters L**, Ceulemans M, Frings D, Lambaerts M, Accarie A, Toth J, Mols R, Augustijns P, De Hertogh G, Van Oudenhove L, Tack J, Vanuytsel T. *Gastroenterology* 2021;160(5):1521-1531.e9.

3 PROTON PUMP INHIBITORS REDUCE DUODENAL EOSINOPHILIA, MAST CELLS AND PERMEABILITY IN PATIENTS WITH FUNCTIONAL DYSPEPSIA

3.1 Abstract

Background & Aims: Despite the growing recognition of duodenal alterations in the pathophysiology of functional dyspepsia (FD), the effect and mechanism of proton pump inhibitors (PPI) or first-line therapy remain unclear. We studied duodenal and systemic alterations in relation to PPI-therapy in FD patients and healthy controls.

Methods: We performed a prospective interventional study assessing symptoms (PAGI-SYM), duodenal alterations and systemic factors in FD patients ("FD-starters") and controls before and after PPI-therapy (pantoprazole 40mg once daily for 4 weeks). Duodenal mucosal eosinophils, mast cells and permeability were quantified. Luminal pH and bile salts were determined in duodenal aspirates. Procedures were also performed in PPI-refractory FD patients ("FD-stoppers") before and 8 weeks after PPI-withdrawal. Between- and within-group changes from baseline and associations with duodenal or systemic factors were analyzed using linear mixed models.

Results: The study was completed by 30 controls, 27 FD-starters and 18 FD-stoppers. Symptoms and duodenal eosinophils, mast cells (all $P < .0001$), and paracellular passage ($P = .02$) were significantly higher in FD-starters vs. controls, and reduced with PPI-therapy. Symptoms and duodenal immune cells also decreased in FD-stoppers off-PPI. In contrast, immune cells and permeability increased in controls on-PPI. Dyspeptic symptoms correlated with eosinophils before and during PPI-therapy, while increased eosinophils and permeability in controls on-PPI were associated with changes in bile salts.

Conclusions: We provide the first prospective evidence for eosinophil-reducing effects as a therapeutic mechanism of PPI in FD, with differential effects in controls pointing to a role of luminal changes.

Clinicaltrials.gov, number: NCT03545243.

3.2 Introduction

Dyspepsia refers to chronic or recurrent upper gastrointestinal (GI) symptoms originating from the gastroduodenal region with a significant impact on patients' lives.[1] According to the Rome IV criteria, functional dyspepsia (FD) comprises the subgroups of epigastric pain syndrome (EPS) with epigastric pain or burning and postprandial distress syndrome (PDS) with meal-related fullness or early satiation, which are unexplained after routine investigation.[2] Despite the common occurrence of FD in up to 20% of the population, the underlying pathophysiology remains unclear and the first-line therapy is acid suppression with proton pump inhibitors (PPI).[1–3] In the Rome IV consensus, PPI were first considered ineffective for the PDS subgroup, and mainly effective in EPS, at least in part through overlapping gastroesophageal reflux disease (GERD).[2] However, a recent Cochrane meta-analysis showed a trend for higher efficacy of PPI in the PDS vs. EPS subgroup.[4] Hence, the exact mechanism of action of PPI in FD and especially PDS, is unknown.

Recently, reports of subtle duodenal pathology with increased mucosal eosinophil and mast cell infiltration in FD patients have shifted the focus to the duodenum.[3] Indeed, activation of duodeno-gastric reflexes has been implicated in gastric sensorimotor dysfunction,[3,5] suggesting a primary role for duodenal pathology in FD symptom generation. We previously demonstrated altered expression of duodenal epithelial adhesion proteins, which correlated with increased mucosal permeability and the number of inflammatory cells.[6] Moreover, these changes were associated with altered neuronal signaling in the submucosal plexus,[7] and systemic immune activation with 'gut-homing' lymphocytes has been linked to gastric emptying and symptoms in FD.[8] Duodenal mucosal changes in FD may be caused by an altered luminal environment, including acidity (pH) and bile salts,[9] but also systemic factors including stress (cortisol) may play a role.[10] Indeed, psychosocial factors and dysregulated hypothalamic-pituitary-adrenal (HPA-)axis responsiveness are potentially implicated in the patho-physiology of functional GI disorders.[2,11]

Besides acid-suppressive effects of PPI, which may reduce duodenal acid exposure, barrier-protective effects similar to their effect in the esophagus may also explain their efficacy in FD.[12] Interestingly, the presence of duodenal eosinophilia was found predominantly in PDS patients,[13,14] which may explain the therapeutic efficacy of PPI by its anti-inflammatory effects, similar to what has been observed in eosinophilic esophagitis (EoE).[15] Considering the potential role of both duodenal and systemic alterations in FD and the fact that PPI-therapy may affect different factors, a comprehensive and prospective study is lacking. We hypothesized that duodenal eosinophilia is a pathophysiological mechanism and a therapeutic target for PPI in FD. Thus, the aims of this study were to (1) confirm duodenal mucosal inflammation and increased permeability in FD, (2) evaluate the role of other duodenal and systemic factors, (3) assess the effect of PPI on both duodenal and systemic alterations including the responsiveness of the HPA-axis and (4) study associations of PPI-related changes in clinical outcomes with duodenal or systemic factors.

3.3 Methods

Study subjects

We included patients with predominant FD symptoms, diagnosed according to Rome IV criteria.[2] Patients were divided in 2 cohorts based on current or previous use of PPI-therapy: (1) "FD-starters" with no standard course of PPI-therapy (4 weeks healing dose) and/or acid suppression < 3 months before inclusion, and (2) "FD-stoppers" with refractory symptoms after > 1 month of at least one daily dose of PPI. All patients were referred to the outpatient clinic of the University Hospitals Leuven (Belgium) for investigation of FD symptoms. In addition, age- and gender-matched healthy volunteers without GI symptoms were recruited as controls by advertisement. All subjects were female or male, aged 18 to 64 years old, with no active psychiatric, atopic, inflammatory or metabolic conditions. The study was conducted according to the Declaration of Helsinki and Good Clinical Practice regulations after approval by the Ethics Committee of the

University Hospitals Leuven (number S60953 and S60984). Written informed consent was obtained from each study participant before inclusion and all data were collected at KU Leuven and University Hospitals Leuven (Leuven, Belgium). All authors had access to the study data and reviewed and approved the final manuscript.

Clinical procedures

Study procedures were performed on visits before and after (1) start PPI-therapy (pantoprazole 40mg once daily for 4 weeks) in controls and FD-starters, and (2) PPI-withdrawal in FD-stoppers (8 weeks).

Duodenal biopsy collection

Biopsies from the second portion of the duodenum (D2) were collected during gastroduodenoscopy at each study visit and in all subjects using a Radial Jaw 3 forceps (Boston Scientific, Natick, MA, USA) by an experienced endoscopist (TV or JT). Biopsies were kept in either ice-cold Hanks' Balanced Salt Solution (HBSS) buffer (Ussing chambers; 4 biopsies) or 10% formalin (histology; 2 biopsies). Gastric biopsies were collected at baseline in all subjects and kept in 10% formalin (2 biopsies).

Duodenal fluid aspiration

After the endoscopy, a double-lumen naso-duodenal aspiration catheter was placed at each visit under fluoroscopic control until the tip was located in the distal part of D2 or in D3.[9] Duodenal fluid aspiration was performed every 30min after placement for 1h (fasting) and every 15min after intake of 200mL nutrient drink (Fortimel Energy, 150kcal in 100mL, 5.8g lipids; Nutricia, Zoetendaal, The Netherlands) for 1h (fed state). After pH-measurement using the Portavo 902 PH portable pH meter (Knick, Berlin, Germany) with a BioTrode electrode (Hamilton, Bonaduz, Switzerland), samples were kept on ice until further processing. Due to potential contamination of the first aspirated sample with gastric fluid, only the fasting sample after 1h, i.e. immediately before the liquid meal (0min), and all fed samples (+15min, +30min, +45min, +60min) were included.

Questionnaires

Symptoms were assessed at each visit and in all subjects using the Patient Assessment of GI Symptom Severity Index (PAGI-SYM), which is specific for upper GI disorders with the total score ranging from 0 (none) to 5 (very severe) over a two-week recall period.[16] Perceived stress in the preceding week was assessed with the 10-item Perceived Stress Scale (PSS).[17]

Experimental procedures

Ussing chambers

Biopsies were mounted in modified 3mL Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) within 30min of collection as previously described.[6] Experiments were performed in triplicate and in open-circuit conditions for 2h with determination of the transepithelial electrical resistance (TEER) and paracellular (mucosal to serosal) passage every 30min after addition of a fluorescein isothiocyanate-labelled 4kDa dextran (FD4, 1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) to the mucosal compartment. The fluorescence level of serially collected serosal samples was measured at 480nm with a FLUOstar® Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). For TEER, the means of all timepoints per biopsy were averaged for each study visit in each subject. For the paracellular FD4 passage, the serosal concentrations after 60, 90 and 120 minutes were averaged.

Immunohistochemistry

Duodenal eosinophil and mast cell counting was done blinded after hematoxylin and eosin (H&E) and c-kit staining, respectively. Stained duodenal biopsy sections were scanned with an Aperio CS2 slide scanner in a random and blinded fashion and visualized using ImageScope software (Leica Biosystems, Wetzlar,

Germany). Duodenal eosinophils (H&E) and mast cells (c-kit) were counted per mm^2 by dividing the number of eosinophils (bilobar nucleus and eosinophilic granules) or mast cells (visible nucleus and cytoplasmic c-kit staining) in 3 separate regions, of which the mean was calculated for each study visit in each subject. Regions were selected in vertically oriented tissue from the base of a villus to the intercryptal region (lamina propria) with exclusion of glandular structures. In addition, duodenal intra-epithelial lymphocytes (IEL) were counted per 50 enterocytes in 2 separate and well-oriented villi (H&E), of which the sum was calculated per 100 enterocytes.[18]

Bile salt measurement

Concentrations of bile salts were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in duodenal aspirates at individual fasting (0min) and fed (+15min, +30min, +45min, +60min) timepoints. The sum of the primary and secondary bile salts (mM) was reported as the total bile salt concentration.

Statistical analysis

Baseline differences between groups were compared with one-way ANOVA for continuous data and chi-square tests for proportions. Changes in duodenal and systemic factors were analyzed using linear mixed models with "group" (FD-starters, controls or FD-stoppers) as between-, "treatment" (off- or on-PPI) and, where applicable, "timepoint" (repeated measurements) as within-subject factors. Between-group differences for FD-starters and controls at baseline (off-PPI) and within-group changes (on- vs. off-PPI) were assessed for all groups. In case of significant treatment-by-group interactions, planned contrasts were conducted to compare PPI-related changes between FD-starters and controls with stepdown Bonferroni adjustment for multiple testing. Since no treatment data on duodenal mucosal alterations is available in FD, no formal sample size calculation was performed. However, a sample size of 25 FD patients starting PPI-therapy would be sufficient to demonstrate a medium effect size (Cohen's d= .6) of PPI on reduction of duodenal eosinophils, which was the main focus of this study. Next, correlations between variables of interest were studied using the Spearman correlation coefficient. Finally, associations were studied between PPI-related changes in outcomes and duodenal or systemic factors. A two-tailed P-value < .05 was considered significant and .05 < P< .1 a trend. All data were analyzed in SAS software version 9.4 (SAS Institute, Cary, USA) and least squares means estimates (β) are given ± standard error (SE). Details are provided in the **supplementary methods**.

3.4 Results

Study population

From April 2018 - April 2020, 79 subjects were included in the study including 30 controls, 30 FD patients off-PPI (FD-starters) and 19 FD patients on-PPI (FD-stoppers) (**Supplementary Figure 3.1**). After exclusion of 2 FD patients with *H. pylori* infection, PPI-therapy was started in 28 and completed in 27 patients. All controls completed the study and PPI-withdrawal was done in 19 and completed in 18 patients. Reasons for drop-out were an unrelated adverse event (pregnancy) and withdrawal by the subject (failure to adhere). Baseline characteristics were similar between groups (**Table 3.1**) and median duration of PPI-therapy in FD-stoppers was 3.2 years (interquartile range 1.5 - 5.1 years).

Table 3.1: Baseline characteristics of healthy controls, FD-starters (off-PPI) and FD-stoppers (on-PPI).

Group and variable	Controls (n= 30)	FD-starters (n= 28)	FD-stoppers (n= 19)	*P*-value
Demographic:				
Age (years)	31.32 ± 1.98	31.70 ± 2.21	36.74 ± 3.01	.25
Female (%)	21 (70)	24 (86)	14 (74)	.35
BMI (kg/m^2)	23.22 ± .68	22.15 ± .65	22.80 ± .78	.5
Caucasian (%)	29 (97)	25 (89)	17 (89)	.51
FD subtypes:				
PDS-subtype (%)	NA	15 (54)	10 (53)	.95
EPS-subtype (%)	NA	3 (11)	6 (32)	.07
Overlap (%)	NA	10 (35)	3 (15)	.13
Lifestyle:				
Alcohol (%)[1]	8 (27)	6 (21)	5 (26)	.88
Smoking (%)[2]	2 (7)	4 (14)	2 (11)	.64

1: > 1 day per week, 2: including cessation in past 3 months. BMI, body mass index; EPS, epigastric pain syndrome; FD, functional dyspepsia; PDS, postprandial distress syndrome; PPI, proton pump inhibitor.

Clinical outcomes

At baseline (off-PPI), symptoms were higher in FD-starters vs. controls (β= 2.08 ± .19, *P*< .0001) and decreased after starting PPI-therapy in FD patients (β= -.65 ± .11, *P*< .0001) but not controls (*P*= .81) (**Figure 3.1A**, **Table 3.2**). This confirms clinical efficacy of a standard course of PPI and in all FD subtypes, although symptoms did not reach levels of controls off-PPI (see **supplementary results**).

Duodenal mucosal alterations

Duodenal mucosal eosinophil counts were higher in FD-starters vs. controls off-PPI (β= 15.29 ± 1.54, *P*< .0001) with a significant decrease after starting PPI-therapy in FD (β= -10.54 ± 1.88, *P*< .0001), compared to an increase in controls (β= 8.36 ± 1.77, *P*< .0001) (**Figure 3.1B**). Duodenal mast cells were also higher in FD-starters vs. controls off-PPI (β= 9.54 ± 1.63, *P*< .0001) with a significant decrease after starting PPI-therapy in FD (β= -3.94 ± 1.58, *P*= .02), compared to an increase in controls (β= 3.78 ± 1.5, *P*= .01) (**Figure 3.1C**). In contrast, duodenal IEL were similar in FD-starters vs. controls off-PPI (*P*= .86) with no change after PPI-therapy in FD patients (*P*= .42) or controls (*P*= .89) (**Table 3.2**). Thus, we confirm the presence of increased duodenal mucosal eosinophil and mast cell infiltration but not IEL in FD patients off-PPI and demonstrate that standard PPI-therapy reduces not only duodenal eosinophils but also mast cells, although not to levels of controls off-PPI (see **supplementary results**).

Regarding mucosal permeability, a trend for higher TEER values on- vs. off-PPI was found in all groups (treatment main effect *P*= .09), without significant between- or within-group differences. Baseline paracellular permeability, quantified by the transmucosal FD4-passage, was higher in FD-starters vs. controls off-PPI (β= .76 ± .32, *P*= .02) with a decrease after starting PPI-therapy in FD (β= -.75 ± .25, *P*= .004), compared to an increase in controls (β= .75 ± .26, *P*= .006) (**Figure 3.1D**). These findings indicate an improvement of mucosal barrier dysfunction after PPI in FD, similar to levels of controls off-PPI (**supplementary results**) with an opposite or barrier-impairing effect of PPI-therapy in controls.

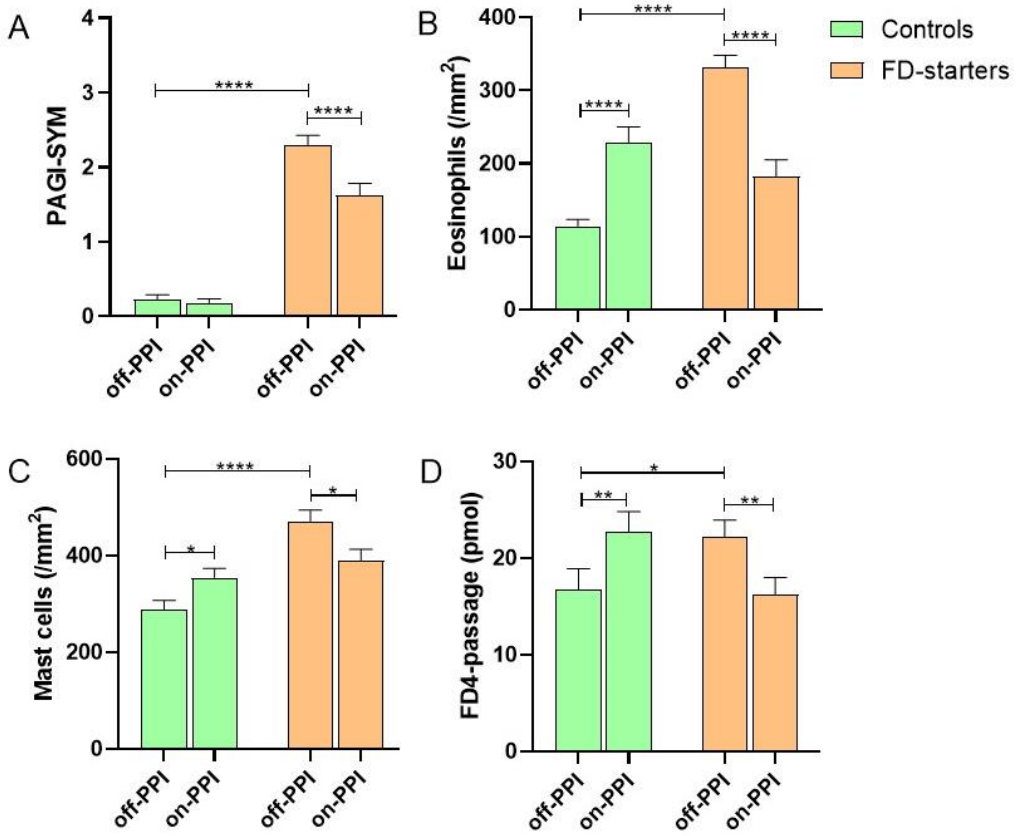


Figure 3.1: Changes in symptoms (A), duodenal mucosal eosinophils (B), mast cells (C) and paracellular passage (D) in controls and FD-starters before and after PPI. **P*< .05, ***P*< .01, *****P*< .0001. Data are presented as means ± standard error. FD4, FITC-Dextran 4kDa; FD, functional dyspepsia; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PPI, proton pump inhibitor.

Table 3.2: PPI-related changes in clinical, duodenal and systemic factors with between-group difference between FD-starters and controls (on- vs. off-PPI).

Group	**Controls**		**FD-starters**		***P*adj-value**
Treatment	**Off-PPI (n= 30)**	**On-PPI (n= 30)**	**Off-PPI (n= 28)**	**On-PPI (n= 27)**	
Clinical:					
PAGI-SYM	.23 ± .06	.19 ± .05	2.3 ± .13	1.62 ± .16	.0003
Duodenal:					
Eosinophils (/mm^2)	114.6 ± 8.83	229.22 ± 21.01	331.07 ± 16.93	182.63 ± 22.62	<.0001
Mast cells (/mm^2)	288.28 ± 18.79	354.31 ± 18.9	470.9 ± 23.24	389.76 ± 22.85	.002
IEL (/100 enterocytes)	8.37 ± .41	8.27 ± .37	8.44 ± .42	9.16 ± .58	1
FD4-passage (pmol)	14.99 ± 2.07	22.11 ± 2.18	20.31 ± 1.83	15.49 ± 1.83	<.001
Systemic:					
hsCRP (mg/L)	.97 ± .19	1.08 ± .22	2.21 ± .66	1.29 ± .3	.1
LBP (pg/mL)	11.99 ± .68	12.38 ± .78	12.86 ± .89	12.62 ± .99	.79
PSS	7.64 ± 1.11	5.16 ± .92	14.29 ± 2.39	12.67 ± 1.59	.55

FD4, FITC-Dextran 4kDa; FD, functional dyspepsia; hsCRP, high-sensitivity CRP; IEL, intra-epithelial lymphocyte; LBP, LPS-binding protein; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PPI, proton pump inhibitor; PSS, Perceived Stress Scale.

Duodenal luminal alterations

Subsequently we investigated luminal alterations, which could potentially drive the mucosal changes. Luminal fasting pH was higher in FD-starters vs. controls off-PPI (β= 1.1 ± .19, *P*< .0001) with an increase in controls (β= 1.33 ± .18, *P*< .0001) but not FD (*P*= .84) after starting PPI (**Figure 3.2A**). Luminal fed pH (60min) was also higher in FD-starters vs. controls off-PPI (β= .53 ± .19, *P*= .007) with an increase in both FD (β= .5 ± .17, *P*= .004) and controls (β= 1.04 ± .17, *P*< .0001) after starting PPI-therapy (**Figure 3.2B**). This indicates a different acid-related luminal environment in FD patients before starting PPI-therapy with PPI-related changes for fed but not fasting pH in FD. Results from the model across all timepoints are described in the **supplementary results**.

Similar to pH, a PPI-induced increase in total bile salts was found for fasting (β= 6.52 ± 1.59, *P*= .0001) and fed (β= 57.73 ± 21.12, *P*= .009) state in controls only and with no differences between FD-starters and controls off-PPI (**Figure 3.2C-D**). Results were similar for the model across all timepoints and for primary or secondary bile salts (**supplementary results**). Thus, luminal bile salts were similar in controls and FD with PPI-related changes in controls but not FD.

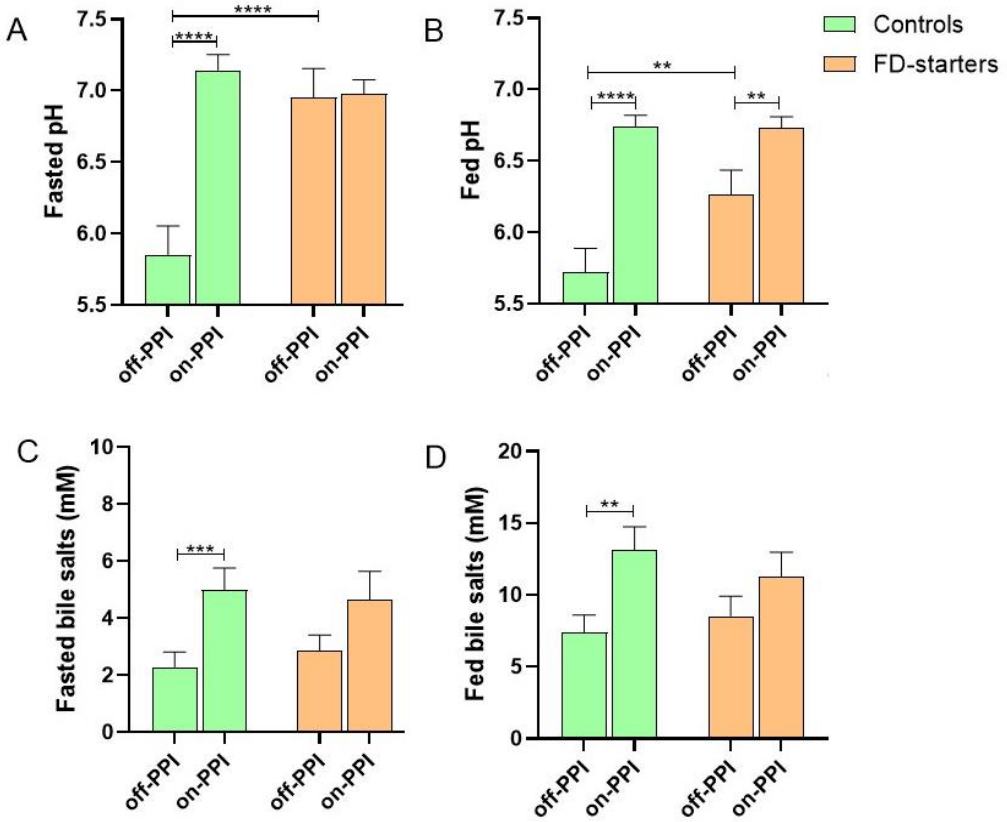


Figure 3.2: Changes in fasted (A) or fed pH (B) and fasted (C) or fed total bile salts (D) in controls and FD-starters before and after PPI. ***P*< .01, ****P*< .001, *****P*< .0001. Data are presented as means ± standard error. FD, functional dyspepsia; PPI, proton pump inhibitor.

Systemic alterations

Finally, we determined markers of systemic immune activation and the stress response. Plasma hsCRP-levels were higher in FD-starters vs. controls off-PPI (β= 1.2 ± .58, *P*= .04) with a significant decrease in FD-starters (β= -.57 ± .25, *P*= .03) but not controls (*P*= .56) (**Figure 3.3A**). This indicates a reduction of systemic immune activation after PPI in FD-starters. Because of similar alterations in intestinal barrier function and the hypothesis that luminal antigens or bacterial products may translocate across the barrier leading to mucosal and systemic inflammation, we evaluated plasma lipopolysaccharide (LPS)-binding protein (LBP) levels.

However, LBP was similar in FD-starters vs. controls off-PPI (*P*= .5) with no changes in FD starters (*P*= .41) or controls (*P*= .7) (**Table 3.2**).

Perceived stress was higher in FD-starters vs. controls off-PPI (β= 1.86 ± .66, *P*= .007) with no change after starting PPI in FD (*P*= .44) but a trend for lower PSS in controls (β= -.85 ± .44, *P*= .06) (**Figure 3.3B**). Salivary cortisol upon awakening (0min) was also higher in FD-starters vs. controls off-PPI (β= .41 ± .15, *P*= .007) with a decrease after start PPI in FD (β= -.46 ± .15, *P*= .002) but no change in controls (*P*= .51) (**Figure 3.3C**). Results were similar for the model across all timepoints (see **supplementary results**). Thus, although subjective stress-levels in FD patients were higher and remained similar, higher awakening cortisol was reduced in FD-starters after PPI.

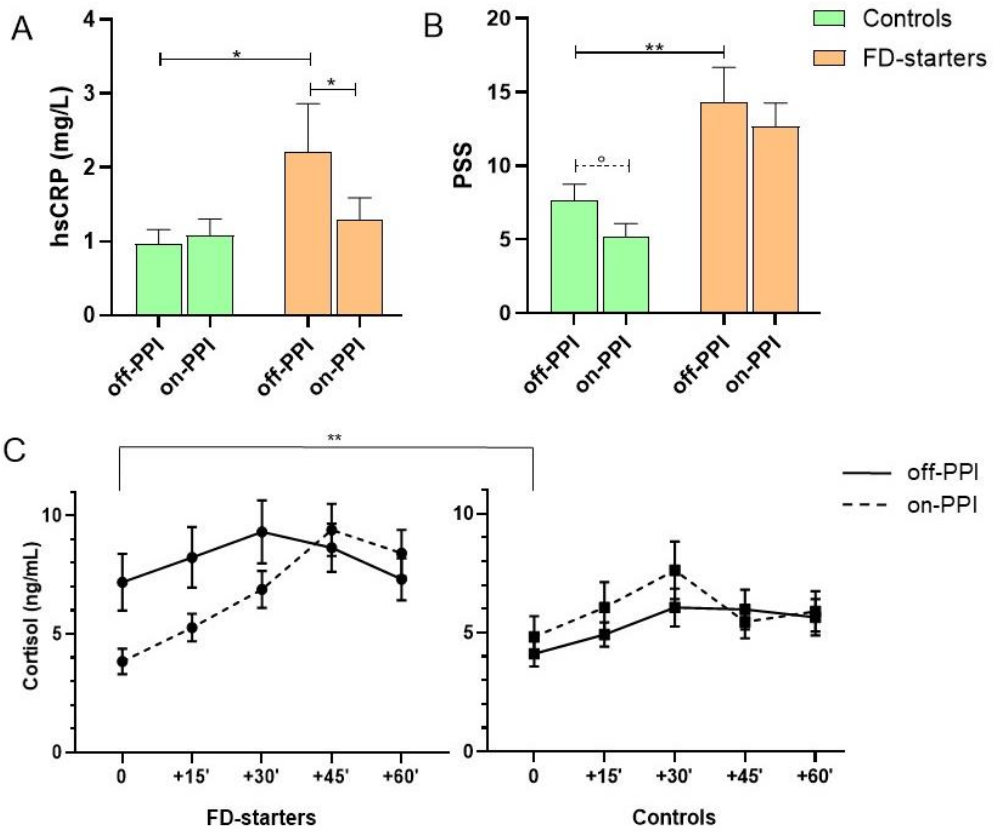


Figure 3.3: Changes in hsCRP (A), perceived stress (B) and cortisol awakening response (C) in FD patients and controls before and after starting PPI. °*P*< .1, **P*< .05, ***P*< .01. Data are presented as means ± standard error. Full line off-PPI, dashed line on-PPI. FD, functional dyspepsia; hsCRP, high sensitivity C-reactive protein; PPI, proton pump inhibitor; PSS, Perceived Stress Scale.

Effect of PPI-withdrawal

Interestingly, symptoms improved after stopping PPI in FD-stoppers (β= -.37 ± .13, *P*< .01), indicating a potential benefit to withdraw PPI in refractory FD patients after long-term PPI, although symptoms did not reach levels of controls off-PPI (see **supplementary results**). In addition, a trend for lower duodenal eosinophil (β= -3.95 ± 2.25, *P*= .08) and significantly lower mast cell (β= -3.84 ± 1.89, *P*< .05) counts but similar paracellular permeability (*P*= .23) were found after stopping PPI.

Associations with clinical outcomes

Following our hypothesis of duodenal eosinophilia as a pathophysiological mechanism and therapeutic target of PPI in FD, correlation analyses were performed. Significant correlations between symptoms and duodenal eosinophils were found at baseline in FD-starters alone (r= .48, *P*= .01) or when combined with controls off-PPI (r= .78, *P*< .0001) (**Figure 3.4A**) and in FD-starters across treatments (r= .48, *P*= .0002) (**Figure 3.4B**) or all FD-patients (on- and off-PPI) (r= .27, *P*= .009). No correlations were found between clinical outcomes and mast cells, permeability, luminal or systemic factors.

Associations between changes in symptoms and duodenal eosinophils in FD were studied by adding the standardized (relative to the mean) PPI-related change in eosinophils (Δeosinophils) in the models. For PAGI-SYM, the treatment*Δeosinophil interaction effect (F= 16.58, *P*= .0002) was explained by lower symptoms for Δeosinophils= 0 (β= -.23 ± .11, *P*= .04), -1 (β= -.71 ± .16) or -2 (β= -1.17 ± .26, both *P*< .0001) and higher symptoms for Δeosinophils= +2 (β= .7 ± .25, *P*= .008) on- vs. off-PPI (**Figure 3.4C**). Subanalyses by FD cohort confirmed that PPI only led to a decrease of both symptoms and mast cells in FD-starters with an average or greater decrease in eosinophils (i.e. Δeosinophils= 0, -1 and -2).

No associations were found between PPI-related changes in symptoms and mast cells (**Figure 3.4D**), FD4-passage, pH, bile salts or cortisol in FD. In contrast, PPI-related changes in eosinophils but not FD4-passage were associated with cortisol in FD. Finally, although PPI-related changes in eosinophils were not associated with FD4-passage or pH in controls, PPI only led to an increase of eosinophils and FD4-passage in controls with an average or greater increase in bile salts (see **supplementary results**).

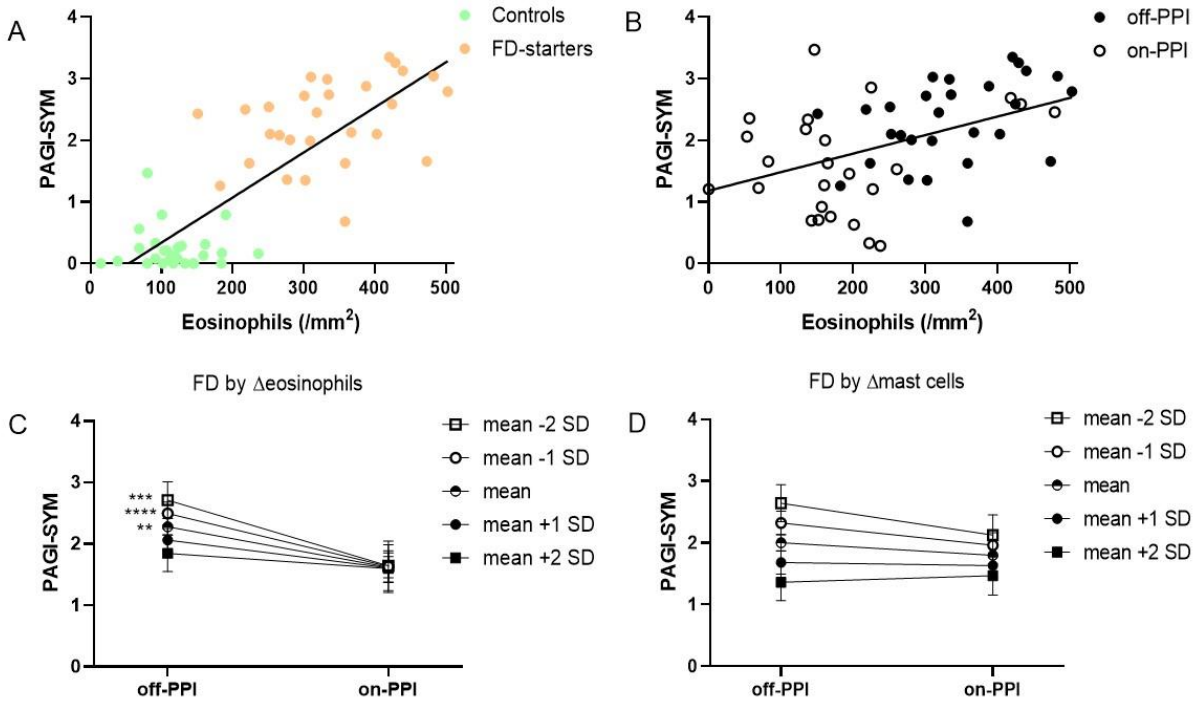


Figure 3.4: Correlation between symptoms and duodenal eosinophils at baseline in FD-starters and controls (A) or across treatments in FD-starters (B) and association between PPI-induced changes in PAGI-SYM with duodenal eosinophils (C) or paracellular passage (D), where mean corresponds to an average change (Δ= 0) and mean +/- 1 or 2 SD to an above or below average change in FD patients. ***P*< .01, ****P*< .001, *****P*< .0001. Graphs C-D show means ± standard error. FD, functional dyspepsia; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PPI, proton pump inhibitor; SD, standard deviation.

3.5 Discussion

FD is a common and unexplained disorder with unknown pathophysiology, hampering a conclusive diagnosis and the development of effective drugs. In this study, we investigated potential underlying duodenal mucosal or luminal and systemic factors, including stress. We also prospectively studied changes with first-line therapy, i.e. PPI, in FD patients, compared to controls as well as a second cohort of PPI-refractory FD patients after PPI-withdrawal. The results confirm the presence of increased mucosal eosinophil and mast cell infiltration and permeability in FD (**Figure 3.5**). Luminal pH was higher but bile salts similar in FD patients compared to controls off-PPI. Systemic inflammation, subjective stress and salivary cortisol levels were also higher in FD patients vs. controls off-PPI. Interestingly, PPI improved not only symptoms but also duodenal mucosal inflammation and barrier dysfunction. Changes in eosinophils but no other duodenal or systemic factors, were associated with clinical efficacy of PPI in FD. In contrast, increased mucosal eosinophil infiltration and permeability in controls on-PPI were associated with changes in bile salts. Thus, we provide the first prospective evidence for eosinophil-reducing effects in the duodenum as a therapeutic mechanism of PPI in FD patients. Moreover, differential effects of PPI in controls point to the role of luminal changes in determining low-grade mucosal immune activation in the duodenum, which may also occur in FD after long-term use and provide arguments against continued use in refractory patients.

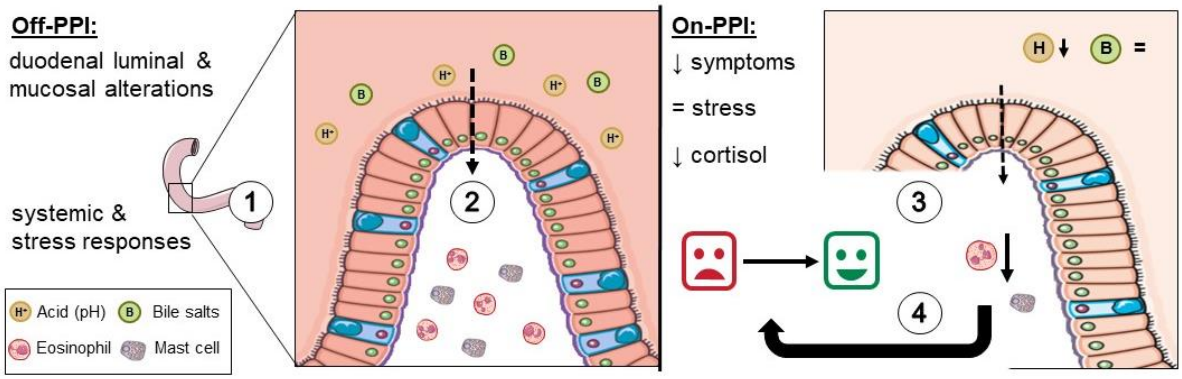


Figure 3.5: Graphical summary. Duodenal (luminal or mucosal) and systemic (immune activation or stress) factors may play a role in FD pathophysiology (1). Increased mucosal permeability, eosinophil and mast cell infiltration was confirmed in FD patients off-PPI (2). Besides improved symptoms, PPI-related changes in luminal and mucosal factors were found during follow-up, with similar stress but lower awakening cortisol (3). However, only decreased duodenal eosinophilia was associated with symptom-reduction in FD patients (4), illustrating its potential role in symptom generation. PPI, proton pump inhibitor.

The presence of duodenal eosinophilia in FD, first reported in children by Friesen *et al.*[19] and adults by Talley *et al.*,[20] was endorsed and expanded by our group in 2 patient cohorts.[6,7] Duodenal micro-inflammation with eosinophils[21] and mast cells[22] in FD was confirmed by systematic reviews and there is also evidence for impaired barrier function in FD.[23,24] We and others have hypothesized that increased antigen penetration via a defective duodenal barrier may result in inflammation.[3,25] Likely causes of the barrier defect in FD include luminal acid and bile salts. While higher fasting and fed pH were found in FD patients vs. controls off-PPI, bile salt concentrations were similar. A less acidic duodenal environment in FD was also found in a previous study, with decreased bile salt concentrations in the 45min preceding a liquid meal.[9] Although baseline differences in fasting or fed bile salts were not detected in this cohort, changes in bile salt receptors and signaling may indeed play a role in FD,[9] but were out of the scope of the present study.

The Th2-immune activation pathway is known to induce eosinophil recruitment and degranulation in asthma or EoE, but evidence is still lacking for similar pathways in FD.[21] While the observed link between FD and atopic or autoimmune diseases may be explained by duodenal eosinophils,[26] these conditions were excluded in the present study and therefore unlikely to drive the results. Changes in the duodenal mucosal microbiome

have been reported in FD patients with increased levels of *Streptococcus.*[27,28] Although a detailed immune and microbiota characterization was outside the scope of the current study, the higher hsCRP levels in FD patients vs. controls off-PPI confirmed previous reports of systemic immune activation in FD.[8,29] Based on the absence of between- or within-group differences for LBP, a role for translocation of bacterial products across an impaired barrier as a trigger of mucosal and systemic inflammation in FD is less likely.

A routine course of PPI (40mg once daily for 4 weeks) reduced not only symptoms and duodenal eosinophilia, but also the higher mast cell infiltration and mucosal permeability in FD patients, with an opposite effect in controls. In addition, symptoms and duodenal eosinophilia were partially restored in FD-stoppers vs. controls off-PPI. Baseline duodenal eosinophil counts were similar between the PDS and EPS subtype in FD-starters, which is in line with a previous systematic review and meta-analysis,[22] even if the number of pure EPS patients was limited in our cohort. Despite lower eosinophilia in the PDS vs. overlap subtype, PPI reduced duodenal eosinophilia in all three subtypes. Although we reported no difference in FD4-passage between patients with or without acid suppression in our previous study,[6] this was likely due to the cross-sectional and not prospective design. The eosinophil-reducing mechanism of action of PPI is unknown, but inhibition of eotaxin-3 expression via STAT6[30] may also be at play in FD. Following our hypothesis, the link between duodenal eosinophils and symptoms was confirmed by significant correlations and the association between PPI-related changes in symptoms and eosinophils in FD. Interestingly, not only symptoms but also mast cells decreased only in FD-starters with greater than average reductions in duodenal eosinophils after PPI. These findings support the concept of the eosinophil-mast cell axis in FD, which is affected by PPI.[3,31]

Despite the differences in PPI-related changes in pH between FD-starters vs. controls, these were not associated with clinical outcomes in FD, arguing against acid-suppressive effects as underlying therapeutic mechanisms of PPI in FD patients. Similar to our previous study,[6] no associations were found between (changes in) duodenal barrier function and symptoms in FD. Interestingly, an opposite effect of PPI was found in controls and although increased gastroduodenal permeability in healthy controls on-PPI was suggested to cause an influx of luminal peptides,[32] it was not linked to duodenal eosinophil infiltration in the present study. In contrast, PPI-related changes in mucosal eosinophils and FD4-passage were associated with either fasting or fed bile salts in controls, which point to the role of luminal changes. Changes after short- vs. long-term PPI are relevant regarding the lower duodenal eosinophils in FD-starters vs. -stoppers on-PPI, which may also be caused by changes in the luminal environment. Indeed, one could argue that anti-inflammatory effects of PPI are counteracted by changes in duodenal luminal acid and bile salts, similar to the distal esophagus.[33] This would however require longer exposure of PPI in FD-starters, which may result in potential adverse effects as recent studies have demonstrated.[34] Further studies should include a longer duration of follow-up after starting but also stopping PPI in FD.

Besides the increasingly recognized duodenal alterations in FD, we also studied the psychological and physiological stress response. Perceived stress was high and similar in both FD cohorts at baseline. Although PSS remained high in FD-starters after PPI-therapy, a trend for lower PSS was found during PPI-withdrawal with similar levels in FD-stoppers vs. controls off-PPI. While the trend for lower PSS in controls on- vs. off-PPI could also suggest baseline stress and an effect of habituation with repeated exposure to the study procedures, this was not observed in FD-starters. Reasons for the reduction of higher awakening cortisol in FD after PPI-therapy in the absence of psychological changes may be explained by common discrepancies between biological and behavioral stress-responses.[35] However, we found no association between PPI-related changes in symptoms and cortisol. Nevertheless, psychological stress may initiate mucosal inflammation,[36] and an association between PPI-induced changes in eosinophils and awakening cortisol but not hyperpermeability was confirmed in FD-starters.

The limitations of this study include limited generalizability of this single center study at the tertiary care level, although baseline characteristics and distribution of FD subtypes were comparable to the general population.[37] Although we excluded subjects with atopy and IgE-mediated responses to potential luminal antigens in the current and our previous study,[6] non-IgE mediated food allergy is possible but not included in routine investigations. Endoscopic investigation was only performed in FD patients with refractory symptoms on-PPI, and direct comparison is not possible due to long-term vs. short-term course of PPI in FD-starters. While higher doses of PPI in FD-starters may be useful to exploit the anti-eosinophil mechanism similar to EoE, recent guidelines advice against dose-increase in FD.[1] The lack of placebo in the current study limits the interpretation of clinical outcomes but would have less impact on duodenal eosinophils or other pathophysiological changes. Immunohistochemistry was limited to D2 and we did not study the bulb (D1) or jejunum. Although we did not study activation status of immune cells, quantification on routine H&E is more feasible for large studies, and advanced immunohistochemistry may lead to under detection due to degranulation.[38,39]

Strengths of this study are the inclusion of clinically well-characterized FD patients (Rome IV) with strict in- and exclusion criteria for both patients and controls. We performed a comprehensive and detailed investigation of potential duodenal and systemic factors in FD, both at baseline and after PPI-therapy, compared to both controls and PPI-refractory patients stopping PPI. Our results demonstrate the limitations of cross-sectional studies and confirm the need for prospective studies when trying to assess the impact of a treatment on pathophysiological mechanisms. While the correlation between eosinophils and symptoms in FD does not prove causality, the association between PPI-induced changes in both players strengthens the link, and confirmation from mechanistic studies is needed.

In conclusion, we confirmed duodenal mucosal inflammation and hyperpermeability as well as luminal and systemic changes in FD, and provide the first prospective evidence for PPI-related changes in both duodenal and systemic alterations. We demonstrated a link between symptoms and duodenal eosinophilia, and show that anti-eosinophil and not acid-suppressive or barrier-protective effects of PPI-therapy are associated with clinical efficacy in FD. Our results suggest that quantification of duodenal eosinophils has the potential to become part of diagnostic work-up and guide therapeutic decisions in FD. Further study of the underlying mediators may lead to the discovery of new potential biomarkers or novel therapeutic targets, potentially allowing the identification of subgroups responding to biologically targeted rather than symptom-based treatments.

3.6 Supplementary data

SUPPLEMENTARY METHODS

Study subjects

All participants were screened for eligibility, including medical history taking and physical examination. Body weight and height (to calculate the body mass index or BMI) were measured and medication intake was noted in accordance with the detailed exclusion criteria (**Supplementary Table 3.1**). While a stable dose of a single antidepressant was allowed, use of prokinetics was limited to 3 times per week in all subjects. Forbidden medication included antibiotics or immunosuppressants (< 3 months before inclusion) and non-steroidal anti-inflammatory drugs, bile acid sequestrants or ursodeoxycholic acid (< 2 weeks of each study visit). All subjects were questioned for allergy, and atopy or intake of anti-allergy drugs including anti-histamines or mast cell stabilizers led to exclusion. Personal or family (first-degree relative) history of diabetes mellitus type 1, celiac disease, inflammatory bowel disease, psoriasis, rheumatic or other autoimmune diseases were also exclusionary. Alcohol use and smoking were not allowed in the 2 days preceding each study visit, with a maximum of 10 units per week for alcohol.

Sample collection and processing

Biopsy samples

After fixation, duodenal and gastric biopsies were embedded in paraffin with processing for hematoxylin and eosin (H&E) staining for routine histological evaluation and eosinophil quantification. Immunohistochemical staining for c-kit (CD117) was performed for mast cell quantification as previously described.[40] CD117 was detected with a 1:250 diluted polyclonal rabbit anti-human antibody solution (Dako, Carpinteria, CA, USA), with antigen retrieval by boiling dewaxed paraffin sections in 0.01 M Tris– EDTA solution (pH 9) for 30min. Next, peroxidase-labelled anti-rabbit EnVision and reagent solution (Dako, Carpinteria, CA, USA) were added with Diaminobenzidine (DAB) as the chromogen, followed by counterstaining with Harris' hematoxylin solution. Finally, Giemsa-staining was done for *Helicobacter pylori* (*H. pylori*)-assessment in gastric biopsies in all subjects.

Bile samples

Duodenal aspirates were centrifuged at maximal speed and stored at -80°C until analysis of bile salts using blinded sample codes with a LC-MS/MS system (ThermoFisher Scientific). Deuterated cholic acid (D4C, 200 nM) was used as internal standard with a calibration curve and reference samples with known concentrations according to standardized operation procedures.[41] For controls, duodenal fluid aspiration was not performed in the 5 last subjects after amendment of the protocol.

Blood samples

Fasting plasma samples were collected at each study visit and in all subjects for determination of high-sensitivity C-reactive protein (hsCRP) using the Latex turbidimetric method on a COBAS 8000 autoanalyzer (HITACHI/Roche, Rotkreuz, Switzerland). In addition, Lipopolysaccharide (LPS)-binding protein (LBP) was determined on separately stored plasma aliquots using a specific enzyme-linked immunosorbent assay (ELISA) with standard 1,000 fold dilution according to manufacturers' instructions (ThermoFisher Scientific, Waltham, MA, USA).

Saliva samples

Salivary cortisol samples were collected to determine the cortisol awakening response (CAR) as a marker of the hypothalamic-pituitary-adrenal (HPA) axis-activation.[42] On the day of each study visit, subjects were first asked to refrain from drinking, eating, smoking or brushing their teeth for 1h with collection of 5 salivary samples every 15min upon awakening. Samples were collected using Salivabio oral swabs (Salimetrics, LCC,

Carslab, USA) and cortisol concentrations determined using an ELISA according to manufacturers' instructions (DRG diagnostics, Marburg, Germany).

Statistical analysis

The assumption of a normal distribution (based on the Kolmogorov-Smirnov test) was checked for all dependent variables, with box-cox or logarithmic transformations to normalize this distribution if needed. Variables which could not be transformed, were analyzed with generalized linear models with the identity link function after exclusion of outliers using the extreme studentized deviate method.[43] Significant 3-way (treatment-by-group-by-timepoint) interaction effects for repeated measurements (luminal pH, bile salts and CAR) were followed by planned contrasts at different timepoints with stepdown Bonferroni adjustment for multiple testing. Non-significant 3-way and 2-way (treatment- or group-by-timepoint) interaction effects were eliminated to generate the most parsimonious model based on the lowest value of Akaike's information criterion (AIC).[44]

Based on previous associations between symptoms and duodenal eosinophils and our hypothesis of duodenal eosinophilia as a trigger of symptoms in FD,[20,45] changes in duodenal eosinophils (Δeosinophils) on- vs. off-PPI and the interaction with treatment in FD were entered in the model of PAGI-SYM. To visualize the association between PPI-related changes in symptoms and eosinophils in FD patients, Δeosinophils was standardized with mean value of 0 and standard deviation of 1. In case of a significant treatment*Δeosinophil interaction effect, changes in symptoms were assessed and plotted for different levels of Δeosinophils including the average (0) and average ± 1 or 2 standard deviations.[46] Similarly, associations were studied between PPI-related changes in symptoms, duodenal eosinophils and permeability with other duodenal or systemic factors in FD or controls.

SUPPLEMENTARY RESULTS

Clinical outcomes

Following the significant treatment*group interaction effect (**Supplementary Table 3.2**), PPI-related changes in PAGI-SYM differed significantly for FD-starters vs. controls (β= -.62 ± .16, *P*adj= .0003). Baseline symptom scores were similar between subtypes (all *P*adj > .6), with a similar decrease in the PDS (β= -.58 ± .18, *P*< .01), EPS (β= -1.15 ± .39, *P*< .01) or overlap subtype (β= -.61 ± .22, *P*= .01) after treatment with PPI and no between-group differences (all *P*adj > .6) (**Supplementary Figure 3.2A**). Compared to controls off-PPI, symptoms remained higher in FD-starters on-PPI (β= 1.42 ± .19, *P*adj< .0001), indicating only partial clinical improvement with PPI-therapy.

Duodenal mucosal alterations

Following the treatment*group interaction effect (**Supplementary Table 3.2**), PPI-related changes in duodenal eosinophils (β= -18.9 ± 2.59, *P*adj< .0001) and mast cells (β= -7.72 ± 2.18, *P*adj= .002) differed for FD-starters vs. controls. Baseline duodenal eosinophil counts were similar between the PDS and EPS (*P*adj= .36) but lower in PDS vs. overlap subtype (*P*adj= .04). PPI treatment resulted in a significant reduction of duodenal eosinophils in the PDS (β= -33.32 ± 11.77, *P*< .01), EPS (β= -59.45 ± 25.45, *P*= .03), and overlap subtype (β= -48.5 ± 13.94, *P*< .01) with no between-group differences (all *P*adj= 1) (**Supplementary Figure 3.2B**). Duodenal mast cells were also similar at baseline between subgroups (all *P*adj > 1) with a trend for a reduction in the PDS (β= -3.9 ± 2.17, *P*= .09) and overlap (β= -4.33 ± 2.51, *P*< .1) but not EPS subtype (*P*= .56) after PPI-therapy and no between-group differences (all *P*adj= 1) (**Supplementary Figure 3.2C**). Additional analyses in FD-starters on- vs. controls off-PPI showed (a trend for) higher duodenal eosinophil (β= 4.74 ± 1.97, *P*adj= .07) and mast cell (β= 5.6 ± 1.65, *P*adj< .01) counts, indicating no complete reversal of eosinophil and mast cell infiltration after start PPI in FD.

For FD4-passage, PPI-related changes differed between FD-starters vs. controls (β= -1.5 ± .36, *P*adj< .001). Baseline FD4-passage was similar between subtypes (all *P*adj > 1) with a trend for reduced passage in the PDS (β= -.68 ± .33, *P*= .06) but not EPS (*P*= .11) or overlap subtype (*P*= .11) after PPI treatment and no between-group differences (all *P*adj= 1) (**Supplementary Figure 3.2D**). Additional analyses in FD-starters on- vs. controls off-PPI showed no difference in FD4-passage (*P*adj= 1), indicating complete reversal of baseline barrier dysfunction after PPI in FD-starters.

Duodenal luminal alterations

Luminal pH changed upon intake of the liquid meal (timepoint) with treatment*group, treatment*time and treatment*group*timepoint interaction effects (**Supplementary Table 3.3**). Luminal pH across all timepoints was lower in FD-starters vs. controls off-PPI (β= -.54 ± .12, *P*< .0001) with an increase in both controls (β= .74 ± .08, *P*< .0001) and FD (β= .16 ± .08, *P*= .04) after starting PPI-therapy. However, PPI-related changes in pH across all timepoints were significantly different for FD-starters vs. controls (β= -.58 ± .11, *P*adj< .0001) (**Supplementary Figure 3.3A**).

Total bile salt concentrations changed upon intake of a liquid meal (timepoint) with a treatment*group interaction effect (**Supplementary Table 3.3**). Total bile salts across all timepoints were similar in FD-starters vs. controls off-PPI (*P*= .47) with an increase in controls (β= 6.52 ± 1.59, *P*= .0001) but not FD (*P*= .18) after starting PPI-therapy. Only a trend was found for differences in PPI-related changes in bile salts across all timepoints for FD-starters vs. controls (β= -4.43 ± 2.21, *P*adj< .1) (**Supplementary Figure 3.3B**). Results were similar for primary or secondary bile salts (**Supplementary Table 3.4**).

Systemic alterations

Following the treatment*group interaction effect for hsCRP, a trend was found for different PPI-related changes between FD-starters vs. controls (β= -.7 ± .34, *P*adj= .1) after correction for multiple testing. For LBP, no main or interaction effects were found (**Supplementary Table 3.2**).

For PSS, no difference was found for PPI-related changes in FD-starters vs. controls (*P*adj= .55). Salivary cortisol levels changed upon awaking (main effect of timepoint) with treatment*time, group*time and treatment*group*timepoint interaction effects (**Supplementary Table 3.3**), explained by differences in the PPI-related change in awakening cortisol (0min) (β= -.56 ± .21, *P*adj= .02) but not other timepoints between FD-starters and controls.

Effect of PPI-withdrawal

Results for FD-stoppers on- vs. off-PPI are shown in **Supplementary Table 3.5**. Compared to controls off-PPI, symptoms remained higher in FD-stoppers off-PPI (β= 1.41 ± .21, *P*adj< .0001), indicating only partial clinical improvement. Duodenal eosinophil (β= 7.86 ± 1.75, *P*adj= .0002) but not mast cell counts (*P*adj= .15) were also higher in FD-stoppers vs. controls off-PPI. Baseline PAGI-SYM was similar in FD–stoppers (on-PPI) vs. FD-starters (off-PPI) (*P*adj= .19) and no differences were found for eosinophils (*P*adj= .34) or mast cells (*P*adj= .76), which contrasts with the within-group changes in FD-starters. Moreover, higher duodenal eosinophil (β= -7.06 ± 2.5, *P*adj= .04) but not mast cell counts (*P*adj= .76) were found in FD-stoppers vs. FD-starters on-PPI, which may be related to the longer duration of PPI-intake in FD-stoppers. Baseline paracellular passage was similar in FD–stoppers (on-PPI) vs. FD-starters (off-PPI) (*P*adj= .21), which also illustrates the limitation of cross-sectional vs. prospective studies.

Regarding luminal alterations, pH across all timepoints decreased (β= -.19 ± .09, *P*< .05) after stop PPI (**Supplementary Figure 3.4A**) but remained higher in FD-stoppers vs. controls off-PPI (β= .41 ± .13, *P*adj= .02) with no baseline difference between FD-stoppers (on-PPI) vs. FD-starters (off-PPI) (*P*adj= 1). In contrast, total

bile salt concentrations across all timepoints were similar after stopping PPI (*P*= .79) (**Supplementary Figure 3.4B**), with no baseline difference between FD-stoppers (on-PPI) vs. FD-starters (off-PPI) (*P*adj= 1). Also, no change was found in hsCRP (*P*= .36) or LBP (*P*= .33) after stop PPI. A trend was found for lower PSS (β= -.99 ± .56, *P*= .08) after stopping PPI, with similar PSS in FD-stoppers vs. controls off-PPI (*P*adj= .18). Baseline PSS was similar (*P*adj= .88) but awakening cortisol (0min) tended to be lower (β= -.48 ± .18, *P*adj= .06) in FD-stoppers (on-PPI) vs. FD-starters (off-PPI), with no change after stopping PPI (*P*= .37) (**Supplementary Figure 3.4C**). The different baseline and changes in objective but not subjective stress in both FD groups also illustrate the discrepancy between the behavioral and biological stress-responses.

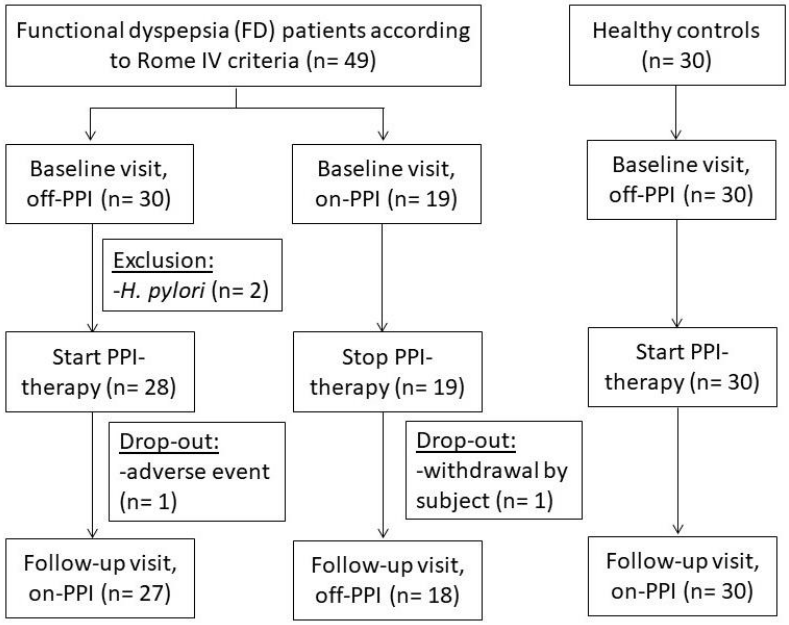
Associations with clinical outcomes

Results for the effect of PPI-therapy on symptoms in FD, including the standardized Δeosinophils, are shown in **Supplementary Table 3.6**. For FD-starters, lower symptoms on- vs. off-PPI were found for Δeosinophils= 0 (β= -.56 ± .15, *P*= .0008), -1 (β= -.78 ± .16, *P*< .0001) and -2 (β= -.99 ± .27, *P*= .002) only. For FD-stoppers, higher symptoms on- vs. off-PPI were found for Δeosinophils= +1 (β= .53 ± .17, *P*= .007) and +2 (β= .93 ± .34, *P*= .02), also indicating an association between PPI-related changes in symptoms and eosinophils. In addition, a treatment*Δeosinophil interaction effect (F= 6.6, *P*= .01) was found for the model with mast cells in FD, explained by lower mast cells for Δeosinophils= -1 (β= -17.12 ± 8.31, *P*= .05) or -2 (β= -32.15 ± 13.14, *P*= .02) and higher mast cells on- vs. off-PPI for Δeosinophils= +2 (β= 27.98 ± 13.01, *P*= .04). This was confirmed in FD-starters, with lower mast cells for Δeosinophils= -1 (β= -33.64 ± 12.6, *P*= .01) or -2 (β= -22.44 ± 7.62, *P*= .007), but not in FD-stoppers. Thus, the PPI-induced decrease in both symptoms and duodenal mast cells was more pronounced in case of greater than average reductions in eosinophils in FD-starters.

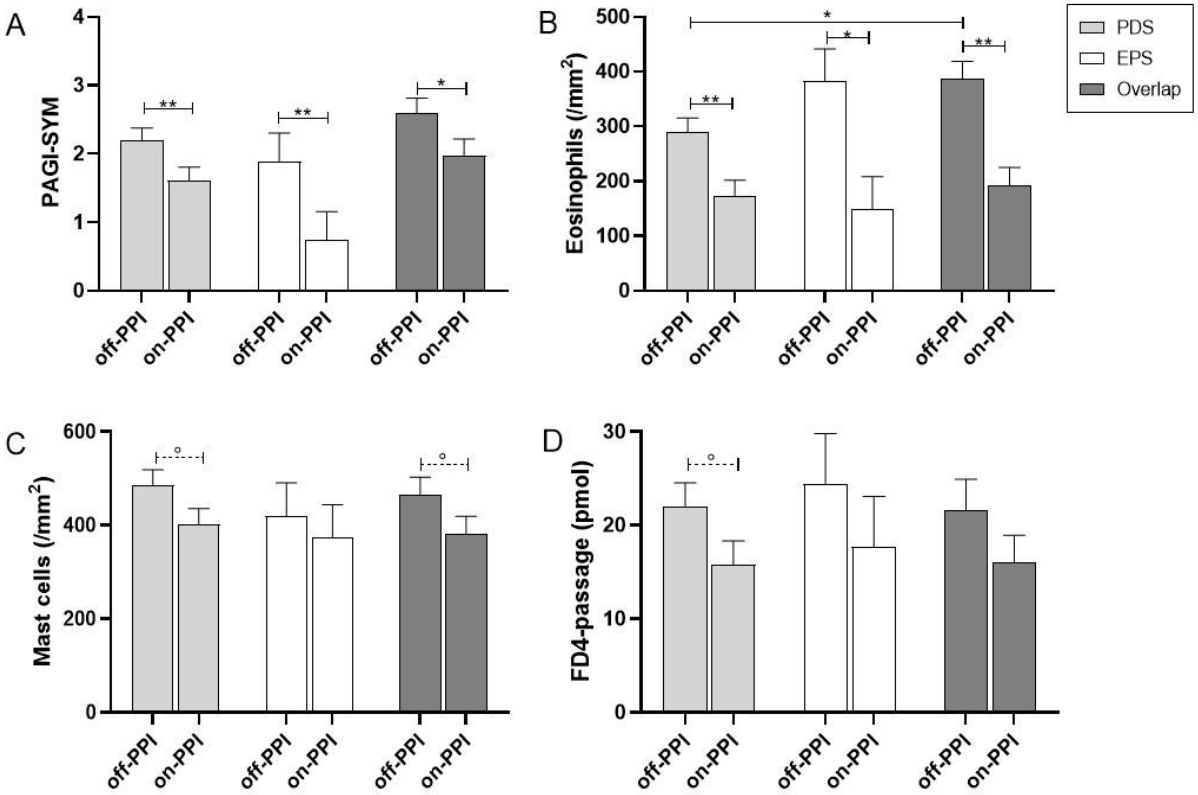
Based on our findings of PPI-related changes in mucosal mast cells and permeability, similar analyses were performed for PPI-effects on symptoms, including the standardized Δmast cells or ΔFD4-passage in FD with no interaction effects (**Supplementary Table 3.6**). Similarly, no associations were found between changes in symptoms and pH, bile salts or cortisol (interaction effects *P*>.05). However, a trend for a treatment*Δcortisol interaction effect (F= 3.22, *P*= .08) was found for awakening cortisol in the model with duodenal eosinophils but not mast cells (*P*= .15) in FD, explained by lower eosinophils for Δcortisol= 0 (β= -13.9 ± 7.5, *P*= .07), -1 (β= -27.45 ± 10.65, *P*= .01) or -2 (β= -41.01 ± 16.87, *P*= .02) (**Supplementary Figure 3.5A**). This was confirmed in FD-starters but not FD-stoppers, indicating a more pronounced decrease in eosinophils after PPI in case of greater than average reductions in awakening cortisol. In contrast, the effect of PPI on FD4-passage was not associated with awakening cortisol in FD (*P*= .86) (**Supplementary Figure 3.5B**).

Finally, despite the finding of a PPI-related increase in mucosal eosinophils and permeability in controls, the effect of PPI on eosinophils was not associated with changes in FD4-passage (interaction effect F= .03, *P*= .86). In addition, no association was found between changes in eosinophils and fasting or fed pH in controls (interaction effects *P* > .05). In contrast, a treatment*Δbile salts interaction effect (F= 7.88, *P*= .01) was found for fasting bile salts in the model with eosinophils in controls, explained by higher eosinophils on- vs. off-PPI for Δbile salts= 0 (β= 1.98 ± .45, *P*= .0006), +1 (β= 3.27 ± .65, *P*= .0001) or +2 (β= 4.57 ± 1.03, *P*= .0005) (**Supplementary Figure 3.6A**). In addition, a treatment*Δbile salts interaction effect (F= 10.79, *P*= .005) was found for fed total bile salts in the model with FD4-passage, explained by higher FD4-passage on- vs. off-PPI for Δbile salts= 0 (β= 1.73 ± .57, *P*= .009), +1 (β= 3.61 ± .8, *P*= .0005) or +2 (β= 5.49 ± 1.26, *P*= .0007) (**Supplementary Figure 3.6B**). Thus, PPI-related changes in mucosal eosinophils and FD4-passage were associated with either fasting or fed bile salts in controls.

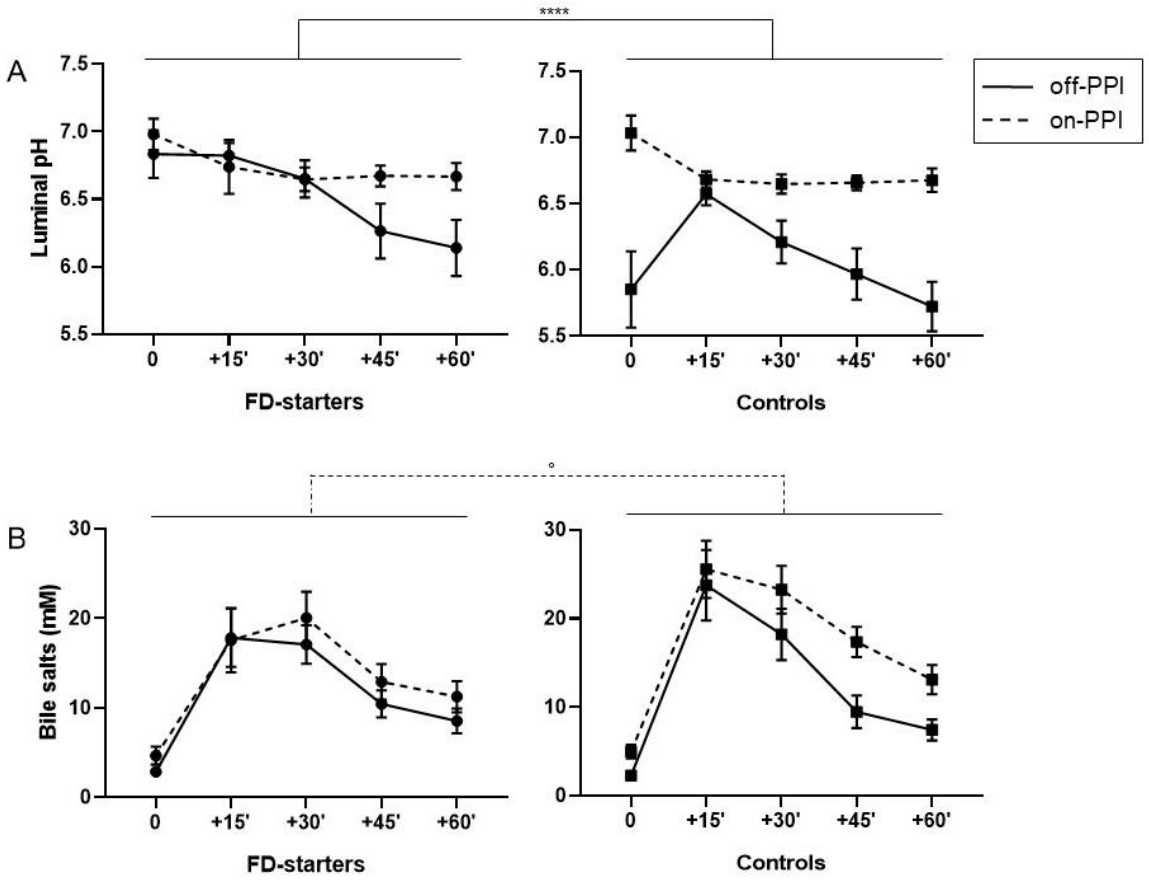
SUPPLEMENTARY FIGURES



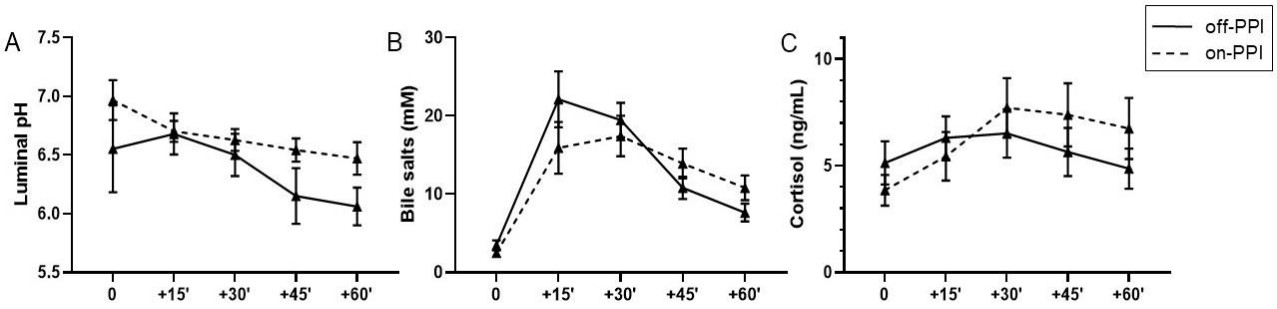
Supplementary figure 3.1: Patient flowchart.



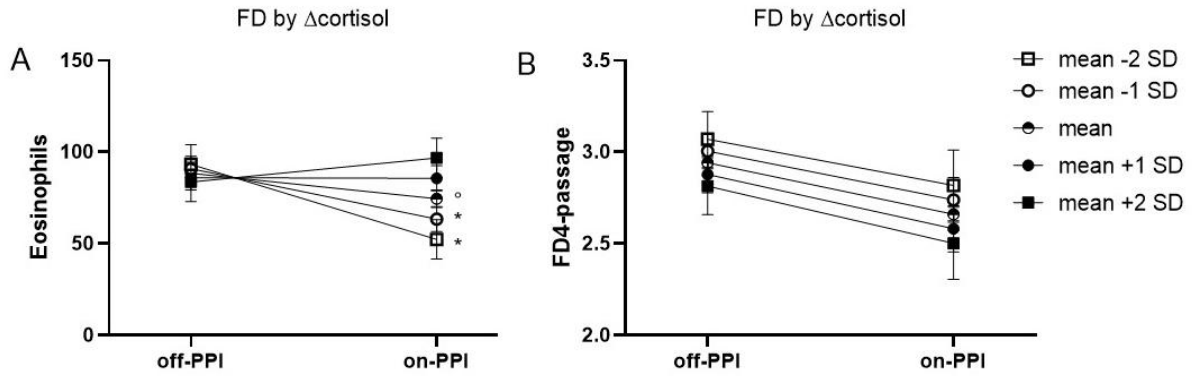
Supplementary figure 3.2: Changes in symptoms (A), duodenal mucosal eosinophils (B), mast cells (C) and paracellular passage (D) in FD subtypes of the FD-starters cohort before and after starting PPI. °*P*< .1, **P*< .05, ***P*< .01. Data are presented as means ± standard error. FD4, FITC-Dextran 4kDa; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PPI, proton pump inhibitor.



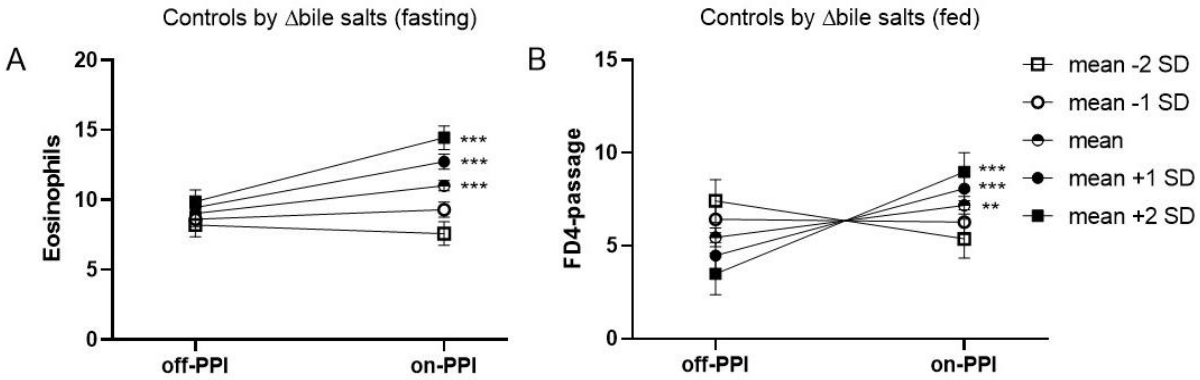
Supplementary figure 3.3: Changes in duodenal luminal pH (A) and total bile salts concentrations (B) in FD patients and controls before and after starting PPI. °*P*< .1, ****P*< .0001. Data are presented as means ± standard error. Full line off-PPI, dashed line on-PPI. FD, functional dyspepsia; PPI, proton pump inhibitor.



Supplementary figure 3.4: Changes in duodenal luminal pH (A), total bile salts concentrations (B) and cortisol awakening response (C) in FD patients before and after stopping PPI. Data are presented as means ± standard error. Full line off-PPI, dashed line on-PPI. FD, functional dyspepsia; PPI, proton pump inhibitor.



Supplementary Figure 3.5: Association between PPI-induced changes in eosinophils (A) or paracellular passage (B) and awakening cortisol (Δcortisol) in FD, where mean corresponds to an average change (Δ= 0) and mean +/- 1 or 2 SD to an above or below average change. Graphs show means ± standard error of Box-Cox transformed eosinophils and FD4-passage. FD4, FITC-Dextran 4kDa; FD, functional dyspepsia; PPI, proton pump inhibitor; SD, standard deviation.



Supplementary Figure 3.6: Association between PPI-induced changes in eosinophils (A) or paracellular passage (B) and either fasting or fed total bile salts (Δbile salts) in controls, where mean corresponds to an average change (Δ= 0) and mean +/- 1 or 2 SD to an above or below average change. Graphs show means ± standard error of Box-Cox transformed eosinophils and FD4-passage. FD4, FITC-Dextran 4kDa; PPI, proton pump inhibitor; SD, standard deviation.

SUPPLEMENTARY TABLES:

Supplementary table 3.1: Exclusion criteria for the present study.

Exclusion criteria	**Remarks**
Active psychiatric condition	Stable dose of single antidepressant allowed
Use of prokinetics (> 3 times per week)	< 2 weeks before study visit
Use of immunosuppressants or antibiotics	< 3 months before inclusion
Use of non-steroidal anti-inflammatory drugs, anti-allergy drugs, bile acid sequestrants or ursodeoxycholic acid	< 2 weeks before study visit
Atopy (eczema, asthma and/or allergic rhinoconjunctivitis)	Including therapy
Personal or family (first-degree relative) history of diabetes mellitus type 1, celiac disease, inflammatory bowel disease, psoriasis, rheumatic or other auto-immune diseases	Including therapy
History of abdominal surgery, including cholecystectomy	Not appendectomy or splenectomy
Kidney, liver or coagulation disorders	Including therapy
Active coronary or peripheral artery disease	Including therapy
Diabetes mellitus type 2	Including therapy
Active malignancy	Including therapy
Known HIV, hepatitis B or hepatitis C infection	Including therapy
Significant alcohol use	> 10 units / week

Supplementary table 3.2: Type 3 effects for linear (mixed) model analyses of dependent variables with treatment as within- and group as between-subject factors of interest.

Effect	**Treatment**	**Group**	**Treatment*group**
Model information	**F value (*P*)**	**F value (*P*)**	**F value (*P*)**
Clinical outcome:			
- PAGI-SYM	2.29 (.14)	63.97 (<.0001)	18.35 (<.0001)
Mucosal permeability:			
- TEER	2.97 (.09)	1.45 (.24)	.96 (.39)
- FD4-passage	.61 (.44)	.04 (.97)	9.1 (.0004)
Mucosal inflammation:			
- Eosinophils	.26 (.61)	10.26 (.0001)	28.18 (<.0001)
- Mast cells	1.63 (.21)	11.35 (<.0001)	7.72 (<.001)
Systemic alterations:			
- hsCRP	.12 (.73)	1.52 (.23)	3.06 (.05)
- LBP	.16 (.69)	.64 (.53)	.86 (.43)
- PSS	.1 (.75)	11.36 (<.0001)	3.44 (.04)

FD4, FITC-dextran 4kDa; hsCRP, high sensitivity C-reactive protein; LBP: LPS-binding protein; PSS, Perceived Stress Scale; TEER, trans-epithelial electrical resistance.

Supplementary table 3.3: Type 3 effects for linear (mixed) model analyses of dependent variables with both treatment and timepoint (repeated measures) as within- and group as between-subject factors.

Variable	**pH**	**Total bile salts**	**CAR**
Model information	**F value (*P*)**	**F value (*P*)**	**F value (*P*)**
Main effect			
treatment	58.23 (<.0001)	3.5 (.07)	.12 (.73)
timepoint	13.41 (<.0001)	80.92 (<.0001)	22.88 (<.0001)
group	2.99 (.06)	.93 (.4)	2.54 (.09)
Interaction effect			
treatment*group	16.74 (<.0001)	3.2 (<.05)	.58 (.57)
treatment*timepoint	6.17 (.0001)	1.9 (.11)	2.3 (.06)
group*timepoint	.56 (.81)	.62 (.76)	2.17 (.03)
treatment*group*timepoint	2.31 (.02)	.74 (.65)	2.61 (.009)

CAR, Cortisol Awakening Response.

Supplementary table 3.4: Type 3 effects for linear (mixed) model analyses of primary and secondary bile salt concentrations.

Variable	**Primary bile salts**	**Secondary bile salts**
Model information	**F value (*P*)**	**F value (*P*)**
Main effect		
treatment	3.6 (.06)	1.44 (.23)
timepoint	81.75 (<.0001)	57.13 (<.0001)
group	.94 (.4)	2.09 (.13)
Interaction effect		
treatment*group	3.04 (.06)	1.89 (.16)
treatment*timepoint	1.84 (.12)	1.6 (.18)
group*timepoint	.53 (.83)	1.05 (.4)
treatment*group*timepoint	.75 (.65)	.69 (.7)

Supplementary table 3.5: PPI-related changes in clinical, duodenal and systemic factors in FD-stoppers (before and after stopping PPI).

Group	**FD-stoppers**		***P*-value**
Treatment	**On-PPI (n= 19)**	**Off-PPI (n= 18)**	
Clinical:			
PAGI-SYM	2 ± .18	1.66 ± .27	<.01
Duodenal:			
Eosinophils (/mm^2)	285.17 ± 32.01	222.67 ± 27.19	.08
Mast cells (/mm^2)	435.13 ± 31.99	366.97 ± 36.11	<.05
IEL (/ 100 enterocytes)	8.37 ± .69	7.94 ± .57	.64
FD4-passage (pmol)	19.16 ± 2.55	16.36 ± 3.08	.23
Systemic:			
hsCRP (mg/L)	1.4 ± .33	1.09 ± .28	.36
LBP (pg/mL)	12.12 ± 1.45	11.06 ± 1.06	.33
PSS	15 ± 1.69	12.17 ± 1.78	.08

FD4, FITC-dextran 4kDa; FD, functional dyspepsia; hsCRP, high sensitivity C-reactive protein; IEL, intra-epithelial lymphocyte; LBP: LPS-binding protein; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PSS, Perceived Stress Scale.

Supplementary table 3.6: Interaction effects for mixed model analyses including the standardized change in eosinophils, mast cells, FD4-passage, fasting pH or awakening cortisol in the model with PAGI-SYM for Functional Dyspepsia patients.

Clinical outcome	**PAGI-SYM**
Model information	**F value (*P*)**
Interaction effect	
Δeosinophils*treatment	16.58 (.0002)
Δmast cells*treatment	1.23 (.27)
ΔFD4-passage*treatment	.02 (.89)
ΔpH*treatment	.12 (.73)
Δcortisol*treatment	1.21 (.28)

FD4, FITC-dextran 4kDa; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index.

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CHAPTER 4

DUODENAL DYSBIOSIS IS UNRELATED TO PROTON PUMP INHIBITOR EFFICACY IN FUNCTIONAL DYSPEPSIA PATIENTS

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4 DUODENAL DYSBIOSIS IS UNRELATED TO PROTON PUMP INHIBITOR EFFICACY IN FUNCTIONAL DYSPEPSIA PATIENTS

4.1 Abstract

Background & Aims: Proton pump inhibitors (PPI) improve symptoms in functional dyspepsia (FD) through duodenal eosinophil-reducing effects. However, the contribution of the duodenal microbiome to FD symptoms and interaction with PPI remains elusive.

Methods: Aseptic duodenal biopsies and brushings were collected before and after PPI-intake (4 weeks Pantoprazole 40mg daily, controls and FD-starters) or -withdrawal (2 months, FD-stoppers) for 16S-rRNA sequencing. Additional duodenal biopsies, fluids and symptoms (PAGI-SYM) were also collected. Microbiome composition and differential genera-abundances were studied between locations (lumen or mucosa) and groups. Between- and within-group changes in genera and diversity (Shannon) and associations with symptoms or duodenal factors were analyzed using linear mixed models.

Results: In total, 30 controls, 28 FD-starters and 19 FD-stoppers were followed. Microbiome-wide shifts were mostly driven by inter-individual variability, with limited group- and only luminal PPI-effects. Luminal *Porphyromonas* was lower in FD vs. controls and correlated with symptoms and duodenal eosinophils. Although clinical and eosinophil-reducing effects of PPI-therapy were unrelated to microbiota changes in FD-starters, increased luminal *Streptococcus* was associated with mucosal and luminal effects of PPI in controls, and remained higher despite PPI-withdrawal in FD-stoppers.

Conclusions: Duodenal microbiome analysis demonstrated differential luminal but not mucosal genera in FD, with a potential role of luminal *Porphyromonas* in FD pathophysiology. Previously observed beneficial effects of PPI were, however, not associated to changes in the duodenal microbiome. In fact, increased *Streptococcus* abundance and its association with potentially negative duodenal effects of PPI suggest a role for inadvertent microbiota changes after long-term PPI-therapy in FD.

Clinicaltrials.gov, number: NCT03545243.

4.2 Introduction

Functional dyspepsia (FD) is a common functional gastrointestinal (GI) disorder defined by epigastric symptoms originating from the gastroduodenal region.[1,2] Although no structural disease is found on routine investigations, the presence of subtle pathology is not excluded by current Rome IV criteria.[1] Indeed, increasing data point towards duodenal alterations in the pathophysiology of FD, including mucosal hyperpermeability and low-grade inflammation.[3,4] The causes are unknown but candidates include luminal acid, bile salts and the gut microbiota.[2,5] Culture-independent microbiome sequencing revolutionized our understanding of the gut microbiota.[6] Gut commensals play an essential role in nutrient acquisition, colonization resistance, epithelial barrier function and immune development.[7,8] Disruption of the gut ecosystem or dysbiosis has been described in different GI disorders.[9,10] However, scientific focus has mainly been on fecal flora, which do not accurately reflect the mucosa-associated microbiota (MAM). Moreover, evidence for dysbiosis is mounting in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) but scarce for FD, despite the latter being even more prevalent than IBS according to a recent global study.[11]

Besides the potential role of the colonic microbiome, several groups have studied small intestinal microbial communities in IBD and IBS,[9,12,13] where bacterial adherence is more important for persistent colonization and host-microbiome interactions.[10,14] Techniques to characterize the duodenal MAM have also been developed.[15] Although a previous pilot study showed changes in the duodenal MAM of FD patients, sample size was small and concomitant therapy including proton pump inhibitors (PPI) was not taken into account.[16] Indeed, acid suppression with PPI profoundly impacts the gut microbiome.[17] Whereas the vast majority of studies included only feces, dysbiosis of the gastric microbiome has also been described with PPI.[18] PPI are the current first-line therapy in FD but long-term efficacy is limited and possibly related to microbiota changes with an increased risk of enteric infections.[19] Cessation of PPI or association of probiotics have been proposed, although clinical and microbial evidence for both approaches is still lacking in FD patients.

Recently, we reported the first prospective evidence for eosinophil-reducing effects as a therapeutic mechanism of short-term PPI in the duodenum of FD patients.[20] In contrast, duodenal changes were also present after long-term PPI treatment and not reversed after withdrawal in refractory FD patients, suggesting a role for persistent luminal alterations. In addition, mucosal immune activation and increased permeability of the duodenum were found and associated with changes in luminal bile salts in healthy controls after PPI, which may be related to duodenal dysbiosis.[20] Therefore, the aims of the present study were to (1) characterize the duodenal luminal and mucosal microbiome of FD patients vs. controls, (2) assess the effect of PPI-therapy on the duodenal microbiome and its reversibility after long-term use in FD and (3) study associations with symptoms and duodenal factors.

4.3 Methods

Study population

Two interventional studies aimed at characterizing the duodenal microbiome and effect of PPI were conducted over 2 years (April 2018 - April 2020) at a single center according to the Declaration of Helsinki and Good Clinical Practice regulations after approval by the Ethics Committee of the University Hospitals Leuven (numbers S60953/S60984). The clinical and duodenal mucosal data of both studies have been reported before (**Chapter 3**).[20] The primary analysis on the duodenal microbiome is presented here, also in relation to symptom- and eosinophil-reducing effects of PPI-therapy in FD. Symptomatic FD patients, diagnosed according to Rome IV criteria,[1] were included in case they had not been treated with routine PPI-therapy (4 weeks healing dose) or other acid suppression < 3 months before inclusion ('FD-starters'), or if refractory to > 1 month of at least one daily dose of PPI ('FD-stoppers'). Age- and gender-matched healthy controls without GI symptoms were also recruited. All subjects were aged 18 to 64 years old, with no active

psychiatric, atopic, inflammatory or metabolic conditions. Use of immunosuppressants, anti- or probiotics < 3 months were also exclusionary. Written informed consent was obtained from all subjects before inclusion and data were collected at KU Leuven and the University Hospitals Leuven (Leuven, Belgium). All authors had access to the study data and reviewed and approved the final manuscript.

Sample collection

The study design and procedures are shown in **Supplementary Figure 4.1**. During upper GI endoscopy, aseptic biopsies from the second portion of the duodenum (D2) were collected using the sheathed and sealed Brisbane aseptic biopsy device (BABD) (MTW, Wesel, Germany)[15], with additional precautions to avoid contamination. Next, a sterile brush (Zhuji Pengtian Medical Instrument Co., Zhejiang, China) was advanced while leaving the sheathed BABD in place for luminal brushing, on the opposite side from where the biopsy sample was taken. Aseptic procedures were repeated after 2-4 weeks to assess the variability of the duodenal microbiota (off-PPI) and after an additional 4 weeks of routine PPI-therapy (Pantoprazole 40mg once daily) in controls and FD-starters (on-PPI). For FD-stoppers, all procedures were performed at baseline (on-PPI) and after 8 weeks of PPI-withdrawal (off-PPI). Routine duodenal biopsies (for histology) and fluids (pH and bile salts) were collected in all subjects at baseline and follow-up. At baseline, *Helicobacter pylori* was excluded in gastric biopsies (Giemsa staining).

Sample and data processing

Aseptic duodenal biopsies (mucosal microbiota) and brushes (luminal microbiota) were transferred in sterile, nuclease-free tubes using sterile needles and wire cutters. Samples were immediately snap-frozen and stored at -80°C. All procedures were performed under sterile conditions in a biohazard type II cabinet using cleaned (RNase AWAY, Molecular Bio-Products, USA) and UV-irradiated equipment.[21] DNA was extracted using the AllPrep® DNA/RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of 1 extraction blank for every 5 samples (random order for brushes and biopsies). Bacterial DNA quantification (Uni16S) was performed before amplification of the 16S rRNA V4 hypervariable region as previously described.[22] Final DNA concentration and fragment lengths were determined before equimolar pooling and dual-index sequencing using the Illumina MiSeq platform, yielding paired-end reads of 250 bases length in each direction.[22] Quality control and annotation of 16S rRNA-sequences was followed by removal of potential contaminants using decontam (prevalence-based method, threshold .5),[23] before further analysis at genus-level using a minimum of 1,000 reads for all samples.

Statistical analysis

The primary analysis was the duodenal microbiome composition in FD patients vs. controls, for both locations (lumen and mucosa). The association of subject, group, treatment and demographics to bacterial community variation was studied using distance-based redundancy analysis (dbRDA, genus-level Aitchison distance) after centered log-ratio (CLR) transformation. Clustering of significant variables were also determined on principle component analyses (permutational MANOVA). Differential genera abundance was assessed using (un)paired t-tests with correction for multiple testing (Benjamini-Hochberg FDR< .1). Next, genera of interest, richness (Observed, Chao1) and diversity (Shannon, Simpson) metrics were compared between and within groups using linear mixed models with group (controls, FD-starters or FD-stoppers) as between- and treatment (off- or on-PPI) as within-subject factors, including their interaction. In addition, spatial and temporal variation were assessed with respectively location (lumen or mucosa) or visit (baseline or variability off-PPI) as within-subject factors in controls and FD-starters. Finally, spearman correlations (FDR< .1) and associations between PPI-induced changes in symptoms or duodenal factors and duodenal microbial variables were determined. All analyses were performed using R (v4.0.3) and SAS software (v9.4) and two-

tailed *P*-values < .05 were considered significant unless otherwise specified. For mixed models, least squares means estimates (β) are given as mean ± standard error. A detailed description of the study procedures including microbiota and statistical analyses can be found in the **supplementary methods**.

4.4 Results

Study cohort and sample overview

In total, PPI-therapy was started in 30 controls and 28 FD-starters and withdrawn in 19 FD-stoppers. Baseline characteristics were similar between groups, except for the estimated intake of proteins and fiber (**Table 4.1**). Median duration of PPI-therapy in FD-stoppers was 3.2 years (interquartile range (IQR) 1.5 - 5.1 years). Median (IQR) number of reads was 58,628 (43,510-75,546) for luminal and 16,358 (7,257-25,931) for mucosal samples (**Supplementary Figure 4.2**). From the 188 brushes and 192 biopsies with >1,000 reads, a total of 785 and 762 annotated genera were obtained after sub-setting (for α-diversity) and CLR-transformation (for β-diversity and genera abundance), respectively. Microbial load of luminal and mucosal samples was similar between groups (**supplementary results**).

Table 4.1: Baseline characteristics of healthy controls, FD-starters (off-PPI) and FD-stoppers (on-PPI).

Group and variable	Controls (n= 30)	FD-starters (n= 28)	FD-stoppers (n= 19)	*P*-value
Demographic:				
Age (years)	27 (24-33.5)	27 (23.5-34.5)	32 (26.8-49.5)	.18
Female (%)	21 (70)	24 (86)	14 (74)	.35
BMI (kg/m^2)	23 (20-25.3)	22 (19-24)	21.5 (20.8-24.3)	.56
FD subtypes:				
PDS-subtype (%)	NA	15 (54)	10 (53)	.95
EPS-subtype (%)	NA	3 (11)	6 (32)	.07
Overlap (%)	NA	10 (35)	3 (15)	.13
Daily food intake:				
Energy (kcal/day)	1419 (1308-1627)	1186 (974.9-1621)	1284 (937.7-1617)	.35
Carbohydrates (g/day)	175.7 (148.2-187.5)	148.3 (115.1-222.5)	143.9 (93.42-194.4)	.25
Fat (g/day)	46.87 (41.7-57.1)	40.8 (34.4-52.5)	45.7 (31.2-61.5)	.51
Fiber (g/day)	18.4 (15.2-21.5)	15.9 (9.3-23)	12.8 (8.3-17.4)*	.02
Protein (g/day)	175.7 (148.2-187.5)	148.3 (115.1-222.5)*	143.9 (93.4-194.4)*	<.01

*P_{adj}< .05 vs. controls (post-hoc Dunn tests) after Kruskal-Wallis test with Chi2=7.58 (fiber) and 10.22 (protein). BMI, body mass index; EPS, epigastric pain syndrome; FD, functional dyspepsia; PDS, postprandial distress syndrome; PPI, proton pump inhibitor.

Duodenal luminal and mucosal microbiome is altered in FD with luminal effects of PPI

We first assessed the relative importance of all variables explaining the duodenal luminal and mucosal microbiota variation (community-wide shifts). Significant inter-individual variation (subject) with limited group- and only luminal PPI-effects were found (**Table 4.2**). In multivariate models, subject had a contribution of 16.01% (P_{adj}= .002) with PPI adding to its contribution (P_{adj}= .01) to reach a total explanatory power of 16.75% for luminal samples, while only a significant and smaller contribution of subject was found for mucosal samples (R^2= 5.81%, P_{adj}= .002). Despite the significant effect of sampling location (lumen or mucosa) when combining all samples (n= 380), mucosal (biopsy) samples were more likely contaminated (see **supplementary results**). Besides the clustering of duodenal luminal and mucosal samples (location), a significant but smaller effect of group was found for all samples (**Figure 4.1A**). In addition to the significant

group effect, an effect of PPI was confirmed for luminal but not mucosal samples (**Figure 4.1B-C**). No association of other host factors or dietary intake with community variation was found using univariate dbRDA. Thus, the duodenal microbiome differed between groups with limited luminal effects of PPI.

Table 4.2: Association of subject (inter-individual variation), group or treatment and demographics with duodenal luminal and mucosal microbiome community composition.

Univariate dbRDA	**Luminal (brush samples)**				**Mucosal (biopsy samples)**			
	F-value	**R^2 (%)**	***P*-value**	**P_{adj}-value**	**F-value**	**R^2 (%)**	***P*-value**	**P_{adj}-value**
subject	1.47	16	**.001**	**.006**	1.16	5.4	**.002**	**.01**
group	2.1	1.16	**.002**	**.006**	1.52	.51	**.03**	**.09**
PPI	1.64	.34	**<.05**	**.09**	0.92	0	.41	.51
gender	1.02	.01	.38	.38	1.05	.03	.4	.4
age	1.16	.09	.23	.28	1.47	.25	.06	.12
BMI	1.52	.28	.07	.1	1.05	.03	.32	.4

Univariate distance-based redundancy analysis (dbRDA) with individual effect sizes assuming covariate independence. Variables which remained significant after adjustment for multiplicity (Benjamini-Hochberg) were entered in a stepwise multivariate model for the luminal and mucosal microbiome variation. BMI, body mass index; PPI, proton pump inhibitor.

Specific effects on genera and diversity after short-term PPI in FD patients and controls

Despite the absence of major shifts in community composition, between-group differences in specific genera were found, including a lower abundance of luminal *Neisseria* (FDR< .001), *Porphyromonas* (FDR= .003), *Selenomonas* (FDR= .02), *Haemophilus* (FDR= .03) and *Fusobacterium* (FDR= .06) in FD-starters vs. controls (**Figure 4.2A**) and decrease in *Prevotella* (FDR= .03) after PPI. The lower *Neisseria* (β= -1.48 ± .59, *P*= .02) and *Porphyromonas* (β= -2.17 ± .65, *P*= .001) abundance was confirmed in FD-starters vs. controls at baseline (off-PPI) using mixed models, with decreased *Porphyromonas* (β= -1.34 ± .56, *P*= .02) in controls after PPI (**Figure 4.2B-C**). In addition, *Prevotella* decreased in both controls (β= -.92 ± .43, *P*= .03) and FD-starters (β= -1.65 ± .47, *P*< .001) after PPI (**Figure 4.2D**). Based on the findings of a recent study,[24] we analyzed luminal *Streptococcus* abundance, which was similar between groups but increased after PPI in controls (β= .31 ± .12, *P*= .01) and FD-starters (β= .22 ± .13, *P*= .03) (**Figure 4.2E**). In contrast, no differentially abundant mucosal genera were found (see **supplementary results**).

Regarding α-diversity, luminal richness was lower in FD-starters vs. controls (β= -.85 ± .39, *P*= .03) at baseline, with a significant decrease in controls (β= -.73 ± .32, *P*= .03) after PPI (**Table 4.3**). Shannon and Simpson's index were similar with a decrease in both controls (β= -.31 ± .11, *P*= .008 and β= -1.06 ± .38, *P*= .007) and FD-starters (β= -.39 ± .13, *P*= .003 and β= -1.26 ± .41, *P*= .003, respectively) after PPI (**Table 4.3**). No significant changes were observed in mucosal α-diversity. Finally, significant spatial (**Supplementary Figure 4.3**) but not temporal variation of the duodenal luminal and mucosal microbiome was found (**supplementary results**).

In summary, baseline differences and effects of short-term PPI-therapy were only found for specific luminal genera and diversity, with stable duodenal luminal and mucosal bacterial communities in the absence of PPI-therapy.

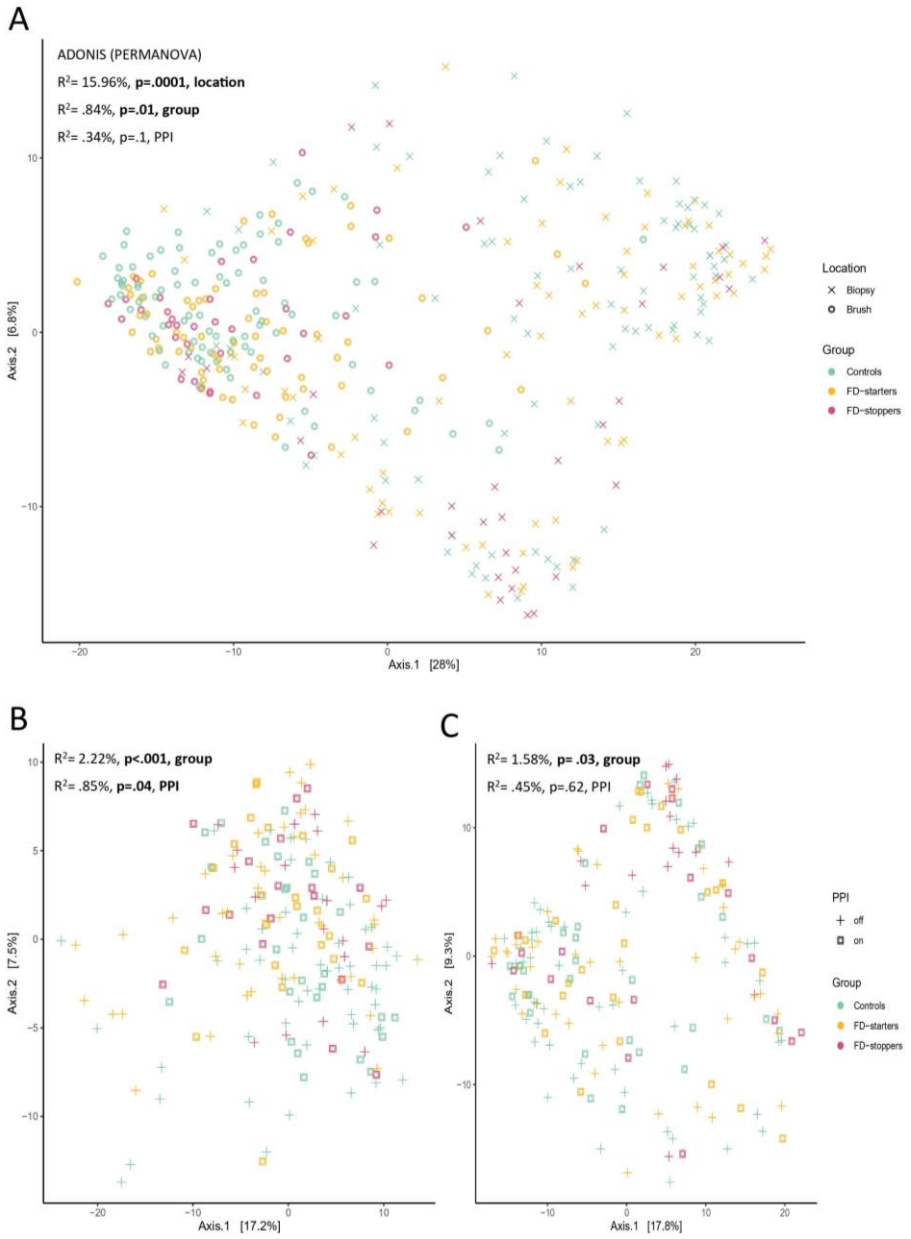


Figure 4.1: Duodenal bacterial community variation for all (A), luminal (B) or mucosal (C) samples with effect sizes (R^2) of location (all samples), group and PPI. Principle component analyses with the percentage of variation explained by the first 2 principal components reported on the axes. Effect sizes were determined using the vegan function adonis (PERMANOVA). FD, functional dyspepsia; PPI, proton pump inhibitor.

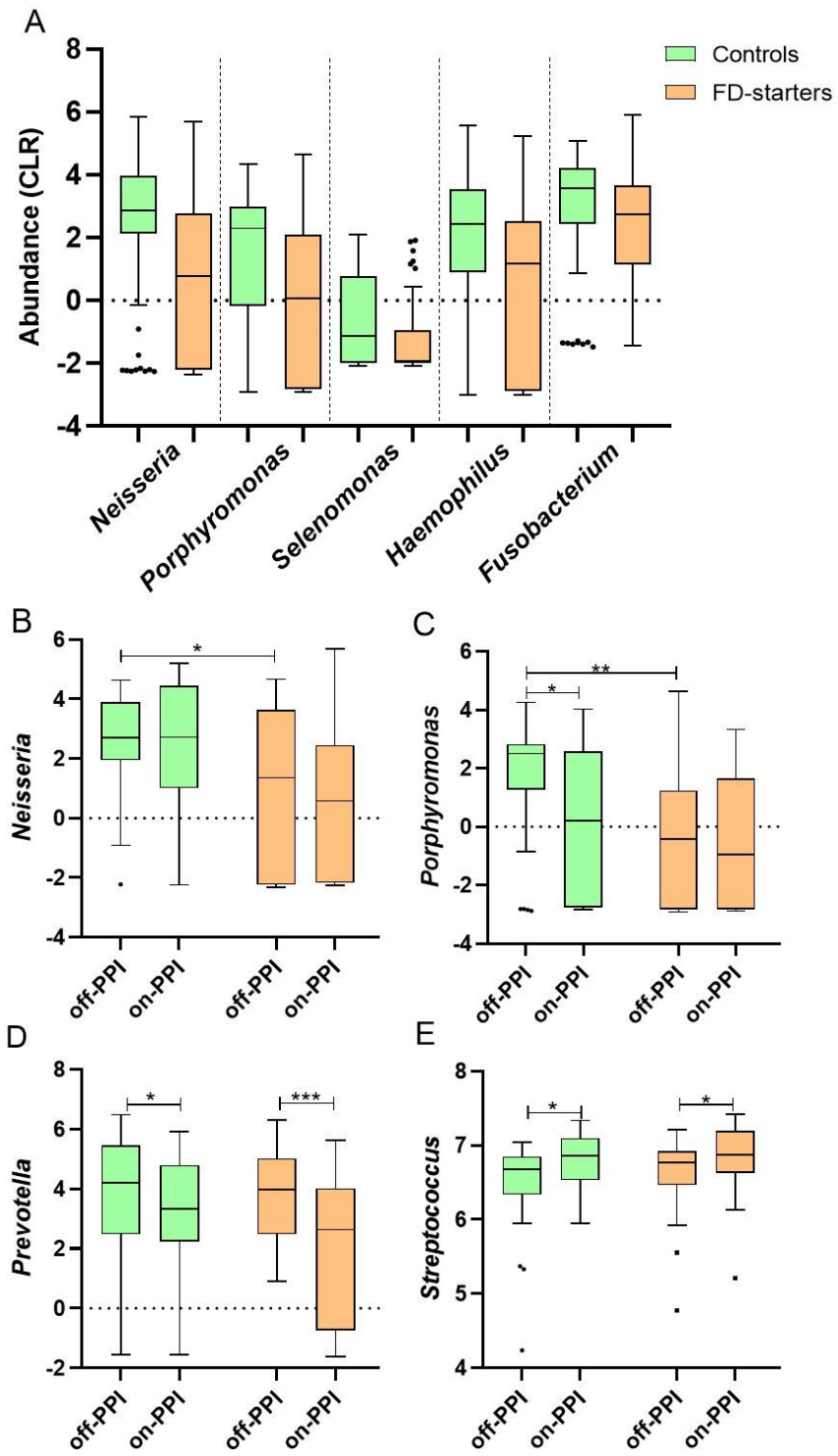


Figure 4.2: Differential genera abundance for duodenal luminal samples of FD-starters vs. controls (A) with changes in luminal *Neisseria* (B), *Por-phyromonas* (C), *Prevotella* (D) and *Streptococcus* (E) according to group and PPI-status. Tukey boxplots of CLR-transformed genera with median, IQR and 1.5*IQR whiskers (outliers beyond). Graph A: FDR< .1 for all genera (between-groups). Graphs B-E: *P< .05, **P< .01, ***P< .001. CLR, centered log-ratio; FD, functional dyspepsia; IQR, interquartile range; PPI, proton pump inhibitor.

Table 4.3: Duodenal luminal and mucosal microbial α-diversity before and after PPI-therapy, with within- and between-group comparisons (p_{adj} for interaction) for controls and FD-starters.

Group	**Controls**		**FD-starters**		P_{adj}-value
Treatment	**Off-PPI (n= 30)**	**On-PPI (n= 30)**	**Off-PPI (n= 28)**	**On-PPI (n= 27)**	
Luminal:					
Observed	42.03 ± 1.36	37.79 ± 1.9 *	36.81 ± 1.6	34.79 ± 1.67	1
Chao1	48.98 ± 1.91	44.81 ± 2.71	43.24 ± 2.28	44.89 ± 2.75	.62
Shannon	2.32 ± .05	2.05 ± .08 **	2.24 ± .07	1.92 ± .1 **	.66
Simpson	.79 ± .01	.72 ± .02 **	.78 ± .02	.68 ± .03 **	.71
Mucosal:					
Observed	43.07 ± 3.33	41.43 ± 6.12	43.36 ± 7.08	42.37 ± 7.32	.97
Chao1	45.43 ± 3.82	43.58 ± 6.95	47.74 ± 8.62	45.36 ± 8.53	.99
Shannon	2.82 ± .07	2.8 ± .1	2.69 ± .12	2.69 ± .12	1
Simpson	.88 ± .01	.89 ± .01	.86 ± .02	.87 ± .02	1

*P< .05, **P< .01 (within-group). FD, functional dyspepsia; PPI, proton pump inhibitor.

Persisting microbiota alterations in FD patients after withdrawal of long-term PPI

In FD-stoppers, luminal *Neisseria* abundance was higher vs. FD-starters (FDR= .09) but not controls. Higher *Neisseria* abundance was confirmed in FD-stoppers vs. FD-starters on-PPI (β= 1.41 ± .69, P= .04) using linear mixed models, pointing to differences between short- and long-term use of PPI in FD. *Streptococcus* abundance was similar in FD-stoppers vs. controls on-PPI but higher off-PPI (β= .31 ± .16, P= .03), suggesting persistent microbial alterations with no changes after PPI-withdrawal (P> .05). In contrast, a decreased abundance of luminal *Rothia* (FDR= .01) and *Stomatobaculum* (FDR= .09) and increased *Prevotella* (β= 1.21 ± .55, P= .03) were found after PPI-withdrawal, the latter of which was consistent with the observed PPI-induced decrease in FD-starters and controls. Despite differentially abundant mucosal genera including *Dyella*, mucosal richness was lower in FD-stoppers vs. FD-starters (both P= .03) and controls (both P< .01) off-PPI and with no effect of PPI-withdrawal (**Supplementary Figure 4.4**). No changes were observed in luminal α-diversity. These findings indicate persisting luminal and mucosal microbial alterations despite withdrawal of long-term exposure to PPI in FD patients.

Duodenal dysbiosis is unrelated to effects of PPI in FD patients but not controls

Based on our findings of luminal microbial alterations in FD-starters vs. controls, correlations with symptoms and duodenal eosinophils were assessed. Baseline abundance of luminal *Porphyromonas* correlated with symptoms (r= -.35) and eosinophils (r= -.43) (**Figure 4.3A-B**) and *Neisseria* with symptoms (r= -.33, all FDR= .04) in controls and FD-starters. No correlations were found with other host factors or dietary intake. Next, we addressed whether symptom- or eosinophil-reducing effects of PPI-therapy were associated with microbial changes, as clinical efficacy was only found in FD patients with an average or greater decrease in eosinophils.[20] However, the reduction in symptoms or eosinophils after PPI was similar for different levels of the standardized (relative to the mean) changes in luminal *Porphyromonas* (**Figure 4.3C-D**), *Neisseria*, *Prevotella* and *Streptococcus*. Although baseline luminal diversity correlated with symptoms (r= -.57, FDR= .02), clinical efficacy of PPI was also not associated with changes in diversity in FD-starters (see **supplementary results**).

In contrast, increased duodenal eosinophils were found in controls after PPI and associated with fasting bile salts, suggesting a potential role of the luminal microbiota.[20] Duodenal eosinophils correlated with luminal *Porphyromonas* (r= -.44, FDR= .04) and *Streptococcus* (r= .4, FDR= .06) in controls. Moreover, increased duodenal eosinophils after PPI were associated with changes in *Streptococcus* (**Figure 4.3E**) but not

Porphyromonas. Interestingly, increased duodenal secondary bile salt concentrations were also associated with changes in *Streptococcus* after PPI (**Figure 4.3F**), while an inverse association of *Streptococcus* with changes in secondary bile salts was not found. Increased luminal *Streptococcus* and decreased diversity were not associated with changes in duodenal pH, in contrast to *Prevotella* (see **supplementary results**). Thus, mucosal and luminal effects of PPI were associated with luminal microbiota alterations in controls and not FD-starters, with a potential role for *Streptococcus* in determining duodenal eosinophilia during PPI treatment.

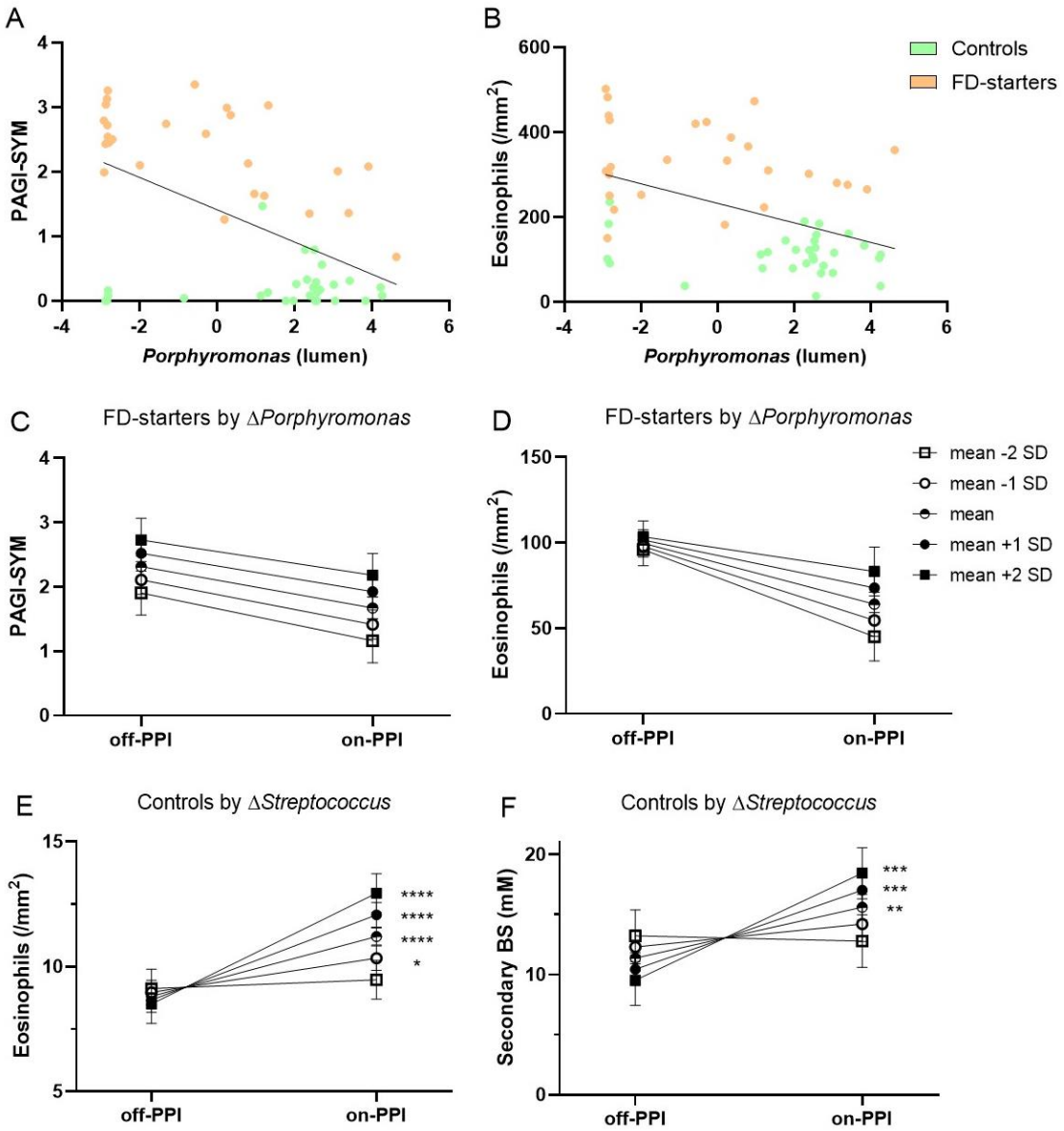


Figure 4.3: Correlations between luminal *Porphyromonas* and symptoms (A) or duodenal eosinophils (B) in controls and FD-starters off-PPI. Association between the evolution of symptoms (C) or duodenal eosinophils (D) after PPI with changes in luminal *Porphyromonas* in FD-starters. Association between the evolution of duodenal eosinophils (E) or fasting secondary bile salts (F) after PPI with changes in luminal *Streptococcus* in controls. Graphs C-F show evolution in symptoms and (Box-Cox transformed) duodenal eosinophils or secondary bile salts by changes in luminal genera, where mean corresponds to an average (Δ= 0) and mean +/- 1 or 2 SD to an above or below average change. **P*< .05, ***P*< .01, ****P*≤ .001. BS, bile salts; FD, functional dyspepsia; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PPI, proton pump inhibitor; SD, standard deviation.

4.5 Discussion

Despite the high prevalence, the pathophysiology of FD is incompletely understood. Duodenal mucosal eosinophilia has been consistently reported in different studies, but the presence and potential role of duodenal dysbiosis are still unclear. Therefore, we studied the luminal and mucosal microbiome of the duodenum, including temporal variation in the absence of PPI. Indeed, PPI are frequently scrutinized due to microbiota changes, which we prospectively studied in the duodenum after a routine course of PPI in naïve patients and controls and after withdrawal of long-term PPI in patients with persistent symptoms. Significant inter-individual variation was found for each location with limited group- and only luminal PPI-effects. Interestingly, specific genera differed between FD patients and controls, including luminal *Neisseria* and *Porphyromonas,* which were less abundant in FD and also correlated with symptoms and duodenal eosinophils. The abundance of luminal *Streptococcus* increased and *Prevotella* and diversity decreased after PPI-therapy in FD and controls. However, symptom- and eosinophil-reducing effects of PPI were not associated with microbial changes in FD patients. In contrast, increased eosinophils in controls were associated with changes in *Streptococcus* after PPI-therapy. These prospective data confirm not only a role of microbial changes in determining potentially inadvertent effects of PPI in controls, but also suggest similar effects with limited reversibility after withdrawal of long-term PPI in FD patients (**Figure 4.4**). Finally, baseline changes in *Porphyromonas* and *Neisseria* may have a role in FD-pathology given their association with eosinophil levels and symptoms, yet any causal role still needs confirmation.

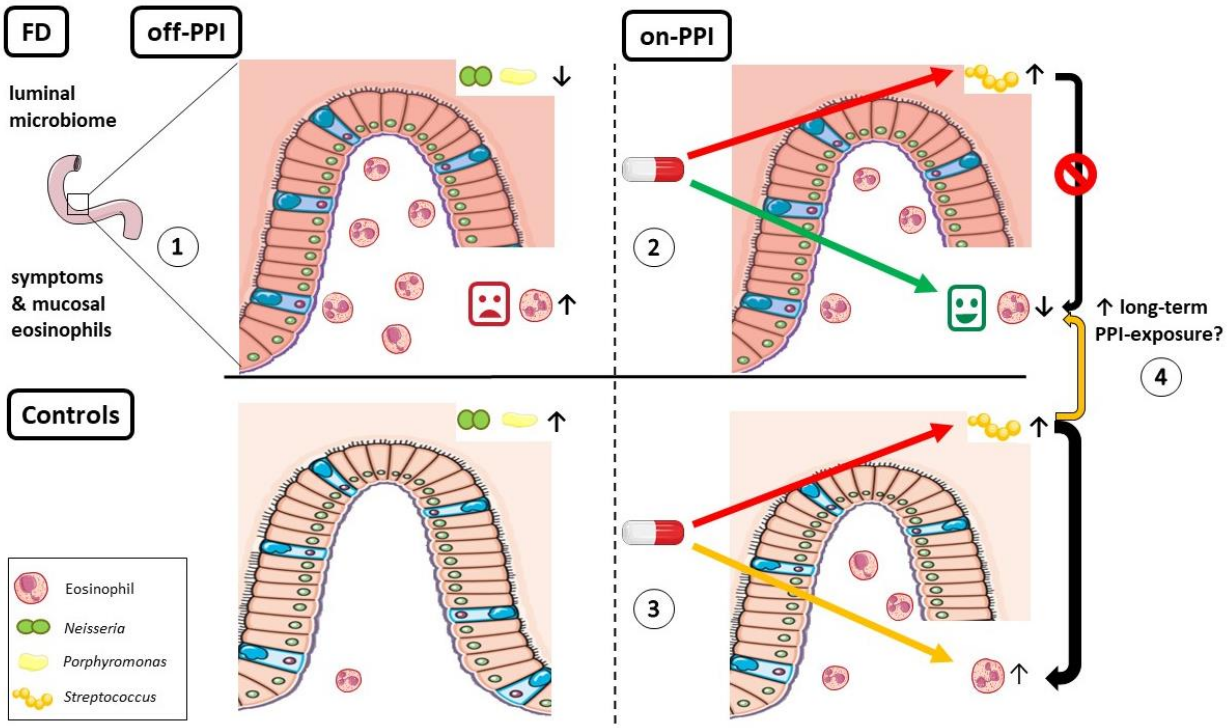


Figure 4.4: Graphical summary. Luminal *Neisseria* and *Porphyromonas* were less abundant in FD patients vs. controls and correlated with symptoms and duodenal eosinophils off-PPI (1). Microbial changes including increased luminal Streptococcus on-PPI were not associated with symptom- and eosinophil-reducing effects of PPI in FD patients (2). In contrast, increased luminal *Streptococcus* was associated with duodenal eosinophil infiltration after PPI in controls (3). The persistently higher *Streptococcus*-abundance also suggested a role for inadvertent microbiota changes with similar duodenal alterations in FD patients after long-term PPI-therapy (4). FD, functional dyspepsia; PPI, proton pump inhibitor.

Due to technical challenges, the small intestinal microbiome has not been studied in sufficient detail and has been neglected compared to the fecal microbiota. Moreover, contamination is a major issue of low-biomass samples.[25] Methodological advances such as the BABD have enabled aseptic sampling of the duodenal MAM, although no significant changes were observed between the BABD and standard biopsy forceps.[15] While this

may be explained by the small sample size, exposure of the mucosa to fluids from the biopsy channel would still be possible when advancing both the aseptic and standard device through the endoscope.[15,26] This could also be the case for sheathed but not sealed brushes, which complicate the interpretation of the recently reported higher *Streptococcus*-abundance from the esophagus to the duodenum of FD patients vs. controls.[24] Besides procedural measures, the risk of contamination during DNA-extraction and PCR-amplification imposes important analytical considerations.[25] Indeed, even removal of sequences present in a larger fraction of negative controls vs. biological samples (prevalence threshold of .5 using decontam)[23] did not fully eliminate the presence of potential contaminants in mucosal samples. Subanalyses with a cut-off of 10,000 reads also illustrated that richness could be falsely inflated using a lower minimum of reads, which was not the case for Shannon and Simpson's index.[27] Thus, changes in diversity after PPI were not driven by rare taxa. The similar richness with lower diversity of duodenal luminal vs. mucosal samples may also indicate less equally abundant taxa. Although this may point to the stronger effect of external influences on the duodenal lumen, both luminal and mucosal communities were stable over time in the absence of PPI. This suggests that bacterial adherence to tissue or mucus may indeed be important for persistent colonization.[14]

Inter-individual variation of the duodenal luminal and mucosal microbiota composition was significant but with no temporal variation in the absence of PPI. Limited PPI-effects were also recently found in a cross-sectional analysis of duodenal aspirates.[28] The lack of group effects in multivariate models of luminal and mucosal samples was not unexpected as the majority of patients with functional GI disorders had a "healthy-like" microbial composition but with decreased α-diversity, *Porphyromonas*, *Neisseria, Haemophilus* and *Fusobacterium* vs. controls in a previous study.[29] The abundance of *Porphyromonas* was also lower in the duodenum of IBS patients[13] and the difference and correlation with symptoms and duodenal eosinophils in our study point to a potential role of this genus in FD pathophysiology. No correlations were found with other (host) factors and although reduced mucosal *Porphyromonas* abundance and an inverse association with small intestinal permeability were found in chronic liver disease,[30] changes in intestinal mucus thickness may affect microbiota-host interactions in these patients.[31] While we observed a PPI-induced increase in luminal *Streptococcus* and decrease in *Prevotella* and diversity in FD patients and controls, only *Prevotella* was associated with changes in duodenal pH. Although pH-effects are attenuated by the distal duodenum, bacterial and direct targets of PPI could also contribute to changes in specific genera including *Streptococcus.*[17,32]

Recently we showed that eosinophil-lowering and not acid-suppressive or barrier-protective effects of short-term PPI-therapy were associated with clinical efficacy in FD patients (**Chapter 3**).[20] Although luminal genera and diversity correlated with symptoms and duodenal eosinophils at baseline, both symptom- and eosinophil-reducing effects of PPI-therapy were not associated with microbial changes in FD patients. In contrast, increased eosinophils and bile salts after PPI were associated with changes in *Streptococcus* in controls. While duodenal dysbiosis could be an epiphenomenon of short-term PPI-therapy, the association between secondary bile salts and changes in *Streptococcus* (but not vice versa) rather point to causal effects. Although anti-eosinophil effects of PPI have been studied,[33] acid suppression was also linked to Th2-type reactions in mechanistic and population-based studies, suggesting a potential role of PPI-induced dysbiosis.[34] Indeed, luminal changes may mask anti-inflammatory effects of PPI in the duodenum of FD patients after long-term exposure, similar to the distal esophagus.[35] As persistent microbial alterations were found after PPI-withdrawal for 2 months, shorter washout periods may not be sufficiently long to interpret differences in duodenal *Streptococcus* in cross-sectional studies.[24] Gastric *Streptococcus*-abundance was also higher with long-term PPI and potentially linked to persisting symptoms in FD.[18] Interestingly, changes in the gastric microbiome have been described after probiotic treatment in FD,[36] but evidence for the clinical efficacy and duodenal effects of probiotics in FD are lacking.[37,38]

This study was performed at a single and tertiary care center, which may limit the generalizability of our findings although baseline characteristics of FD patients were comparable to the general population.[39] While the duodenal lumen and superficial mucus were sampled with brushes, separating the inner mucus layer from the mucosa would require more advanced techniques such as laser capture micro-dissection, which showed similar results in the colon.[40] Despite the different procedural and analytical preventive measures, contamination could still influence the results as statistical removal of potential contaminants is also not intended to detect cross-contamination.[23] However, cross-contamination with true signals would also preclude manual removal of sequences present in negative controls. Because of the greater risk of contamination with biopsies, results for mucosal samples need to be interpreted with caution. We studied bacterial composition and not function (meta-genomics) or other micro-organisms. Also, baseline correlations and associations with symptoms and duodenal factors do not prove causality.

Strengths of this prospective study include the homogenous FD patient and control populations with repetitive sampling of both the duodenal luminal and mucosal microbiome, which have not yet been compared. Besides the interventional design with short-term PPI-therapy, temporal variation was also studied in the absence of PPI. Methodological optimizations were done for sampling and storage procedures, as contamination may arise from PBS or RNA-later solutions, and we included more than the suggested number of negative controls for the detection of contaminants.[23] Potential effects of diet were measured but not expected as duodenal samples were taken in fasted state, with limited substrate availability compared to the colon. Although a longer duration of PPI-intake and -withdrawal would be needed to study the potential role and reversibility of microbial changes in FD patients, our results also illustrate the limitations of cross-sectional studies of the duodenal microbiome.

In conclusion, we showed significant inter-individual variation of the duodenal microbiome of the lumen and mucosa, which was stable over time in the absence of PPI. Specific microbial changes were only found for the duodenal lumen, with a potential role of *Porphyromonas* although symptom- and eosinophil-reducing effects of PPI were not associated with microbial changes in FD patients. The magnitude of the increased luminal *Streptococcus* was associated with potentially inadvertent mucosal and luminal effects of PPI in controls. Indeed, the persistently increased *Streptococcus* after long-term exposure may eventually counteract the observed anti-inflammatory effects of PPI in FD patients. Whether this could be prevented or treated with novel or microbiota-directed treatments should be further studied, especially in FD patients with progressive or refractory symptoms on first-line therapy.

4.6 Supplementary data

SUPPLEMENTARY METHODS

Sample collection

Based on pilot experiments with the BABD, the procedure was adapted before this study to prevent the evacuation of oral and gastric fluids from the working channel of the endoscope when advancing the device only in the duodenum. Thus, the sheathed and sealed (glycerol plug) BABD was already advanced when arriving at the gastric antrum, and again retracted in the working channel after the tip was visible, allowing evacuation of fluids before passage through the pylorus. After collection of the aseptic duodenal biopsy as previously described,[15] the sheath was kept in place and the forceps fully retracted, allowing introduction of the brush (diameter 1.8mm and length 230cm) through the sheath of the BABD (diameter 2.6mm and length 180cm). This technique also prevented exposure of the sheathed (but not sealed) brush device to oral and gastric fluids present in or evacuated from the working channel, in contrast to previous studies.[15,24]

Routine duodenal biopsies (D2) and fluids were processed as previously described.[20] In brief, duodenal eosinophils (H&E) and mast cells (c-kit) were counted per mm^2 in a random and blinded fashion. Transepithelial electrical resistance and paracellular passage of a fluorescein isothiocyanate-labeled 4kDa dextran (FD4, 1 mg/mL; Sigma-Aldrich, St Louis, USA) were determined in modified 3mL Ussing Chambers (Mussler Scientific Instruments, Aachen, Germany). After the endoscopy, duodenal fluids were obtained via a double-lumen naso-duodenal aspiration catheter, which was positioned in D2 under fluoroscopic control. Luminal pH was determined using a Portavo 902 PH portable pH meter (Knick, Berlin, Germany) with a BioTrode electrode (Hamilton, Bonaduz, Switzerland) before measuring primary and secondary bile salts using liquid chromatography-tandem mass spectrometry.[41] Fasting samples were used for analyses in relation to the luminal microbiota. The PAGI-SYM questionnaire, specific for upper GI disorders, was collected at each visit with the total score ranging from 0 (none) to 5 (very severe) over a two-week recall period.[42] Finally, a validated and online Food frequency questionnaire (FFQ) was completed to estimate the total energy and macro-nutrient (carbohydrates, fat, fiber and protein) intake at baseline and follow-up.[43]

Sample and data processing

First, qPCR (Uni16S) of diluted brush (1:5) and biopsy (1:2) samples was performed using the KAPA SYBR® Fast qPCR Kit (Roche, Pleasanton, USA). Dilutions with nuclease-free water were based on pilot experiments and adapted if needed. For the microbiota analysis, the V4 region of the 16S rRNA gene was amplified with the primer pair 515F and 806R (GTGYCAGCMGCCGCGGTAA and GGACTACNVGGGTWTCTAAT, respectively), modified to contain a barcode sequence between each primer and the Illumina adaptor sequences to produce dual-barcoded libraries.[21] Internal PCR-controls included negative (no-template) and no-primer controls to assess potential contamination of the primer plate and master mix, respectively. Positive controls included a standard diluted fecal sample on each run and predefined universal combination of bacterial strains (Zymo Research, Irvine, USA). PCR amplification was performed in triplicate and DNA concentration and fragment lengths of individually pooled amplicons were determined using a 5200 Fragment Analyzer (Agilent, Santa Clara, USA) according to the manufacturer's instructions. Following equimolar pooling and clean-up of the library with QIAquick® PCR Purification kit (Qiagen, Hilden, Germany), the final concentration was confirmed using the Qubit® dsDNA BR Assay Kit (Invitrogen) before sequencing on the Illumina MiSeq platform (500 cycles, 20% PhiX; MiSeq Reagent Kit, version 2) at the VIB Nucleomics core laboratory (KU Leuven, Belgium).[21]

Sequences were processed using the LotuS and DADA2 pipelines (v. 1.6) with taxonomic annotation formatted RDP training set 'rdp_train_set_16'.[44,45] Sequences unclassified at phylum level or annotated to the class Chloroplast or family mitochondria were removed as previously described.[46] Data from all brush or

biopsy samples and negative controls (extraction blanks and no-template controls) were then filtered using the open-source R package decontam.[23] Statistical removal of contaminants was done using the prevalence-based method, which is based on the assumption of contaminants appearing in a smaller fraction of the biological samples vs. negative controls due the presence of competing true bacterial DNA. The classification threshold of .5 was used, allowing removal of sequences present in a higher fraction of negative controls compared to brush and biopsy samples.[23]

Statistical analysis

As there is no previous study investigating effects of PPI on the duodenal microbiome, no reasonable power analysis was possible. However, a number of 60 subjects would allow reproduction of previous findings on microbiota covariates,[22] with repeated sampling in all subjects in the current study. Between-group analysis of baseline covariates was done with Kruskal-Wallis test and post-hoc Dunn test (if applicable) for continuous data and chi-square tests for proportions. Beta-diversity and genus relative abundances were studied after CLR-transformation as required for compositional data with a minimum number of 1,000 reads and proportion of .001.[47] Univariate dbRDA was followed by a stepwise multivariate model including those variables which remained significant after adjustment (Benjamini-Hochberg FDR< .1). Permutational MANOVA (PERMANOVA) was performed with the vegan function adonis, using 10,000 permutations. Comparisons of genus relative abundance between and within groups (including spatial and temporal variation) and correlations were done for taxonomically assigned genera with a prevalence of >20%.[48] Calculation of α-diversity metrics was performed after sub-sampling to 1,000 reads using phyloseq.[49]

For linear mixed models, box-cox or logarithmic transformations of the dependent variables were done depending on normality (Kolmogorov-Smirnov test). Variables which could not be transformed, were analyzed with generalized linear models with the identity link function. Between-group differences and the effect of initiation (controls and FD-starters) or withdrawal (FD-stoppers) of PPI-therapy were studied using planned contrasts. Following a treatment-by-group interaction effect, differences in PPI-related changes between groups were adjusted (stepdown Bonferroni). Based on our previous findings of differential effects of PPI in FD patients and controls, associations between changes in symptoms or duodenal eosinophils and the microbiota were determined in FD-starters and controls.[20] To this end, changes (Δ) in microbial variables were standardized (mean value 0 and standard deviation (SD) of 1) and entered in the model of symptoms and eosinophils, including the interaction with treatment (PPI). In case of significant interaction effects, the evolution in symptoms and eosinophils were plotted for different levels of the mean ± 1 or 2 SD changes (Δ) in microbial variables after PPI.

SUPPLEMENTARY RESULTS

Study cohort and sample overview

An overview of subjects and samples collected with high-quality sequences is given in **Supplementary Table 4.1**. Besides 2 drop-outs (1 FD-starter and 1 FD-stopper with no follow-up visits), variability was assessed in 19 FD-starters and 25 controls after amendment of the protocol. Due to technical difficulties during endoscopy, brushes were missing for 2 baseline and 3 follow-up visits. After sequencing and quality control, 1 additional luminal and mucosal sample were lost for each visit. Based on the high number of reads with brushes (**Supplementary Figure 4.2A**), a subanalysis was done with minimal 10,000 reads, similar to fecal microbiota analyses.[22,48] While only 1 mucosal sample was discarded using a cut-off of 1,000 reads, only 3 luminal but 62 mucosal samples were discarded using a cut-off of 10,000 reads (**Supplementary Table 4.1**). From the 185 brushes and 130 biopsies with >10,000 reads, a total of 696 and 564 annotated genera were obtained after sub-setting and CLR-transformation, respectively.

Median (IQR) read number was 4,496 (1,501-11,204) for negative controls (**Supplementary Figure 4.2A**) with no sequenced reads for no-primer controls. Variability between plates was assessed using the Bray-Curtis dissimilarity of rarefied (1,000 reads) positive and universal controls, which was < .3 (**Supplementary Figure 4.2B**). Microbial load of luminal and mucosal samples was studied in mixed models with no significant main effects of group and treatment or interaction effects.

Duodenal luminal and mucosal microbiome is altered in FD with luminal effects of PPI

Significant associations of sampling location (R^2= 15.74%, P_{adj}= .007), subject (R^2= 2.39%, P_{adj}= .07) and group (R^2= .35, P_{adj}< .1) but not PPI or demographics with duodenal community variation were found using univariate dbRDA for all samples (n= 380). Although only the contribution of location remained significant in a multivariate model (R^2= 22.14%, P_{adj}= .002), this could be driven by the persisting presence of potential contaminants in mucosal samples even after decontam (see below). A significant contribution of location (R^2= 17.84%, P_{adj}= .007) but not subject was also found using a cut-off of 10,000 reads (n= 315), and possibly driven by the presence of contaminants in mucosal samples.

Specific effects on genera and diversity after short-term PPI in FD patients and controls

Main and interaction effects of mixed model analyses for luminal genera of interest and α-diversity are shown in **Supplementary Table 4.2**. Using a cut-off of 10,000 instead of 1,000 reads, the lower abundance of luminal *Neisseria* (FDR< .001), *Porphyromonas* (FDR< .01), *Selenomonas* (FDR= .06) and *Haemophilus* (FDR= .06) was confirmed in FD-starters vs. controls with decreased *Prevotella* (FDR= .09) after PPI. Although changes in luminal richness were lost, the decrease in Shannon and Simpson's index remained significant in controls (all *P*< .01) and FD-starters (all *P*= .01) after PPI. In the absence of differentially abundant mucosal genera after correction for multiple testing, no mixed model analyses were done. For mucosal α-diversity, a significant group effect was found for richness (F= 3.42, *P*= .04), driven by changes in FD-stoppers (see below). No other main or interaction effects were found.

Regarding spatial variation, all 45 genera (taxonomically assigned and prevalence >20%) were differentially abundant (all FDR< .1) but with a higher abundance of potential contaminants in the duodenal mucosal samples (**Supplementary Figure 4.3**).[25] In addition, similar richness but lower diversity was found for brush vs. biopsy samples in both groups and according to PPI (**Supplementary Table 4.3**). In contrast, genera abundance was similar between baseline and variability visits off-PPI (FDR≥ .1) and with no within- or between-group differences in α-diversity metrics for duodenal luminal or mucosal samples (**Supplementary Table 4.4**). Results were similar when using a cut-off of 10,000 reads, with significant spatial variation of all genera (all FDR< .1) and lower Shannon and Simpson's index in luminal vs. mucosal samples of controls (all *P*< .0001) and FD-starters (*P*= .001 and *P*= .0001, respectively) and no significant temporal variation.

Persisting microbiota alterations in FD patients after withdrawal of long-term PPI

Lower abundances of mucosal *Mesorhizobium* (FDR< .01), *Sediminibacterium* (FDR= .06) and *Dyella* (FDR< .1) was found in FD-stoppers vs. controls, although the first 2 are potential contaminants.[25] *Dyella* decreased after PPI-withdrawal (β= -2.09 ± .93, *P*= .03) and was lower in FD-stoppers vs. controls off-PPI (β= -3.56 ± 1.08, *P*=.002) with a significant difference between the changes in FD-stoppers vs. controls (β= -2.98 ± 1.17, P_{adj}= .04) or interaction effect (**Supplementary Figure 4.4A**). Using a minimum number of 10,000 reads, the decrease in luminal *Rothia* (FDR= .02) and *Stomatobaculum* (FDR= .08) but not mucosal *Dyella* was confirmed after PPI-withdrawal in FD-stoppers.

Changes in luminal and mucosal α-diversity metrics after PPI-withdrawal in FD-stoppers and the comparison with controls and FD-starters are shown in **Supplementary Table 4.5**. The group effect for mucosal richness was explained by lower values in FD-stoppers vs. FD-starters (β= -.17 ± .08, *P*= .03) and controls (β= -.2 ± .07,

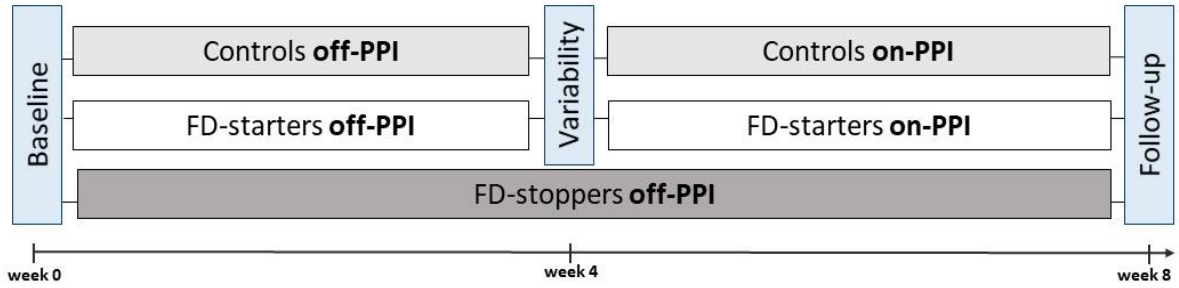
P< .01) off-PPI but with no within-group changes (treatment effect) (**Supplementary Figure 4.4B**). Differences were confirmed in FD-stoppers vs. FD-starters (β= -7.22 ± 3.12, *P*= .03) and controls (β= -9.76 ± 3.01, *P*= .003) off-PPI using a cut-off of 10,000 reads.

Duodenal dysbiosis is unrelated to effects of PPI in FD patients but not controls

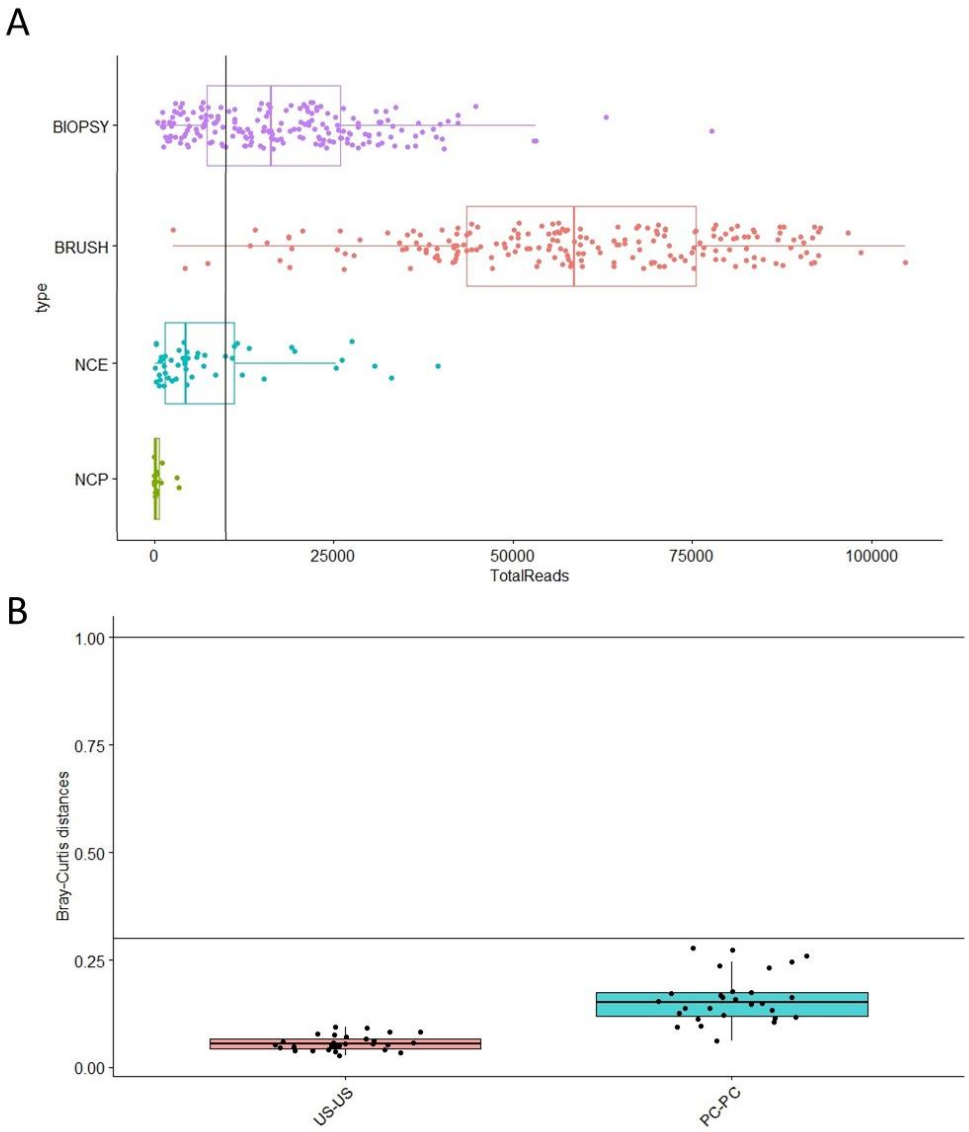
Following the addition of the standardized (mean value= 0 and standard deviation= 1) change (Δ) in luminal genera or diversity and the interaction with treatment in the models of symptoms and duodenal eosinophils in FD-starters, no significant interaction effects were found for Δ*Porphyromonas*, Δ*Neisseria*, Δ*Prevotella*, Δ*Streptococcus* or ΔShannon index (**Supplementary Table 4.6**).

Similar models were used to study associations between PPI-induced changes in host factors and microbiota in controls. The treatment*Δ*Streptococcus* interaction effect for duodenal eosinophils was explained by significantly increased eosinophils for mean -1 (β= 1.37 ± .5, *P*= .01), mean (β= 2.39 ± .35), mean +1 (β= 3.41 ± .5) and +2SD (β= 4.43 ± .8, all *P*< .0001) changes in luminal *Streptococcus* after PPI (**Supplementary Table 4.6**). A treatment*Δ*Streptococcus* interaction effect was also found for secondary (F= 5.64, *P*= .03) but not primary bile salts (*P*= .06), with significantly increased secondary bile salts for the mean (β= 4.24 ± 1.15, *P*= .002), mean +1 (β= 6.57 ± 1.5, *P*< .001) and +2SD (β= 8.91 ± 2.26, *P*= .001) changes in *Streptococcus* after PPI. In contrast, increased luminal *Streptococcus* was not associated with changes in secondary (*P*= .2) or primary (*P*= .17) duodenal bile salts. Increased *Streptococcus* and decreased diversity were not associated with changes in duodenal pH (*P*= .4 and *P*= .37, respectively). However, a treatment*ΔpH interaction effect was found for *Prevotella* (F= 4.27, *P*< .05), explained by significant decreases for the mean -2SD (β= -3.72 ± 1.21, *P*= .004), -1SD (β= -2.64 ± .77, *P*= .002) and mean (β= -1.53 ± .55, *P*=.009) changes in duodenal pH after PPI.

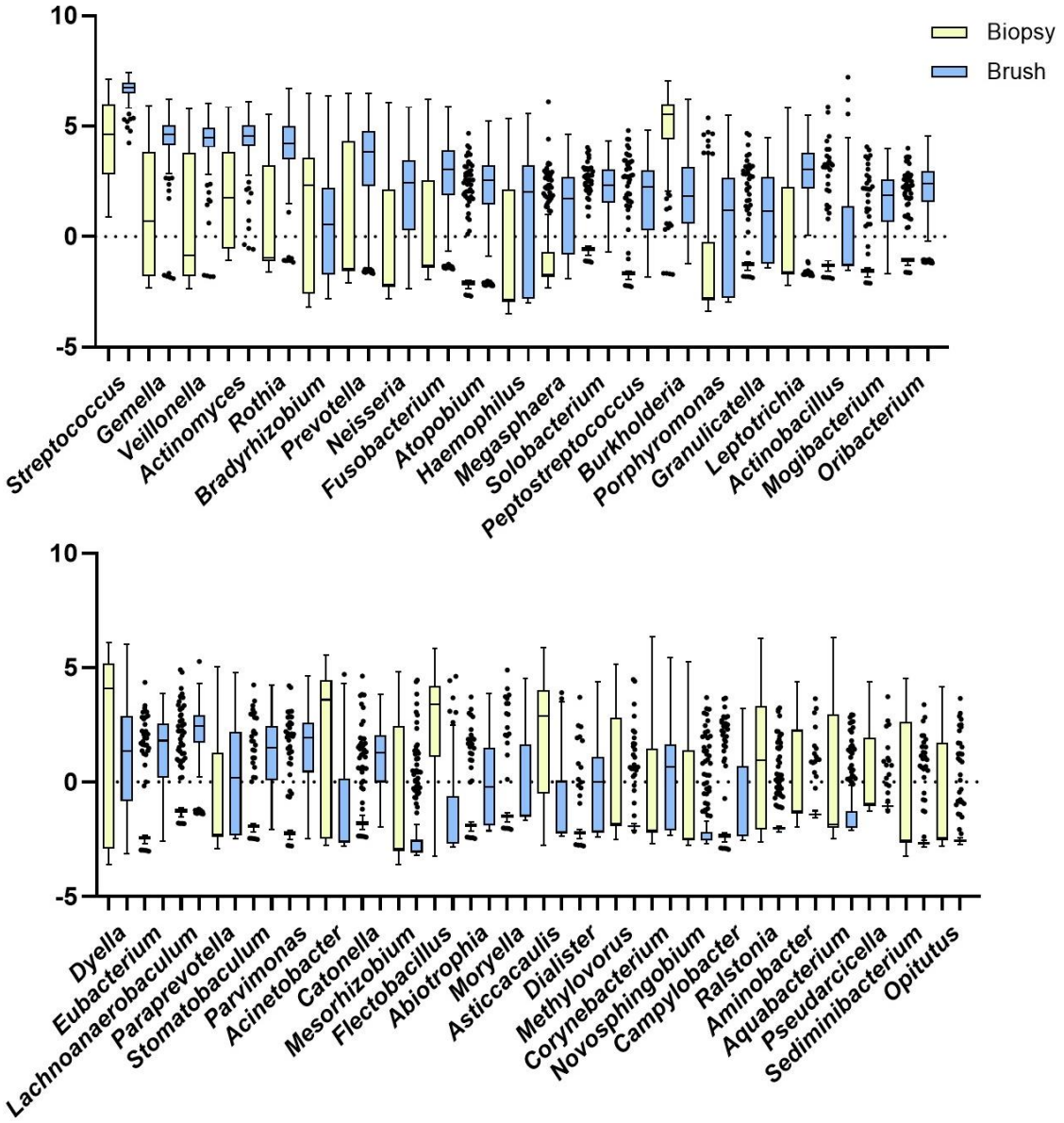
SUPPLEMENTARY FIGURES:



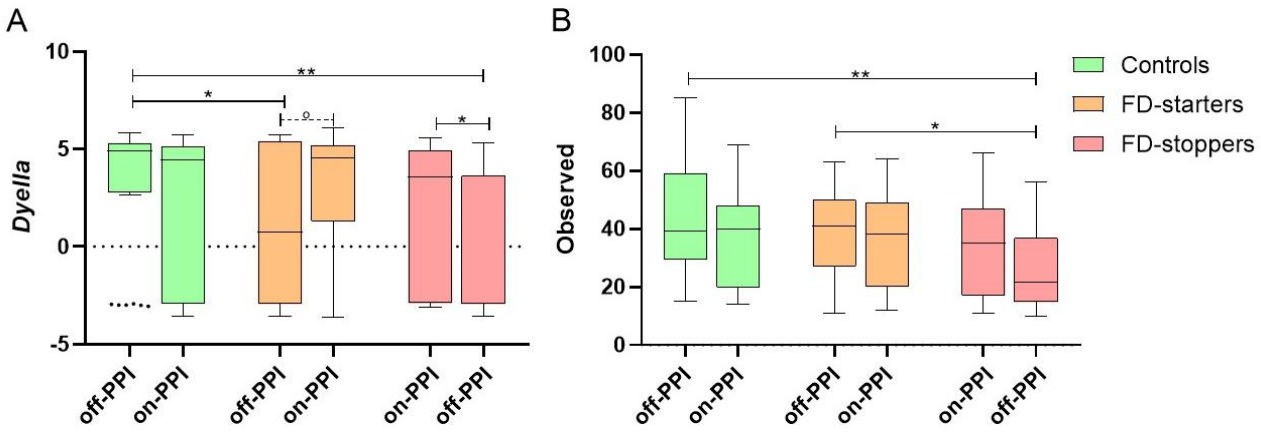
Supplementary Figure 4.1: Study design and procedures. Questionnaires and aseptic samples (for microbiota analysis) were collected at baseline, after 2-4 weeks (variability) and after 4 weeks of Pantoprazole 40mg once daily (follow-up) in controls and FD-starters. For FD-stoppers, procedures were performed at baseline and after 8 weeks of PPI-withdrawal (off-PPI). Routine duodenal biopsies (for histological and permeability analyses) and fluid aspirates (pH and bile salts) were collected at baseline and follow-up visits in all subjects. FD, functional dyspepsia; PPI, proton pump inhibitor.



Supplementary Figure 4.2: Quality control including number of reads for mucosal or luminal samples and negative controls (A) and between-run variability (B). A: number of reads (TotalReads) in all mucosal or luminal samples and negative controls are shown in relation to the additional cut-off of 10,000 reads (vertical line). B: Bray-Curtis dissimilarity of positive and universal controls (rarefied to 1,000 reads) are shown in relation to a maximum of 0.3 (horizontal line). NCE, negative extraction controls; NCP, PCR-controls; PC, positive control; US, universal standard.



Supplementary Figure 4.3: Spatial variation or differential genera-abundance for all paired (luminal and mucosal) samples. FDR< .1 (between-locations) for all genera (>20% prevalence).



Supplementary Figure 4.4: Changes in mucosal *Dyella* (A) and Observed taxa or richness (B) for controls, FD-starters and –stoppers according to PPI-status. Tukey boxplots of CLR-transformed genera with median, IQR and 1.5*IQR whiskers (outliers beyond). °*P*< .1, *P< .05, **P< .01. CLR, centered log-ratio; FD, functional dyspepsia; IQR, interquartile range; PPI, proton pump inhibitor.

SUPPLEMENTARY TABLES

Supplementary table 4.1: Number of subjects and samples collected and with high-quality sequences after quality control for different minimum of reads.

Location and visit	Luminal (brush)				Mucosal (biopsy)			
	baseline	variability	follow-up	total	baseline	variability	follow-up	total
Subjects	77	44	75	196	77	44	75	196
Samples	75	44	72	191	77	44	75	196
Sequenced	74	43	71	188	76	43	74	193
Reads >1,000	74	43	71	188	76	43	73	192
Reads >10,000	74	42	69	185	51	33	46	130

Supplementary Table 4.2: Type 3 effects for linear mixed model analyses of luminal genera of interest and diversity metrics, with treatment as within- and group as between-subject factors of interest.

Effect	Treatment	Group	Treatment*group
Model information	**F value (*P*)**	**F value (*P*)**	**F value (*P*)**
Genera of interest:			
Neisseria	.74 (.39)	4.82 (.01)	.12 (.89)
Porphyromonas	4.45 (.04)	5.1 (.008)	.89 (.41)
Prevotella	20.33 (<.0001)	.76 (.47)	.66 (.52)
Streptococcus	5.44 (.02)	1 (.37)	2 (.14)
Diversity:			
Observed	6.2 (.02)	2.7 (.07)	.29 (.75)
Chao1	.55 (.46)	.7 (.5)	.82 (.44)
Shannon	10.89 (.002)	.58 (.56)	1.65 (.2)
Simpson	7.76 (.007)	.42 (.66)	3.3 (.04)

Supplementary Table 4.3: Spatial variation (luminal vs. mucosal samples) of α-diversity metrics, with within- and between-group differences according to PPI-status.

Group	Controls		FD-starters		*P*-value
Location	**brush**	**biopsy**	**brush**	**biopsy**	
Off-PPI					
Observed	43.07 ± 3.33	42.03 ± 1.36	43.36 ± 7.08	36.81 ± 1.6	.66
Chao1	48.98 ± 1.91	45.43 ± 3.82	43.24 ± 2.28	47.74 ± 8.62	.59
Shannon	2.32 ± .05	2.82 ± .07 ****	2.24 ± .07	2.69 ± .12 ****	.71
Simpson	.79 ± .01	.88 ± .01 ****	.78 ± .02	.86 ± .02 ****	.72
On-PPI					
Observed	37.79 ± 1.9	41.43 ± 6.12	34.79 ± 1.67	42.37 ± 7.32	.71
Chao1	44.81 ± 2.71	43.58 ± 6.95	44.89 ± 2.75	45.36 ± 8.53	.99
Shannon	2.05 ± .08	2.8 ± .1 ****	1.92 ± .1	2.7 ± .12 ****	.75
Simpson	.72 ± .02	.89 ± .01 ****	.68 ± .03	.87 ± .02 ****	.96

*P< .05, **P< .01, ***P< .001, ****P< .0001 (within-group) with lower α-diversity for luminal (brush) samples. FD, functional dyspepsia; PPI, proton pump inhibitor.

Supplementary Table 4.4: Temporal variation (baseline vs. variability visit off-PPI) of luminal and mucosal α-diversity metrics, with within- and between-group differences in controls and FD-starters.

Group	**Controls**		**FD-starters**		***P*-value**
Visit	**baseline**	**variability**	**baseline**	**variability**	
Luminal					
Observed	42.03 ± 1.36	42.24 ± 1.87	36.81 ± 1.6	38.89 ± 2.02	.45
Chao1	48.98 ± 1.91	47.93 ± 2.51	43.24 ± 2.28	43.72 ± 2.33	.68
Shannon	2.32 ± .05	2.32 ± .08	2.24 ± .07	2.27 ± .11	.82
Simpson	.79 ± .01	.78 ± .02	.78 ± .02	.78 ± .02	.72
Mucosal					
Observed	43.07 ± 3.33	46 ± 4.78	44.56 ± 7.25	54.83 ± 10.41	.68
Chao1	45.43 ± 3.82	49.5 ± 5.71	49.1 ± 8.83	59.36 ± 11.57	.78
Shannon	2.82 ± .07	2.85 ± .08	2.71 ± .12	2.91 ± .14	.44
Simpson	.88 ± .01	.89 ± .01	.86 ± .02	.89 ± .01	.6

FD, functional dyspepsia; PPI, proton pump inhibitors.

Supplementary Table 4.5: Duodenal luminal and mucosal α-diversity before and after PPI-withdrawal in FD-stoppers, with within- and between-group comparisons.

Group	**FD-stoppers**		**P_{adj}-value (controls)**	**P_{adj}-value (FD-starters)**
Treatment	**On-PPI (n= 19)**	**Off-PPI (n= 18)**		
Luminal				
Observed	37.94 ± 2.05	40.78 ± 2.72	1	1
Chao1	45.11 ± 2.64	46.89 ± 3.7	1	1
Shannon	2.12 ± .1	2.16 ± .12	.34	.28
Simpson	.75 ± .03	.73 ± .03	.07	.06
Mucosal				
Observed	33.21 ± 3.58	36.31 ± 11.72	.58	.58
Chao1	34.84 ± 4.06	38.71 ± 13.16	.65	.65
Shannon	2.5 ± .11	2.63 ± .18	1	1
Simpson	.84 ±.02	.87 ± .02	1	1

FD, functional dyspepsia; PPI, proton pump inhibitors.

Supplementary table 4.6: Interaction effects for mixed model analyses including the standardized PPI-induced change in luminal genera of interest or diversity in the model with symptoms (FD-starters) and duodenal eosinophils (FD-starters and controls).

Group	**FD-starters**		**Controls**
Outcome	**Symptoms**	**Eosinophils**	**Eosinophils**
Interaction	**F value (*P*)**	**F value (*P*)**	**F value (*P*)**
Luminal:			
Δ*Porphyromonas*	.12 (.74)	.84 (.37)	2.5 (.13)
Δ*Neisseria*	1.6 (.22)	.06 (.81)	.62 (.44)
Δ*Prevotella*	0 (.96)	.07 (.79)	.16 (.69)
Δ*Streptococcus*	.32 (.58)	2.37 (.14)	8.18 (.008)
ΔShannon	.64 (.43)	1.45 (.24)	1.65 (.21)

FD, functional dyspepsia; PPI, proton pump inhibitors.

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CHAPTER 5

EFFICACY AND SAFETY OF SPORE-FORMING PROBIOTICS IN FUNCTIONAL DYSPEPSIA: A PILOT RANDOMIZED PLACEBO-CONTROLLED TRIAL

This chapter has been adapted from: **Wauters L**, Slaets H, De Paepe K, Ceulemans M, Wetzels S, Geboers K, Toth J, Thys W, Dybajlo R, Walgraeve D, Biessen E, Verbeke K, Tack J, Van de Wiele T, Hellings N, Vanuytsel T. *The Lancet Gastroenterology and Hepatology, in press.*

5 EFFICACY AND SAFETY OF SPORE-FORMING PROBIOTICS IN FUNCTIONAL DYSPEPSIA: A PILOT RANDOMIZED PLACEBO-CONTROLLED TRIAL

5.1 Abstract

Background: Current treatments have limited efficacy or safety issues in patients with functional dyspepsia (FD). We studied spore-forming probiotics in FD as monotherapy or add-on to long-term intake of proton pump inhibitors (PPI).

Methods: In this single-center randomized, double-blind, placebo-controlled pilot trial, FD patients (Rome IV, on- or off-PPI) aged ≥ 18 years were randomized 1:1 to receive 8 weeks of treatment with probiotics (*Bacillus coagulans* MY01 and *subtilis* MY02, 2.5 x 10^9 CFU) or placebo consumed twice daily, followed by an open-label extension phase of 8 weeks. History of abdominal surgery, diabetes mellitus, celiac or inflammatory bowel disease, active psychiatric conditions and use of immunosuppressant drugs, anti- or probiotics in the last 3 months were exclusionary. Randomization was stratified to PPI-status using computer-generated blocked lists and all patients and on-site study personnel were blinded to treatment allocation of the first 8 weeks. Symptoms (daily diary), immune activation and fecal microbiota were determined. The primary endpoint was a decrease ≥ .7 of weekly postprandial distress (PDS)-symptoms after 8 weeks (response) in patients with baseline PDS≥ 1 (at least mild). Intention-to-treat analysis was done for all patients randomized and exposed to study products, using an extreme case approach (missing subjects were considered non-responders). This trial is registered with clinicaltrials.gov (number NCT04030780), and is completed.

Findings: Between June 3, 2019, and March 11, 2020, we included 68 FD patients (51 (75%) female, age 40.1 ± 14.4 years, 34 on-PPI) of which 32 were randomized to probiotics and 36 to placebo. Response was higher with probiotics (12 (48%) of 25) vs. placebo (6 (20%) of 30) (RR= 1.95, 95% CI [1.07;4.11], *P*= .03) (baseline PDS≥ 1). The decrease in PDS- and epigastric pain (EPS)-scores at 8 weeks was greater with probiotics vs. placebo and maintained at 16 weeks. The number of patients with adverse events was similar between probiotics (5 (16%) of 32) vs. placebo (12 (33%) of 36). Two serious adverse events occurring during the open-label phase (appendicitis and syncope) were assessed as unlikely related to the study product.

Interpretation: In this exploratory study, *Bacillus coagulans* MY01 and *subtilis* MY02 were effective and safe in FD, with beneficial immune and microbial changes, providing insights into potential underlying mechanisms as future predictors or treatment targets.

Funding: The study is an investigator-initiated study funded by an unrestricted research grant from MY®HEALTH (Kermt, Belgium).

Clinicaltrials.gov, number: NCT04030780.

5.2 Introduction

Functional dyspepsia (FD) is a common chronic gastrointestinal (GI) disorder defined by upper abdominal symptoms originating from the gastroduodenal region with no structural disease on routine investigations.[1] However, the presence of subtle pathology is not excluded by the current Rome IV criteria and increasing evidence points to local duodenal and systemic changes in FD.[2,3] Indeed, impaired duodenal mucosal integrity and low-grade inflammation have been reported in FD patients, correlating with gastric emptying and meal-related symptoms.[4,5] Moreover, systemic immune activation and increased small bowel homing T cells (CD4+ α4β7+ CCR9+) have been reported and correlated with gastric emptying rate and symptom severity.[6] Different underlying mechanisms have been studied, including gastric dysfunction, hypersensitivity to duodenal luminal content and central factors such as gut-brain signaling.[2,3] Despite the socio-economic impact and decreased quality of life, the pathophysiology is incompletely understood and treatment options are limited.[3,7]

Currently, first-line therapy for FD is acid suppression with proton pump inhibitors (PPI) and although guidelines advise against dose escalation, inappropriate use of PPIs, even in the absence of clinical benefit, is frequently reported.[2] Prolonged intake of PPIs may increase the risk of enteric infections (including *Clostridioides difficile*),[8] and changes in the fecal microbiota or dysbiosis have been reported.[9] Probiotics are live micro-organisms that exert a health benefit on the host.[10] Previous studies suggested efficacy of probiotics for PPI-related side effects and uninvestigated dyspeptic symptoms, which may be caused by an altered small intestinal microbiome.[11–13] Indeed, an intestinal-like bacterial profile in the gastric fluid suggested the presence of small intestinal bacterial overgrowth in at least a subset of FD patients.[12] Nevertheless, placebo-controlled studies on probiotics in FD are scarce.[14] Interestingly, gram-positive and spore-forming probiotic strains may outperform traditional probiotic supplements because of gastric-acid resistant endospores with improved storage conditions and survival in the intestine.[15,16] Despite beneficial effects of *Bacillus coagulans* and *subtilis* strains on gut permeability and inflammation in *in vitro* models (M-SHIME®),[17] clinical trials on the effect of spore-forming probiotics are lacking in human disorders with similar alterations, including FD.

To bridge this gap, we conducted a randomized, double-blind, placebo-controlled trial (RCT) to study the efficacy and safety of the combination of *B. coagulans* MY01 and *subtilis* MY02 strains in patients with FD. We hypothesized that FD-symptoms, measured with a validated daily diary, would be improved by these spore-forming probiotics compared to placebo in FD patients as add-on to PPI or as monotherapy. Besides a comprehensive clinical and safety evaluation, biological markers of immune activation and both relative and quantitative microbiota composition were studied to assess potential underlying mechanisms.

5.3 Methods

Study design and participants

The study design of this single-center study with a randomized, double-blind, placebo-controlled and parallel-group design with open-label extension is shown in **Supplementary Figure 5.1**. Male and female patients with FD, diagnosed according to Rome IV criteria with normal endoscopy including *Helicobacter pylori*-testing,[1] were included and divided in 2 predefined cohorts based on current PPI-status: “on-PPI” (daily PPI-therapy of any type and dose during the last 4 weeks with in-sufficient efficacy) or “off-PPI” (no PPI during at least the last 8 weeks). Patients were recruited from the outpatient department of the University Hospitals Leuven (Leuven, Belgium), to which they were referred. All patients were ≥ 18 years old, with no history of abdominal surgery, diabetes mellitus, celiac or inflammatory bowel disease and active psychiatric conditions (stable dose of single neuromodulator was allowed). Use of immunosuppressant drugs, anti- or probiotics in the last 3 months and alcohol use of > 10 units per week were also exclusionary.

The trial was conducted according to the Declaration of Helsinki and Good Clinical Practice regulations after approval by the Ethics Committee of University Hospitals Leuven (number S62043). Written informed consent was obtained from each patient before inclusion. All data were collected at KU Leuven and University Hospitals Leuven (Leuven, Belgium). All authors had access to the study data and reviewed and approved the final manuscript. The trial was registered on clinicaltrials.gov (number NCT04030780) and the protocol was not previously published but accessible via http://targid.eu.

Randomization and masking

Randomization was performed using an online randomization tool (http://www.randomization.com/) by staff not otherwise involved in the study. Randomization was performed 1:1, stratified to PPI-status and the list was generated with a block size of 5. Double-blinding was achieved by packaging probiotics and placebo in the same sealed and consecutively numbered bottles with capsules similar in packaging, smell, and taste. All study patients and on-site study personnel remained blinded for the treatment allocation (RCT-phase) until database lock and signature of the statistical analysis plan.

Procedures

The probiotics treatment consisted of a 1:1 combination of spray-dried *B. coagulans* MY01 and *Bacillus subtilis* MY02 endospores (total of 2.5 x 10^9 CFU per capsule) in a mixture of 50mg with 300mg maltodextrin per capsule, taken twice daily with meals. Placebo was 350mg maltodextrin per capsule, also taken twice daily. Both products were manufactured by MY RESEARCH (Diepenbeek, Belgium). FD patients on-PPI were treated with placebo or probiotics in combination with their daily PPI therapy (no change in dose or type) during the entire study period. Compliance of the study products was determined by counting capsules and defined as good if ≥ 80% was used after each treatment phase.

After screening, a run-in period of 1 week took place with completion of the daily diary. Study procedures were performed at baseline (visit 1), after 8 weeks of treatment with probiotics or placebo (visit 2) and after 8 additional weeks of open-label extension (OLE) treatment with probiotics (visit 3). The Leuven Postprandial Distress Scale (LPDS) was used as a validated daily diary, including 8 items (cardinal PDS- and EPS-symptoms, nausea, belching and heartburn).[18] Monthly questionnaires included patient assessment of upper GI-disorders symptom severity index (PAGI-SYM) and quality of life (PAGI–QOL).[18]

Fasting plasma samples were collected for determination of high-sensitivity C-reactive protein (hsCRP) (baseline, week 8) and lipopolysaccharide (LPS)-binding protein (LBP) (baseline, week 8 and 16). Also, systemic cytokines and peripheral blood mononuclear cells (PBMC) were analyzed at each study visit, with subtyping of CD4+ and gut homing (CD4+ α4β7+ CCR9+) T cell subsets after *ex vivo* stimulation.

Stool samples were collected and transported <24h of each visit under cooled (4-8°C) and anaerobic conditions (AnaeroGen, Thermo Fisher Scientific, Hants, UK) for 16S rRNA gene amplicon sequencing and flow cytometry based quantification of fecal microbiota. In FD patients on-PPI, glycocholic acid breath tests (BT) were done at baseline and week 8 to detect small intestinal bacterial overgrowth, as PPI have been shown to affect the gut microbiome.[9] No BT were performed in FD patients off-PPI.

Outcomes

The primary endpoint was the proportion of clinical responders, defined as a decrease (Δ) of ≥ .7 for weekly postprandial distress syndrome (PDS)-symptoms at week 8 in FD patients with baseline scores ≥ 1 (at least mild) on the LPDS diary in the entire cohort (on- and off-PPI). This diary was chosen due to the recall period of 24 hours, with good reliability, validity and responsiveness for PDS-symptoms.[18] The responder definition was higher than the reported minimum clinically important difference of 0.5 and calculated as the weekly

average of the cardinal PDS or first 3 questions (early satiation, post-prandial fullness, upper abdominal bloating) of the LPDS, scored from 0 (none) to 4 (very severe).[18]

Secondary endpoints were the proportion of minimal clinical responders or Δ≥ .5 for PDS symptoms, the proportions of (minimal) responders (PDS) in ≥ 3 of the last 4 weeks (RCT-phase) and the evolution of weekly (minimal) responder rates (PDS) or symptom scores (PDS, epigastric pain syndrome (EPS) and individual questions). Cardinal EPS-symptoms were defined as the weekly average of epigastric pain and burning. Changes in PAGI-SYM and PAGI–QOL were also assessed and secondary biological endpoints included changes in plasma hsCRP, LBP, cytokines, PBMC and fecal microbiota. Safety was assessed by grading adverse events (AE) at every study visit or in case of premature termination using the Common Terminology Criteria for Adverse Events v4.0, with the relationship for all subjects randomized and exposed to the study products (full analysis set). Results from spore-forming probiotics during the OLE-phase and the glycocholic acid BT (on-PPI) were the pre-specified exploratory endpoints.

Statistical analysis

As there is no previous study investigating the effect of spore-forming probiotics in FD, no reasonable power analysis was possible. Based on an assumed response rate of 50% with probiotics and 20% with placebo using the higher cut-off of the primary endpoint (ΔPDS≥ .7), sample size would be 36 per group (power of 80% and alpha= .05). Based on feasibility, we aimed to include 30 patients completing the RCT-phase per group in this pilot study.

Data from the full analysis set were analyzed according to the intention-to-treat (ITT) principle. Responder-analyses were done following an extreme case approach (missing subjects were considered non-responders) in subjects with at least mild (≥ 1) baseline PDS scores as predefined in the statistical analysis plan, which was finalized and signed before unblinding. Proportions were compared with chi-square or Fisher exact tests and ratio's or relative risks (RR) were calculated and presented with 95% confidence intervals (CI). Mean changes from baseline in continuous clinical and biological endpoints were analyzed using linear mixed models with "group" (probiotic, placebo) as between- and "visit" or "week" (LPDS) as within-subject factors of interest with their interaction. The interaction effect or between-group difference in changes from baseline (RCT) was the main effect of interest and within-group changes from baseline were also assessed for both groups at week 16 (OLE-phase). Finally, associations were studied between changes in clinical and biological endpoints. No imputation was done for missing data. Significance tests were based on a two-sided α= .05 for the primary outcome in this exploratory study. Analyses were implemented using SAS software v9.4 (SAS Institute, Cary, USA) and least squares means estimates (β) are given with 95% CI. Graphs were created with GraphPad Software v8.0 (GraphPad, San Diego, USA). Results are reported in accordance with 2010 CONSORT guidelines, and additional details can be found in the **supplementary methods**. The trial was registered with Clinicaltrials.gov, NCT04030780.

Role of the funding source

The study is an investigator-initiated study funded by an unrestricted research grant from MY®HEALTH (Kermt, Belgium). The company provided feedback on the protocol, which was drafted by the first and last author. The company provided the spore-forming probiotics and placebo control products as well as information about the probiotics. The company was not involved in the collection, analysis, or interpretation of the data and had no access to the individual subject data or samples in agreement with the university policy for investigator-initiated studies. The corresponding author was in charge of collection and analysis of the data and had final responsibility for the decision to submit for publication. All authors had access to the study data and reviewed and approved the final manuscript.

5.4 Results

Study population

Between June 3, 2019, and March 11, 2020, 68 subjects were included and randomized (**Figure 5.1**). Baseline characteristics of patients randomized to probiotics or placebo are shown in **Table 5.1**. Mean duration of PPI-therapy in FD patients on-PPI was 3.14 years. During the first 8 weeks (RCT-phase), drop-out occurred in 1 patient on probiotics (adverse event) and 7 patients on placebo (4 adverse events, 3 withdrawal of consent). LPDS-scores were missing for an additional 4 patients on probiotics and 1 patient on placebo due to failure to adhere to study guidelines (**Figure 5.1**).

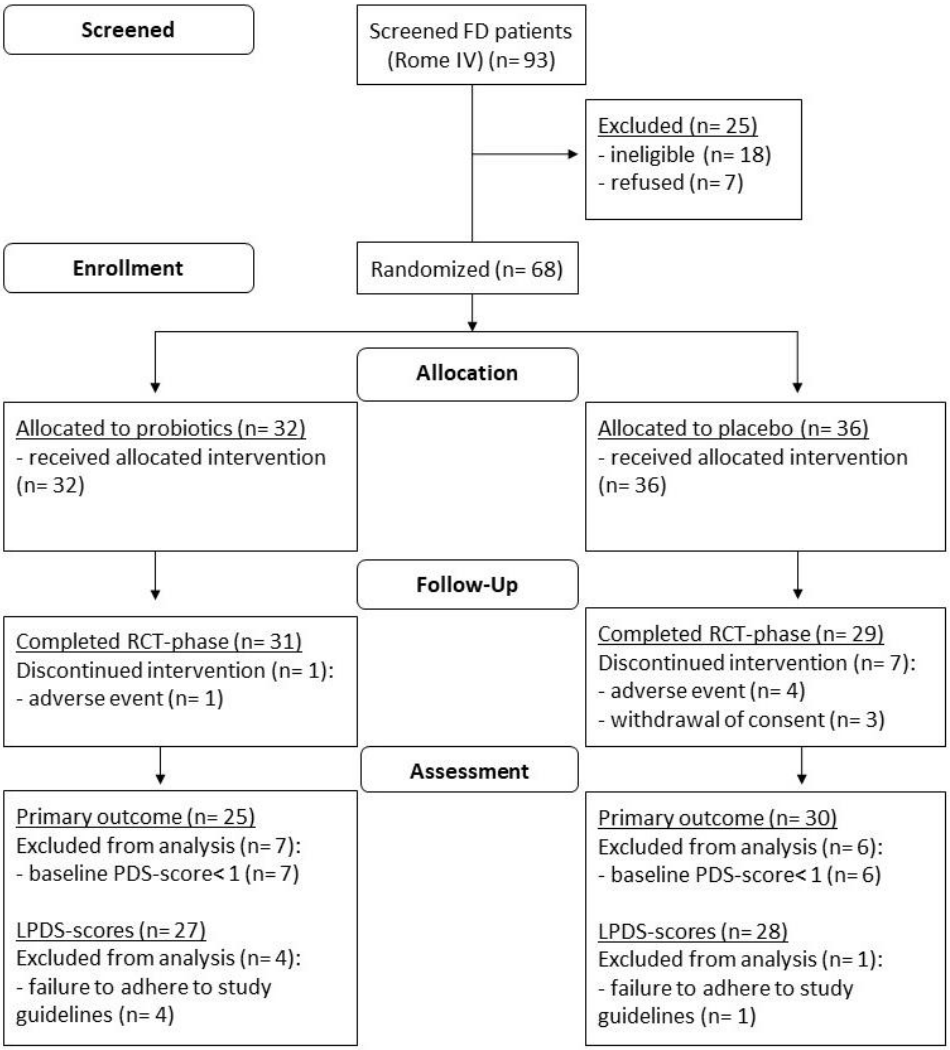


Figure 5.1: Study flow. FD patients discontinuing the intervention or with failure to adhere to study guidelines were regarded as non-responders for the primary endpoint (intention-to-treat analysis). FD, functional dyspepsia; PDS, postprandial distress syndrome; RCT, randomized-controlled trial.

Table 5.1: Baseline characteristics of FD patients randomized to probiotics or placebo (full analysis set).

Group	Probiotics (n= 32)	Placebo (n= 36)
Demographic:		
Age (years)	39.63 ± 15.15	40.51 ± 13.85
Female (%)	24 (75)	27 (75)
BMI (kg/m^2)	23.26 ± 3.51	22.58 ± 3.45
Caucasian (%)	31 (96.97)	32 (88.89)
FD subtypes/IBS:		
PDS (%)	20 (62.5)	22 (61.11)
Overlap (%)	6 (18.75)	8 (22.22)
EPS (%)	6 (18.75)	6 (16.67)
IBS (%)	14 (43.75)	20 (55.56)
Clinical scores:		
Cardinal PDS	1.53 ± 1	1.65 ± .83
Cardinal EPS	.95 ± .82	.91 ± .79
PAGI-SYM	2.08 ± .83	2.15 ± .81
PAGI-QOL	3.24 ± .96	3.39 ± .97
Immune/Microbiota:		
hsCRP (mg/L)	2.46 ± 5.22	2.75 ± 5.77
LBP (pg/mL)	14.1 ± 6.21	12.27 ± 5.53
Richness	142.6 ± 49.37	163.45 ± 79.77
Shannon	37.37 ± 15.46	39.02 ± .17.75
Inverse Simpson	17.56 ± 8.90	17.2 ± 8.78

Continuous data are presented as mean ± SD. BMI, Body Mass Index; EPS, epigastric pain syndrome; FD, functional dyspepsia; hsCRP, high-sensitivity C-reactive protein; IBS, irritable bowel syndrome; LBP, lipopolysaccharide binding protein; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PAGI-QOL, patient assessment of upper GI-disorders quality of life; PDS, postprandial distress syndrome.

Primary endpoint

The proportion of clinical responders (ΔPDS≥ .7) was higher for probiotics (12 (48%) of 25) vs. placebo (6 (20%) of 30) (RR= 1.95, 95% CI [1.07;4.11], *P*= .03) in the ITT-analysis (7 patients randomized to probiotics and 6 on placebo with baseline PDS< 1 were not included) (**Table 5.2, Figure 5.2**). Even when including subjects with low PDS-scores (baseline PDS< 1) as non-responders, efficacy of probiotics (12 (38%) of 32) was still greater than placebo (6 (17%) of 36) (RR= 1.8 [1;3.79]). Responses with probiotics were not significantly higher in FD patients on-PPI (6 (46%) of 13 vs. 2 (13%) of 15, RR= 2.6 [.98;9.36]) or off-PPI (6 (50%) of 12) vs. 4 (27%) of 15), RR= 1.62 [.78;4.05]). Results for the per-protocol (PP) analysis were similar, indicating higher efficacy of probiotics in all randomized subjects and those successfully completing the study (**Supplementary Figure 5.1**, see **supplementary results**).

Secondary endpoints

Minimal clinical response (ΔPDS≥ .5) was higher with probiotics vs. placebo (14 (56%) of 25 vs. 8 (27%) of 30, RR= 1.83 [1.07;3.5]) (**Table 5.2, Figure 5.2**). Weekly responder-rates (ΔPDS≥ .7) tended to be higher at week 7 (RR= 1.88 [.99;4.24]) besides week 8 (cf. primary endpoint), whereas minimal responder-rates (ΔPDS≥ .5) tended to be higher at week 3 (RR= 1.56 [.93;2.86]), week 4 (RR= 1.79 [.99;3.77]) and week 6 (RR= 1.7 [.99;3.24]) (**Figure 5.2**).

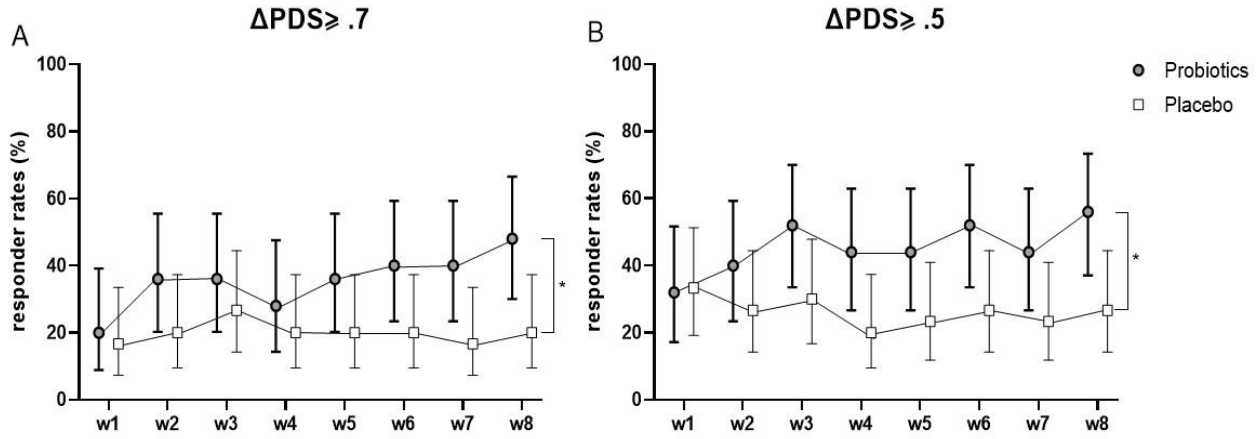


Figure 5.2: Weekly evolution of clinical (A) and minimal clinical responders (B) in FD patients randomized to probiotics vs. placebo (intention-to-treat analysis). Proportions with 95% CI for FD patients with baseline PDS-scores≥ 1 and a decrease (ΔPDS) of ≥ .7 (clinical response) or ≥ .5 (minimal clinical response) at each week and per group (n= 25 probiotics, n= 30 placebo). Significance is given for the difference at week 8 (primary and key secondary endpoint) (*P< .05). FD, functional dyspepsia; PDS, postprandial distress.

When assessing changes in PDS-scores from baseline, the decrease with probiotics was higher than placebo after 8 weeks (β= -.3 [-.95;-.001]) (**Table 5.2**, **Supplementary Figure 5.2**). The decrease in EPS-scores was significant with probiotics but not placebo after 8 weeks (β= -.28 [-.55;-.001]) (**Table 5.2**, **Supplementary Figure 5.2**). The decrease in PAGI-SYM increase in PAGI-QOL from baseline with probiotics and placebo after 8 weeks was similar between groups (**Table 5.2**). Open-label probiotics decreased symptoms in the original placebo group, with maintained clinical effects after 16 weeks in the original probiotics group (see **supplementary results**). Thus, probiotics improved not only PDS- but also EPS-scores after 8 weeks, and this effect was maintained during the OLE-phase.

Table 5.2: Changes in clinical and biological endpoints from baseline after 8 weeks within the probiotics and placebo group (full analysis set).

Group	**Probiotics (n= 32)**		**Placebo (n= 36)**	
Variables	**Estimate**	**95% CI**	**Estimate**	**95% CI**
Clinical scores:				
Clinical response (%)(§)	48	30;66.5	20	9.5;37.3
Minimal clinical response (%) (¶)	56	37.1;73.3	26.7	14.2;44.4
Cardinal PDS	-.53	-.74;-.32	-.23	-.44;-.02
Cardinal EPS	-.39	-.58;-.19	-.11	-.31;.08
PAGI-SYM	-.42	-.66;-.17	-.45	-.69;-.21
PAGI-QOL	1.16	.35;1.96	1.46	.67;2.24
Immune/Microbiota:				
hsCRP (mg/L)	.32	-1.46;2.09	-.64	-2.37;1.1
LBP (pg/mL)	.01	-.12;.14	.07	-.07;.2
Richness	-.01	-.06;.05	.05	-.01;.1
Shannon	.19	-.68;1.05	.39	-.5;1.29
Inverse Simpson	-.05	-.78;.68	.1	-.66;.85

(§) ΔPDS≥ .7 at week 8 in FD patients with baseline scores ≥ 1 (n= 25 probiotics, n= 30 placebo), (¶) ΔPDS≥ .5 at week 8 in FD patients with baseline scores ≥ 1 (n= 25 probiotics, n= 30 placebo). EPS, epigastric pain syndrome; hsCRP, high-sensitivity C-reactive protein; LBP, lipopolysaccharide binding protein; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PAGI-QOL, patient assessment of upper GI-disorders quality of life; PDS, postprandial distress syndrome.

Systemic immune activation

No within- or between-group differences were found for hsCRP or LBP in the first 8 weeks (**Table 5.2**). Based on the clinical efficacy of probiotics during the OLE-phase, changes in systemic cytokines and stimulated CD4+ T cells were also assessed after 16 weeks of probiotics with a significant decrease in IL17A (**Supplementary Figure 5.3**). Although circulating Treg cells decreased after 8 weeks with probiotics and not placebo, effects on CD4+ T cells were mainly found after 16 weeks with probiotics, including significantly decreased Th17 cells (**Supplementary Figure 5.3**). Thus, effects of probiotics included decreased Th17-signaling with an additional decrease in Th2-signaling and gut-homing T cells in FD patients on-PPI (see **supplementary results**).

Microbiota analysis

No within- or between-group differences were found for α-diversity after 8 and 16 weeks (**Table 5.2**). Partial redundancy analyses showed that spore-forming probiotics did not significantly contribute to the conditional variation (on subject) in relative or quantitative microbial community composition when combining samples after 8 and 16 weeks of probiotics in both groups over the entire study period (**Supplementary Figure 5.4**, see **supplementary results**). Nevertheless, a proportional but not absolute significant increase in *Faecalibacterium* with increased abundances of *Roseburia* and the family *Leuconostocaceae* were found with spore-forming probiotic (after 8 and 16 weeks of probiotics) vs. control (at baseline and after 8 weeks of placebo) samples (**Supplementary Figure 5.5**). Interestingly, the proportion of positive BT on PPI with probiotics vs. placebo was similar at baseline (18% vs. 25%, RR = .8 [.28;1.66]) but significantly lower after 8 weeks (7% vs. 38%, RR= .26 [.05;.96]), suggesting a reduction of small intestinal bacterial overgrowth with spore-forming probiotics.

Mediation analysis

Based on decreased Treg cells or Th17-signaling and proportionally increased *Faecalibacterium* or *Roseburia* with probiotics, changes in these biological endpoints were entered in the models of PDS-symptoms within the probiotics and placebo group (RCT-phase). While no association was found for Treg cells, the decrease in PDS-symptoms was only significant in case of average or greater reductions in IL17A or Th17 cells with probiotics and not placebo (**Supplementary Figure 5.3**). In addition, decreased PDS-symptoms were only found with average or greater increases in *Faecalibacterium* but not *Roseburia* with the probiotics and not placebo treatment (see **supplementary results**). Thus, changes in Th17-signaling and *Faecalibacterium* were associated with efficacy of probiotics.

Safety

Treatment with probiotics was safe compared to placebo, with a similar incidence of all (5 (16%) of 32 vs. 12 (33%) of 36) and GI-specific AE (1 (3%) of 32 vs. 5 (15%) of 36) (**Table 5.3**). Besides the single adverse event leading to drop-out with probiotics during the RCT-phase (skin infection), 4 adverse events led to drop-out with placebo (diarrhea in 2 patients, skin or lung infection in 2 other patients) (**Table 5.3**). Two serious adverse events occurred during the OLE-phase (appendicitis and syncope), which were all assessed by the investigators as unlikely related to the study product. There were no treatment-related deaths.

Table 5.3: Patients with adverse events per system organ class in the first 8 weeks for the probiotics and placebo group (full analysis set).

Group	Probiotics (n= 32)	Placebo (n= 36)	Total (n= 68)
Number of patients with adverse events (%)	5 (16)	12 (33)	17 (25)
Cardiac disorders:			
- palpitations		1 (3) (§)	1 (1) (§)
Gastrointestinal disorders:			
- diarrhea		2 (6) (¶)	2 (3) (¶)
- gastritis	1 (3) (¶)	2 (6) (¶)	3 (4) (¶)
- vomiting		1 (3) (¶)	1 (1) (¶)
General disorders:			
- fever		1 (3) (§)	1 (1) (§)
- flu-like symptoms	2 (6) (§)	1 (3) (§)	3 (4) (§)
Infections and infestations:			
- skin infection	1 (3) (§)	1 (3) (§)	2 (3) (§)
- lung infection		1 (3) (§)	1 (1) (§)
Renal/urinary disorders:			
- renal colic		1 (3) (§)	1 (1) (§)
Respiratory/thoracic disorders:			
- allergic rhinitis		1 (3) (§)	1 (1) (§)
Skin/subcutaneous tissue:			
- rash maculo-papular	1 (6) (§)		1 (1) (§)

(§) unlikely related to study product, (¶) possibly related to study product. All adverse events in the first 8 weeks were mild (grade 1) or moderate (grade 2). Underlined text denotes drop-outs due to adverse events in the probiotics or placebo group.

5.5 Discussion

In this exploratory study, we demonstrated the efficacy and safety of *B. coagulans* MY01 and *subtilis* MY02 spore-forming probiotics in FD patients compared to placebo. The primary endpoint was met, with reduced PDS-symptoms in all FD patients with probiotics vs. placebo (RCT-phase). The superior effects of probiotics on PDS- and EPS-symptoms were confirmed for the key individual symptoms of the daily diary compared to placebo. The beneficial effects were also maintained with probiotics during the OLE-phase (week 16). Despite the absence of between-group differences in systemic immune activation at 8 weeks, changes in T cells were evident after longer-term probiotic treatment with mainly decreased Th17-signaling, which was associated with clinical efficacy. Despite the absence of major shifts in relative or quantitative fecal microbiota community composition, the proportional increase in *Faecalibacterium* was also associated with probiotic efficacy. Moreover, spore-forming probiotics lowered the percentage of positive glycocholic acid BT in FD patients on-PPI, suggesting a reduction of small intestinal bacterial overgrowth. Finally, treatment with spore-forming probiotics was safe and well tolerated.

Despite the high prevalence of FD, current treatment options are limited in efficacy and/or safety due to potential side effects.[2] Although we recently showed that routine or short-term PPI-therapy reduced eosinophils, mast cells and permeability in FD, luminal effects of PPI could also provoke similar duodenal alterations in long-term PPI-users.[5] As these changes were not fully reversible during PPI-withdrawal, this would also justify the search for alternative treatments as reflected by the lack of consensus for effective therapies.[14] While a Japanese RCT showed similar overall efficacy with some improvement of postprandial fullness with daily intake of *L. gasseri* OLL2716 (LG21) in uninvestigated dyspepsia,[13] the combination of the

B. coagulans MY01 and *subtilis* MY02 strains in the current study was effective for PDS, EPS and key individual symptoms. Analyses in FD patients on- and off-PPI also require replication in larger and multicenter studies. Despite a number of drop-outs due to (mainly unlikely related) adverse events and a small number of withdrawals of consent, positive outcomes from the ITT-analyses were confirmed in the PP-analysis, with the 20-30% improvement exceeding the suggested 10-15% over placebo as clinically meaningful outcomes.[19] Weekly responder rates were highest at the end of the RCT-phase and the lack of significant effect of probiotics on PAGI-SYM and –QOL scores at 8 weeks may be explained by the two-week recall period, which would not capture the highest efficacy of probiotics in the final week of the RCT-phase. A significant effect of probiotics was evident using the PAGI-SM and -QOL scores at 16 weeks.

Several studies have reported immune activation in FD.[2] On the one hand, increased IL5- and IL13- production after stimulation of PBMC from FD patients suggested a shift from a Th1- to Th2-type inflammation.[20] On the other hand, increased IL1β-production of cultured PBMC pointed to a Th17-response and production of GM-CSF from Th17 cells may drive mucosal eosinophil recruitment in FD,[21] which was significantly increased in the duodenum of FD patients.[5,22] In the present study, decreased Th17-signaling was found after 16 weeks of probiotics. In FD patients on-PPI, there was an additional decrease in Th2, gut-homing and GM-CSF+ T cells after *ex vivo* stimulation of PBMC. CD4+ T cells co-expressing integrin α4β7 and CCR9, indicative of small intestinal mucosal migration, were previously reported to be upregulated in FD.[6] Similar to the inverse association between intestinal and systemic gut-homing T cells in inflammatory small bowel diseases,[6] the decrease in Tregs after 8 weeks of probiotics could be related to increased intestinal recruitment of Treg cells. Indeed, increased CD45RA+ Treg cells were detected after 16 weeks of probiotics. Although CD45RA is not exclusively expressed on naïve T cells, this may point to immuno-regulatory properties of these probiotics, which may be more pronounced in patients on-PPI due to microbiome-related side effects of PPIs.[11] However, only the decreased IL17A or Th17 and not Treg cells were associated with efficacy of probiotics and not placebo during the RCT-phase.

While a previous study reported beneficial changes in individual genera with multispecies probiotics (including *B. coagulans* and *subtilis*) in long-term PPI-users, no changes were found in alpha-diversity or overall fecal microbiota composition.[11] Similarly, spore-forming probiotics had only minor and non-significant effects on the genus-level relative and quantitative community composition, but with a relative increase of *Faecalibacterium* and *Roseburia* compared to control samples. While the lower concordance between relative and absolute abundances of *Faecalibacterium* is known, increased enumeration of *F. prausnitzii* was also found and possibly related to increased antigen-stimulated production of IL10 by PBMC with intake of *B. coagulans*.[23] Although both commensal bacteria have anti-inflammatory activity with decreased Th17-signaling,[24,25] only the increased *Faecalibacterium* was associated with probiotic efficacy in the current study. Interestingly, proportional increases in the family *Leuconostocaceae* have also been reported after treatment with anti-inflammatory proteins of *L. plantarum* or *L. paracasei* LC-37, with effects on the gut barrier and inflammation or metabolites.[26,27] In FD, the reduced abundance of intestinal-like bacteria in the gastric fluid suggested a reduction of bacterial overgrowth with LG21.[12] Although concomitant intake of *L. reuteri* DSM 17938 reduced bacterial overgrowth after 3 months of PPIs,[28] the systematic prevention of PPI-related side effects is not recommended despite the association. In the present study, reduction of bacterial overgrowth as evaluated by the glycocholic acid BT was also found in long-term PPI-users after 8 weeks, pointing to additional benefits of spore-forming probiotics on-PPI.

The limitations of this exploratory study include the limited duration and generalizability of a single center and tertiary care study, although baseline characteristics and distribution of FD subtypes were comparable to the general population.[7] As we did not select patients based on PDS-severity, numbers of eligible patients for the analysis of (minimal) clinical responders were lower but probiotic efficacy was also confirmed when assessing changes in scores from baseline. Although the LPDS-diary is mainly used for PDS (the primary

outcome), it was one of the most promising outcome measures for symptom evaluation in clinical trials in FD.[18] Confirmation of our preliminary findings is needed, especially for EPS as coexisting or predominant symptom or subgroup. We studied systemic and not local immune activation, thus providing only indirect evidence for (changes in) duodenal inflammation in FD.[2] Dietary intake was not accounted for and although changes in the fecal microbiota are not representative of the small bowel microbiome, similarities exist between both and in particular for *Faecalibacterium*.[29] Finally, besides common limitations inherent to all non-invasive BT, substrate availability and low levels of radiation limit the use of ^{14}C-glycocholic acid BT.

The strengths include the rigorous study design with additional information on longer-term efficacy and safety from the OLE-phase. We included clinically well-characterized FD patients (Rome IV criteria) with strict in- and exclusion criteria and assessment by a single study physician, reducing other potential sources of variability. Spore-forming probiotics offer the advantage of high stability and long shelf life, similar to heat-inactivated but non-viable strains.[30] The use of the validated daily diary was more robust for clinical endpoints compared to the questionnaires and we performed a detailed immune and fecal microbial characterization, including co-expression of markers for small bowel homing and relative and quantitative microbiota profiling. As changes in the microbiome were more prominent with PPI than antibiotics or other commonly used drugs in previous population-based studies,[9] the potential for a reduction in bacterial overgrowth with spore-forming probiotics in FD patients who cannot be weaned off PPI deserves further study.

In conclusion, the current combination of *B. coagulans* MY01 and *subtilis* MY02 spore-forming probiotics was effective and safe in FD patients. Both a decreased Th17-signaling and an increased *Faecalibacterium* relative abundance were associated with probiotic efficacy. Although spore-forming probiotics could be considered as mono-therapy, changes in immune activation were more pronounced with probiotics in FD patients on-PPI, suggesting additional beneficial effects on chronic alterations with PPI-therapy. This pilot study underscores the potential role of microbiota in FD and provides effect sizes, which are informative to design larger and multicenter trials. Future studies should strengthen this preliminary evidence for spore-forming probiotics in different populations and FD-subtypes, including immune activation and the microbiome as possible underlying mechanisms, which will help to establish the positions of probiotics as add-on to PPI or monotherapy in FD.

5.6 Supplementary material

SUPPLEMENTARY METHODS

Sample collection and processing

Blood samples

High-sensitivity or hsCRP was determined using the Latex turbidimetric method on a COBAS 8000 autoanalyser (HITACHI/Roche, Rotkreuz, Switzerland). At each visit, LBP was determined using a specific enzyme-linked immunosorbent assay (ELISA) with standard 1,000 fold dilution according to manufacturers' instructions (Thermo Fisher Scientific, Waltham, MA, USA). Systemic cytokines from the Proinflammatory and Cytokine (IL5 and IL17A) Panel 1 were determined at each visit using V-PLEX (Meso Scale Diagnostics, Rockville, MD, USA).

PBMC were isolated from whole blood by Ficoll density gradient centrifugation (Lympholyte, Cedarlane Laboratories, Uden, The Netherlands) and cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, Overijse, Belgium) and fetal bovine serum (FBS, Life Technologies, Ghent, Belgium) using a slow temperature-lowering method (Coolcell, VWR, Haasrode, Belgium). After 24h, cryovials were transferred to liquid nitrogen (University Biobank Limburg, Hasselt, Belgium) until analysis.[31] For flow-cytometry, PBMC were thawed by bringing the temperature of the cryovials to 0°C in a water bath (37°C), followed by addition of cold thawing medium consisting of 20% FBS in RPMI 1640 (Lonza, Basel, Switzerland) and centrifugation at 4°C. The pellet was then resuspended in thawing medium (10 x 10^6 cells/ml) supplemented with DNAse (Sigma-Aldrich, 1/100 diluted) and incubated at 37°C for 10min. Subsequently, cells were washed twice and suspended in RPMI supplemented with 5% FCS, 0.5% Pen/Strep, 1% non-essential amino acids, and 1% sodium pyruvate. PBMC were stimulated with phorbol 12-myristate 13-acetate (PMA, 25 ng/ml) and calcium ionomycin (Sigma-Aldrich, 1 µg/ml) in the presence of GolgiPlug (BD Biosciences, 1 µl/ 1 x 10^6 cells) for 4h. Viable cells were identified with Zombie Aqua Fixable Viability dye (BioLegend, Antwerp, Belgium). Next, cells were stained with the following antibody cocktail: anti-human CD4 BV785, CD45RA BV711, CD3 AF700, Integrin β7 PE-Dazzle594, CD49d PerCP-Cy5,5, CD199 BV421, and CD25 BV605 (BioLegend). After cell surface staining, cells were fixed and permeabilization with Cytofix/Cytoperm (BD Biosciences) and stained intracellularly with anti-human IL-17A AF488, IFN-γ APC-Fire750, IL-4 PE-Cy7, GM-CSF PE, and FoxP3 AF647 (BioLegend). Samples were analyzed on an LSRFortessa flow cytometer with FACSDiva sofware (BD Biosciences). Subsequent analyses were performed using FlowJo software (BD Biosciences).

Microbiota analysis

Fecal pellets (100mg stored at -80°C) were used for DNA extraction and 16S rRNA gene amplicon sequencing on an Illumina MiSeq Platform with v3 chemistry. The V3-V4 region of the 16S rRNA gene was amplified by PCR using the 341F-785R primer pair derived from Klindworth *et al*.,[32] with a slight modification to the reverse primer by introducing another degenerated position (K) to make it more universal. Read assembly and clean-up was performed following the Mothur standard operating procedures.[33,34] Mothur version 1.44.3 was used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED alignment, v.138),[35] remove chimeras (vsearch v2.13.3), assign taxonomy using a naïve Bayesian classifier and SILVA NR v138 and cluster contigs into operational taxonomic units (OTU) at 97% sequence similarity.[36] Next, sequences that were classified as Eukaryota, Archaea, Chloroplasts and Mitochondria or unclassified sequences were removed. The OTU table was further filtered to remove OTUs according to the arbitrary cut-offs described by McMurdie and Holmes.[37]

Enumeration of microbial cells was performed on fecal suspension supernatant with 1,000 fold dilution to allow quantitative microbiota profiling. Samples were filtered through a 20µm cell strainer (Filcon syringe-type filter, Becton Dickinson) with storage at -80°C until flow cytometric analysis as previously described.[38]

In brief, samples were analyzed on a Thermo Scientific Attune NXT 2019 (BVxx configuration) equipped with a default Attune Autosampler after dilution of 10^5 times and addition of SYBR Green I in DMSO 1% (v:v) concentration (final 1X SYBR green I). Sample acquisition was performed at 100 µL/min, using a pre-set threshold (based on several controls) on the BL1 (530/30) bandpass filter for the 488nm laser. The stability of fluorescence over time was carefully monitored to assure correct concentration determination, and the occurrence of doublets and higher n-lets was checked in a peak height vs. area plot of the BL1 fluorescence. To minimize carry-over, an appropriate number of rinse-steps was allowed between wells and a 15-sec lead time was allowed before acquisition, which also enabled flow stabilization. Cell counts were inferred by automatic gating on the BL-1 versus BL-3 plots to capture the events corresponding to the SYBR green labelled cells. Collected cell counts and sample volumes were exported and converted to cell counts g^{-1}, taking into account the sample dilution factor during sample processing.[39] This approach enabled quantitative microbial community profiling,[38,39] besides standard compositional analysis.

For the BT, a gelatin-capsule containing the marked substrate (^{14}C-glycocholic acid) was ingested with breakfast after an overnight fast with collection of breath samples every 30min during 6h.[40] Exhaled $^{14}CO_2$-excretion was measured using a Tricarb 2910 liquid scintillation analyser (PerkinElmer, Waltham, MA, USA) and a normal test was defined as a cumulative $^{14}CO_2$-excretion of <3% after 6h.

Data handling and deposition

The database was kept in Microsoft Excel (Office Professional Plus 2016) and locked by the Data Manager before unblinding and independent analysis. The raw fastq files used to create the taxonomic table, which served as a basis for the microbial community analysis in this paper, have been deposited in the National Center for Biotechnology Information (NCBI) database (number PRJNA720325).

Statistical analysis

For proportions, CI were computed using Wilson's method and the Koopman asymptotic score was used for RR. In addition to the ITT-analysis, a per-protocol (PP) analysis was done for the proportions in subjects randomization and exposed to treatments with at least mild (≥1) baseline scores and presenting no protocol deviations. For mixed model analyses, a restricted maximum likelihood (REML)-based repeated measures approach was used and the assumption of a normal distribution (based on the Kolmogorov-Smirnov test) was checked for all dependent variables, with Box-Cox or logarithmic transformations to normalize this distribution if needed. Variables which could not be transformed were analyzed with generalization linear models with the identity link function after exclusion of outliers using the extreme studentization deviate method. The (co)variance structure providing the best fit (based on the lowest value of the Akaike information criterion) was chosen with transition from unstructured (un) to compound symmetry (cs), auto-regression (ar(1)) or heterogeneous auto-regression (arh(1)) if necessary.

For microbiome analysis, within-sample or α-diversity was estimated by calculating Hill numbers using the iNEXT package (version 2.0.20) with a bootstrap method (50 iterations) to calculate 95% confidence intervals of Chao1 richness, the Shannon and Inverse Simpson index (Hill numbers 0, 1 and 2, respectively).[41] Similar to clinical endpoints, within- and between-group changes were assessed for α-diversity metrics. Further microbiota analyses were performed in R-software (version 4.0.3).[42] The overall effect of probiotics on microbial community composition was assessed by combining samples after 8 and 16 weeks of probiotics in both groups ('sporebiotic') and samples at baseline and after 8 weeks of placebo ('control ') over the entire study period.[43] First, microbial community composition was studied at genus-level using Principal Coordinates Analysis (PCoA; stats 4.0.3) based on the relative or quantitative abundance based jaccard dissimilarity matrix. Next, effect sizes of sporebiotics were calculated through partial distance based redundancy analyses (dbRDA, vegan 2.5.7), after modelling the scores obtained by a PCoA in function of the

subject and sporebiotics (constraints) with variation partitioning (varpart, vegan 2.5.7). The conditional variation (on subject) explained by sporebiotics was adjusted by a subtractive procedure and permutational multivariate analysis of variance (PERMANOVA) and results were visualization in type II scaling correlation triplots. Finally, differences in individual genera between sporebiotics and control samples were assessed with Linear discriminant analysis Effect Size (Lefse) for proportions and log10 fold changes (log10FC) for quantitative data using a Kruskal-Wallis Rank Sum Test, followed by pairwise Wilcoxon Rank Sum Tests with Holm correction for multiple testing and visualization in heatmaps.[44]

Associations between changes in PDS-symptoms and biological endpoints within the probiotics or placebo group were assessed by standardizing the change (Δ) in immune or microbiota variables with a mean value of 0 and standard deviation (SD) of 1 as previously described.[5] Changes in symptoms were then plotted for the average (0) and average ± 1 or 2 SD of the change (Δ) in biological endpoints to visualize the association with clinical effects of probiotics or placebo.

SUPPLEMENTARY RESULTS

Study population

In addition to FD patients with dropout or failure to adhere to study guidelines, those with compliance < 80% (2 on placebo) and use of prohibited treatments (1 on probiotics, 3 on placebo) during the first 8 weeks were excluded for the PP-analysis.

Primary endpoint

In the PP-analysis, the proportion of clinical responders (ΔPDS≥ .7) was higher (11 (55%) of 20) for the probiotics vs. placebo group (3 (17%) of 18) (RR= 2.9, 95% CI [1.2;8.54]) (**Supplementary Figure 5.1A**).

Secondary endpoints

In the PP-analysis, minimal clinical response (ΔPDS≥ .5) was also higher with probiotics vs. placebo (RR= 2.34 [1.12;5.45]) (**Supplementary Figure 5.1B**). Weekly responder-rates (ΔPDS≥ .7) were higher with probiotics at week 7 (RR= 3.26 [1.14;11.86]) and week 8 (cf. primary endpoint), while a higher minimal clinical response was found at week 4 (RR= 2.6 [1.08;7.61]) in PP-analyses (**Supplementary Figure 5.1**). In contrast, clinical (RR= 1.88 [.99;4.24]) and minimal clinical response (RR= 1.73 [.99;3.46]) were not significantly higher with probiotics vs. placebo when including sustained responses in ≥ 3 of the last 4 weeks, which was confirmed in the PP-analysis (RR= 2.31 [.97;6.75] and RR= 1.89 [.91;4.41], respectively).

Besides the greater decrease in PDS- and EPS-symptoms with probiotics vs. placebo after 8 weeks, superior probiotic effects were also found for postprandial fullness (β= -.37 [-.71;-.03]) and epigastric pain (β= -.42 [-.78;-.07]) (**Supplementary Figure 5.2 and Table 5.1**). Despite decreased early satiation, upper abdominal bloating, epigastric burning and belching within the probiotics but not placebo group after 8 weeks, no interaction effects were found (*P*>0.05) (**Supplementary Table 5.1**).

Open-label treatment

Open-label probiotics decreased PDS- but not EPS- symptoms in the original placebo group vs. baseline, whereas both PDS- (β= -.003 [-.23;.22]) and EPS- (β= .1 [-.12;.31]) symptoms remained stable in the original probiotics group (**Supplementary Table 5.2**). Moreover, early satiety, postprandial fullness, upper abdominal bloating and belching symptoms decreased with open-label probiotics in the original placebo group, with no further decrease in the original probiotics group.

Open-label probiotics decreased PAGI-SYM and increased PAGI-QOL scores in the original placebo group (**Supplementary Table 5.2**), with a further decrease in PAGI-SYM (β= -.21 [-.41;-.02]) and increase in PAGI-

QOL (β= .61 [.07;1.15]) in the original probiotics group. Also, the PDS, bloating, upper abdominal pain, lower abdominal pain and reflux domains improved with open-label probiotics in the original placebo group. A further decrease in the original probiotics group was only found for PDS (β= -.53 [-.83;-.23]) and upper abdominal pain (β= -.52 [-1.01;-.04]) domains, while the other symptoms remained stable.

Systemic immune activation

No within- or between-group differences were found for cytokines in the first 8 weeks (**Supplementary Table 5.3**). Only Treg cells decreased after 8 weeks with probiotics but not placebo with no interaction effect (**Supplementary Table 5.3**). The decrease in Th17 cells after 16 weeks of probiotics was present within the gut-homing (CD4+ α4β7+) IL17+ subset (**Supplementary Figure 5.3C**). In FD patients on-PPI, probiotics increased CD45RA+ Treg cells (β= .17 [.005;.33]) and decreased Th2 cells (β= -.08 [-.16;-.004]) (**Supplementary Figure 5.3D**), gut-homing (β= -.11 [-.18;-.03]) and CD4+ α4β7+ GM-CSF+ (β= -.82 [-1.48;-.17]) or IL17+ (β= -.06 [-.12;-.01]) subsets after 16 weeks.

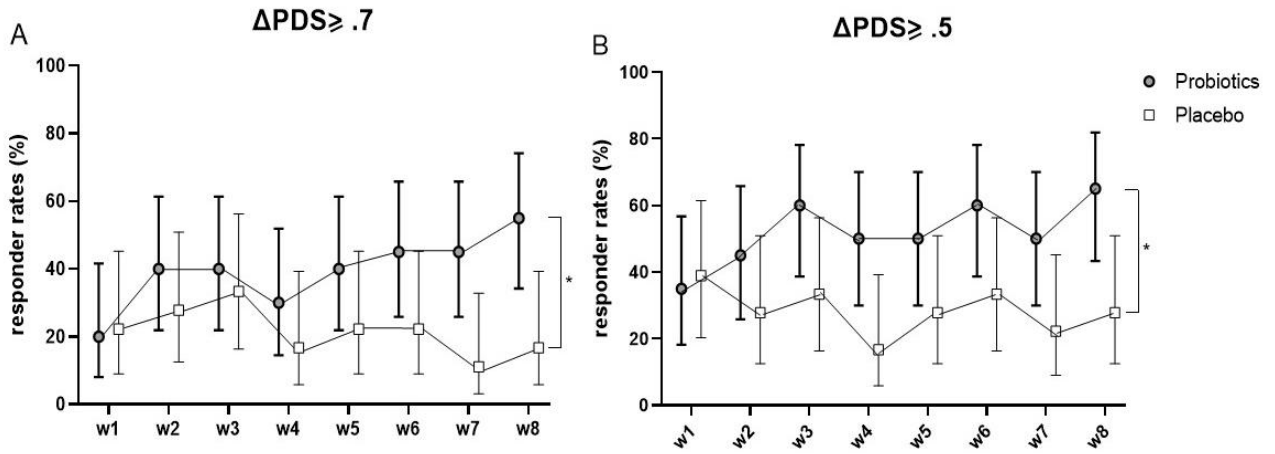
Microbiota analysis

The contribution of sporebiotics to the conditional variation in relative or quantitative genus-level community composition was studied after partialling out inter-individual variability (subject) in a partial dbRDA. Despite some clustering and an adjusted R^2 of 0.4%, treatment with spore-forming probiotics had no significant effect on relative (PERMANOVA *P*= .14) or quantitative (PERMANOVA *P*= .68) microbial community composition (**Supplementary Figure 5.4A-B**). Nevertheless, proportionally increased *Faecalibacterium, Roseburia, Weissella* and *Terrisporobacter* as well as reduced *Romboutsia, Coprobacillus* and *Intestinibacter* were found with all probiotic-exposed vs. control (baseline and 8 weeks of placebo) samples (**Supplementary Figure 5.5**). Although the relative increase in *Faecali-bacterium* was not confirmed with quantitative analyses, increased *Roseburia* (log10FC= .19) was found with reduced *Romboutsia* (log10FC= -.35) and *Propionibacterium* (log10FC= -1.17) with sporebiotics vs. control samples.

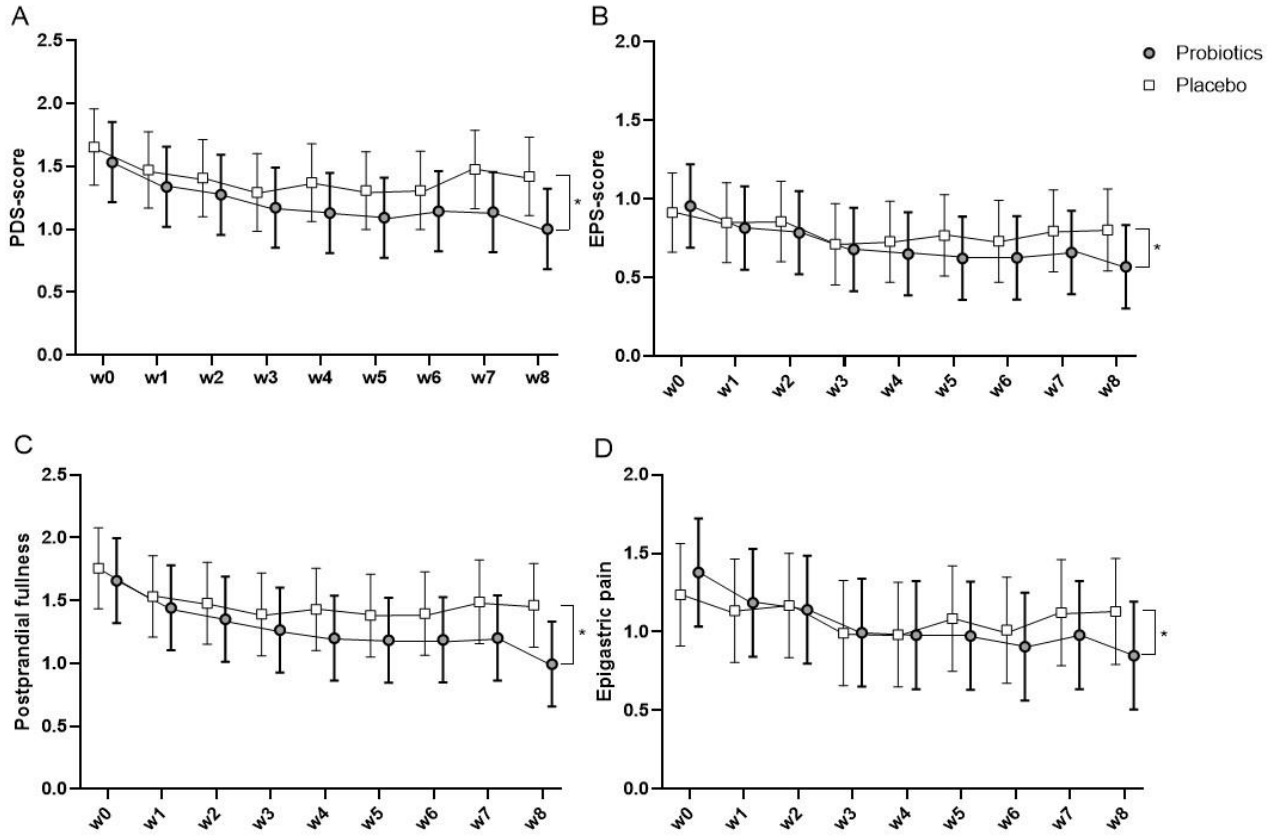
Mediation analysis

Results from the mediation analysis are shown for the placebo and probiotics group during the first 8 weeks (**Supplementary Table 5.4**) and illustrated for changes in Th17-signaling (**Supplementary Figure 5.3E-F**).

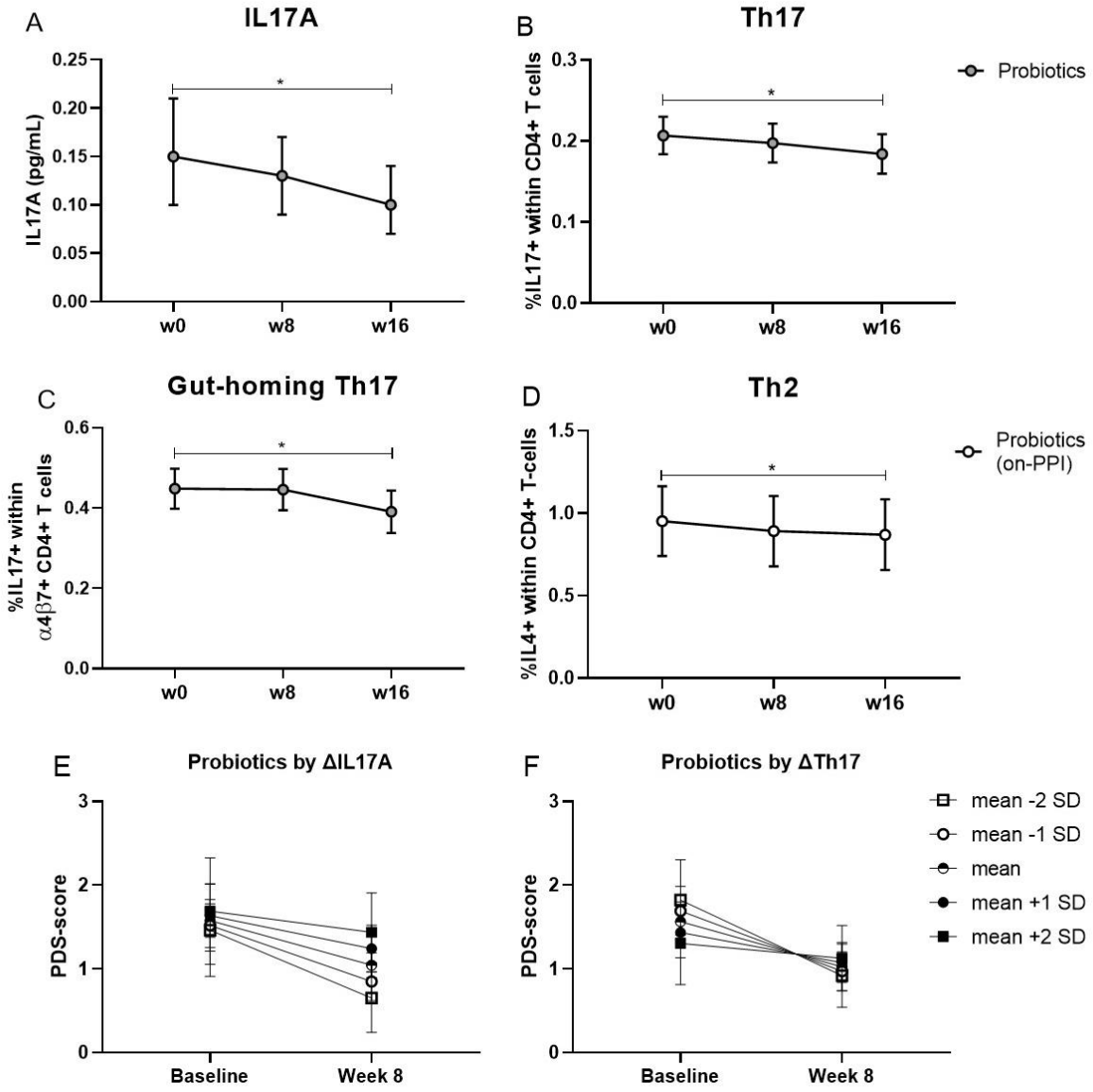
SUPPLEMENTARY FIGURES



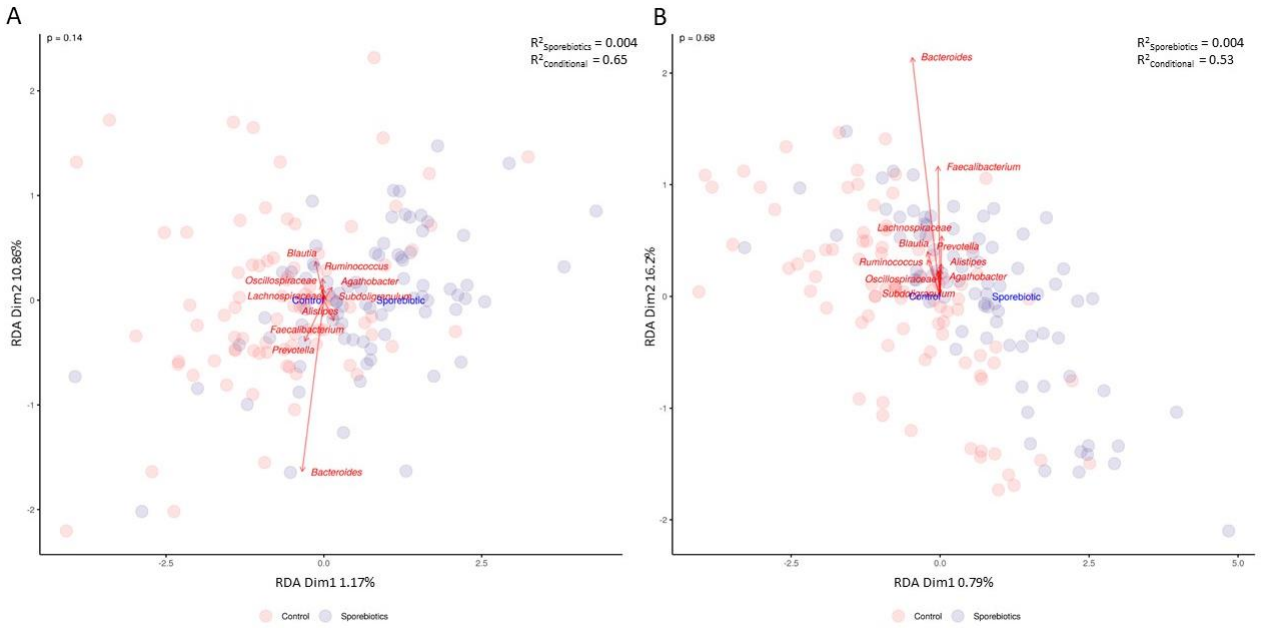
Supplementary figure 5.1: Weekly evolution of clinical (A) and minimal clinical response (B) in FD patients from the entire cohort with probiotics and placebo (per-protocol analysis). Proportions with 95% CI for FD patients with baseline PDS-scores≥ 1 and a decrease (ΔPDS) of ≥ .7 (clinical response) or ≥ .5 (minimal clinical response) at each week and per group (n= 20 probiotics, n= 18 placebo). Significance is given for the difference at week 8 (*P< .05). FD, functional dyspepsia; PDS, postprandial distress syndrome.



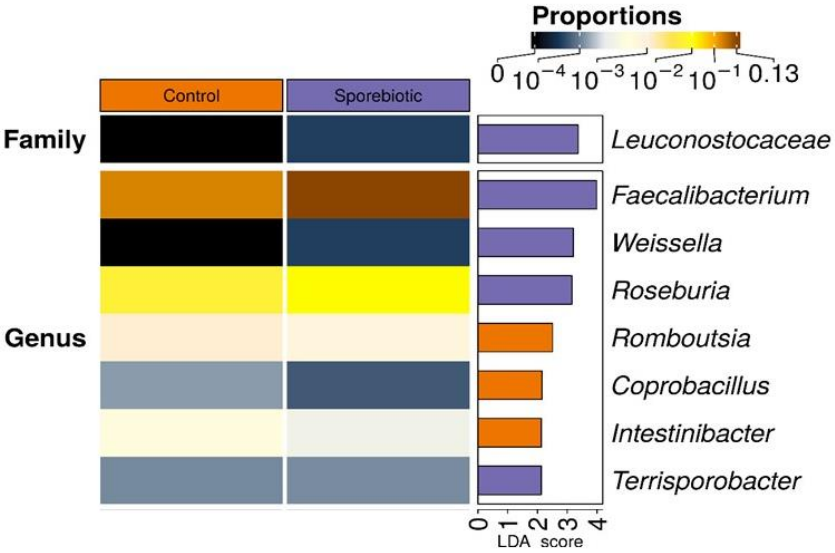
Supplementary figure 5.2: Weekly evolution of cardinal PDS- (A) or EPS-symptoms (B), postprandial fullness (C) and epigastric pain (D) in FD patients for probiotics and placebo. Clinical scores with 95% CI for FD patients at each week and per group (n= 32 probiotics, n= 36 placebo). Significance is given for the between-group difference in changes from baseline at week 8 or interaction effect (*P< .05). EPS, epigastric pain syndrome; FD, functional dyspepsia; PDS, postprandial distress syndrome.



Supplementary figure 5.3: Reduction in IL17A (A) and the frequency of Th17 cells (B), gut-homing IL17+ T cells (C) and Th2 cells (D) after 16 weeks of probiotics. Association between clinical efficacy of probiotics (PDS-symptoms) and changes (Δ) in IL17A (E) and Th17 cells (F) after 8 weeks. Systemic levels of IL17A-cytokine (V-PLEX) and frequency of IL17+ within CD4+ (Th17) and within CD4+ α4β7+ (gut-homing Th17) cells (flow-cytometry after *ex vivo* stimulation) at baseline (w0) and the end of the RCT- (w8) and OLE-phase (w16) in the probiotics group. Frequency of IL4+ within CD4+ (Th2) cells is shown for FD patients on-PPI. Significance is given for the within-group difference in changes from baseline at week 16 (*P< .05). Graphs B-D show means and 95% CI after Box-Cox transformation. Graphs E-F show means and standard error of PDS-symptoms for different levels of the standardization (mean= 0 and SD= 1) change in Th17-signaling after 8 weeks of probiotics. FD, functional dyspepsia; OLE, open-label extension; PPI, proton pump inhibitor; RCT, randomized controlled trial; SD, standard deviation.



Supplementary figure 5.4: Effect of sporebiotics (after 8 and 16 weeks of probiotics) vs. control (at baseline and after 8 weeks of placebo) samples on the conditional variation of relative (A) and quantitative (B) genus-level community composition. Partial distance based redundancy analysis (RDA) type II scaling correlation triplot with response variables (most abundant genera) in red and sporebiotics or control samples (centroid factor levels) in blue. Sporebiotics ($R^2_{Sporebiotics}$) did not significantly contribute to the conditional variation (on subject, $R^2_{Conditional}$) after partialling out inter-individual variation. Significance (p-value) is given for both relative (A) and quantitative (B) microbial community composition (PERMANOVA).



Supplementary figure 5.5: Differences in family- and genus-level relative abundances with sporebiotics (after 8 and 16 weeks of probiotics) compared to control (at baseline and after 8 weeks of placebo) samples. Mean relative abundances with sporebiotics or controls samples are shown in heatmaps with a histogram of LDA scores for significantly increased taxa at family and genus-level in either the sporebiotics (purple) or control (orange) samples. Linear discriminant analysis Effect Size (Lefse) was used to obtain LDA scores from a model with the sporebiotics vs. control class as a dependent variable and the fecal microbiota relative abundances as independent variables, as described by Segata *et al.* (2011). LDA scores indicate which taxonomic signatures among all those detected as statistically differential explain the greatest differences between sporebiotic and control samples. LDA, linear discriminant analysis.

SUPPLEMENTARY TABLES

Supplementary table 5.1: Changes in individual LPDS questions and PAGI-SYM clusters in the first 8 weeks for probiotics and placebo (full analysis set).

Group	**Probiotics (n= 32)**		**Placebo (n= 36)**	
Variables	**Estimate**	**95% CI**	**Estimate**	**95% CI**
Questions LPDS:				
Early satiety	-.35	-.56;-.13	-.15	-.37;.07
Postprandial fullness	-.66	-.9;-.43	-.29	-.54;-.05
Upper abdominal bloating	-.58	-.83;-.34	-.26	-.51:-.02
Epigastric pain	-.53	-.78;-.28	-.11	-.36;.14
Epigastric burning	-.24	-.43;-.05	-.11	-.3;.08
Nausea	-.19	-.42;.04	-.09	-.32;.14
Belching	-.34	-.53;-.15	-.19	-.38;-.01
Heartburn	-.13	-.32;.06	-.17	-.36;.02
Clusters PAGI-SYM:				
Nausea and vomiting	-.08	-.55;.4	-.16	-.62;.31
Postprandial distress	-.64	-1.04;-.25	-.62	-1;-.24
Bloating	-.98	-1.41;-.56	-.48	-.9;-.06
Upper abdominal pain	-.78	-1.23;-.34	-1	-1.44;-.56
Lower abdominal pain	-.35	-.95;.25	-.69	-1.27;-.1
Reflux	-.37	-.68;-.05	-.49	-.79;-.18

LPDS, Leuven Postprandial Distress Scale; PAGI-SYM, patient assessment of upper GI-disorders symptom severity.

Supplementary table 5.2: Changes in clinical endpoints over the entire study (16 weeks) in the original probiotics and placebo group (full analysis set).

Group	**Probiotics (n= 32)**		**Placebo (n= 36)**	
Variables	**Estimate**	**95% CI**	**Estimate**	**95% CI**
Cardinal PDS	-.53	-.76;-.31	-.36	-.58;-.14
Cardinal EPS	-.29	-.5;-.08	-.09	-.3;.12
PAGI-SYM	-.63	-.87;-.39	-.47	-.71;-.24
PAGI-QOL	1.46	.72;2.21	1.14	.42;1.87
Questions LPDS:				
Early satiety	-.42	-.71;-.13	-.34	-.62;-.06
Postprandial fullness	-.6	-.92;-.28	-.4	-.72;-.08
Upper abdominal bloating	-.54	-.87;-.21	-.46	-.78;-.13
Epigastric pain	-.39	-.7;-.08	-.11	-.41;.19
Epigastric burning	-1.17	-.46;.12	-.08	-.36;.2
Nausea	-.1	-.38;.17	-.02	-.29;.25
Belching	-.41	-.72;-.09	-.37	-.68;-.07
Heartburn	-.31	-.59;-.03	-.11	-.38;.16
Clusters PAGI-SYM:				
Nausea and vomiting	-.34	-.76;.07	-.29	-.68;.11
Postprandial distress	-1.17	-1.59:-.74	-.73	-1.14;-.32
Bloating	-1.07	-1.62;-.51	-.8	-1.33;-.26
Upper abdominal pain	-1.31	-1.79;-.82	-.74	-1.2;-.27
Lower abdominal pain	-.22	-.72;.28	-.7	-1.18;-.22
Reflux	-.57	-.95;-.19	-.39	-.75;-.03

EPS, epigastric pain syndrome; PAGI-SYM, patient assessment of upper GI-disorders symptom severity index; PAGI-QOL, patient assessment of upper GI-disorders quality of life; PDS, postprandial distress syndrome.

Supplementary table 5.3: Changes in systemic cytokines and peripheral blood mononuclear cells in the first 8 weeks for probiotics and placebo (full analysis set).

Group	**Probiotics (n= 32)**		**Placebo (n= 36)**	
Variables	**Estimate**	**95% CI**	**Estimate**	**95% CI**
Cytokines (pg/mL)				
IFNγ	.04	-.01;.08	.02	-.02;.07
IL2	-.01	-.04;.01	-.02	-.04;.01
IL4	.0001	-.003;.003	-.003	-.01;.0003
IL6	-.002	-.04;.04	-.01	-.05;.03
IL8	-.01	-.12;.1	.09	-.02;.2
IL10	.005	-.004;.01	-.002	-.01;.01
IL12p70	-.002	-.02;.01	.003	-.01;.02
IL13	-.05	-.12;.02	.05	-.02;.12
TNFα	.003	-.01;.02	-.001	-.02;.01
IL5	.02	-.08;.11	.02	-.05;.08
IL17A	-.03	-.09;.04	.03	-.04;.1
CD4 T cells (%):				
Tregs	-.08	-.15;-.01	-.04	-.11;.03
Treg CD45RA+	.09	-.06;.24	.03	-.12;.19
Th GM-CSF+	-.16	-.56;.24	-.09	-.48;.3
Th2	-.01	-.08;.05	-.01	-.07;.06
Th17	-.02	-.04;.01	-.03	-.05;.002
Th1	-.07	-.38;.23	.13	-.18;.44
α4β7+ CCR9+	.005	-.05;.06	-.04	-.09;.01
Gut-homing (%):				
GMCSF+	.08	-.48;.64	.05	-.52;.61
IL4+	-.0005	-.09;.08	-.01	-.1;.07
IL17+	-.001	-.05;.05	-.03	-.08;.02
IFNg+	.11	-1.22;1.44	-.07	-1.41;1.28

Supplementary table 5.4: Changes in PDS-symptoms with placebo and probiotics for different levels of the standardization change (Δ) in selected biological endpoints (RCT-phase).

Δ	**IL17A**	**Th17 cells**	***Faecalibacterium***
-2 SD			
Placebo	-.44 [-.99;.11]	-.21 [-.71;.29]	-.03 [-.6;.54]
Probiotics	-.81 [-1.59;-.03]	-.9 [-1.59;-.21]	-.12 [-.94;.7]
-1 SD			
Placebo	-.34 [-.72;.04]	-.17 [-.51;.17]	-.12 [-.5;.26]
Probiotics	-.67 [-1.11;-.24]	-.72 [-1.14;-.3]	-.34 [-.81;.13]
mean			
Placebo	-.24 [-.52;.04]	-.13 [-.38;.13]	-.21 [-.49;.07]
Probiotics	-.53 [-81;-.25]	-.54 [-.82;-.26]	-.56 [-.83;-.29]
+1 SD			
Placebo	-.14 [-.46;.19]	-.08 [-.41;.25]	-.3 [-.66;.06]
Probiotics	-.39 [-.93;.14]	-.36 [-.8;.08]	-.78 [-1.27;-.29]
+2 SD			
Placebo	-.04 [-.51;.44]	-.04 [-.53;.45]	-.39 [-.94;.15]
Probiotics	-.25 [-1.15;.65]	-.18 [-.89;.53]	-1 [-1.85;-.16]

PDS, postprandial distress syndrome; RCT, randomized-controlled trial; SD, standard deviation.

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CHAPTER 6
GENERAL DISCUSSION

6 GENERAL DISCUSSION

FD is a common disorder with unknown pathophysiology, hampering a conclusive diagnosis and development of effective drugs. In this PhD-project, we studied duodenal and systemic factors in FD. We focused on the duodenal lumen and mucosa, as luminal changes have been proposed to initiate or maintain mucosal alterations. We also separated the duodenal luminal and mucosa-associated microbiota composition. Effects of first-line therapy or PPI were prospectively studied in FD patients compared to controls and a second cohort of PPI-refractory FD patients after PPI-withdrawal. Finally, the clinical efficacy and underlying immune and microbial mechanisms of spore-forming probiotics were studied in FD patients on- or off-PPI, which is relevant regarding the potential cumulative effect of PPI and spore-forming probiotics on luminal and systemic changes in FD.

6.1 Duodenum is key in FD

6.1.1 Evidence for a 'leaky gut'

Baseline comparisons between FD patients and healthy controls confirmed the presence of increased duodenal mucosal permeability (**Chapter 3**). Although both transepithelial electrical resistance (TEER) and Fd4-passage measure paracellular passage, the former reflects ion transport through the pore pathway, while the latter also reflects the leak pathway.[1,2] The size- and charge-selective pore pathway enables high-capacity transport of solutes with a radius up to 4 Å and is predominant near the tips of the villi, whereas the low-capacity leak pathway is mostly found in the crypts, where ions and molecules with a radius of ± 20 Å can permeate, regardless of charge.[1,3] Regulators of the pore pathway include the CLDN family, while OCLN and ZO proteins are mainly involved in the leak pathway. Tight-junctions are supported by adhesive forces from adherens junctions and desmosomes, of which alterations with dilated intercellular spaces have also been described in FD patients.[4]

In our FD cohort, no changes in TEER were observed, which was similar to a recent study showing also altered epithelial secretion in FD.[5] Indeed, the applied current for measuring the generated potential and thus resistance is carried by the common ions Na^+ and Cl^-, of which the altered duodenal secretion and absorption may influence TEER.[1,5] Moreover, we previously found no changes in the CLDN family.[6] This is in contrast with studies showing decreased CLDN1,[7,8] although the expected apical staining was absent, and increased CLDN3.[9] Interestingly, CLDN3-expression may be affected by duodenal acid since duodenal acid perfusion decreased CLDN3-expression in healthy subjects.[10] We found a higher fasting and fed duodenal pH in FD patients vs. controls, similar to a previous study.[11] In contrast, using continuous and catheter-based methods, increased acid exposure was found during the daytime and late post-prandial phase but with no correlation to symptoms.[12] Therefore, duodenal pH-fluctuations are likely and their effect on the duodenal micro-environment are still unclear.

Besides duodenal acid, bile may influence permeability. Although decreased duodenal BS were previously reported in the 45min preceding a liquid meal,[11] we deliberately included only 1 fasted sample after 60min as contamination with gastric fluid may influence measurements early after positioning the nasoduodenal tube. As the aspirated gastric fluid from FD patients commonly contained bile due to reflux from the small intestine, this should be differentiated from the true duodenal content.[13] Despite the potential cytotoxic effects of hydrophobic secondary bile acids in human colonic biopsies and the murine small intestine,[14,15] co-administration of conjugated BS is known to diminish the cytotoxic effects of bile acids.[16] We found no correlation between duodenal (conjugated) BS and permeability, as these direct but non-physiological epithelial effects are only expected for (unconjugated) bile acids.[17] In our analysis on this subgroup of FD patients, gastric emptying of solids was measured and correlated with duodenal BS, suggesting a therapeutic role for bile signaling, which should be studied in relation to the microbiome.[17]

In addition to paracellular permeability, increased transcellular passage of bacteria was described in the colonic epithelium of IBS patients.[18] While duodenal transcellular bacterial translocation was not increased,[19] the importance of the paracellular leak pathway is supported by decreased expression of OCLN and ZO-1 in FD patients.[6,20] This would suggest an increased influx of larger molecules or antigens into the duodenal mucosa, with immune activation as discussed below. We studied plasma LBP as a marker of bacterial translocation across an impaired barrier and found no between-group differences. Although passage of luminal microbes is unlikely in the absence of cellular changes, these were recently described in the third portion of the duodenum using CLE.[7] However, interpretation of cell extrusions (epithelial gaps) caused by inflammatory cell death (pyroptosis) is hampered by the lack of correlation with *ex vivo* permeability.[7] In contrast, a gradual reduction of *in vivo* permeability, measured with impedance, was found from the duodenum to jejunum in FD patients and controls, probably due to lower exposure to luminal content.[21] Challenges remain in the interpretation and reproducibility of experimental methods, including *ex-vivo* studies which measure only the integrity of the epithelial layer and not the superficial mucus layer or enteric nervous system, which also affect mucosal barrier function. Recently, microRNA (miRNA) in exosomes (gastric fluid) or targeting genes associated with ionic transport (duodenal mucosa) were proposed as novel markers but not yet validated in FD.[5,22]

6.1.2 Eosinophil-mast cell axis

Baseline duodenal eosinophil and mast cell infiltration was confirmed in our FD cohort (**Chapter 3**). Although increased antigen penetration via a defective barrier may result in mucosal inflammation,[23] duodenal eosinophils were not correlated with *ex vivo* permeability in a previous study.[24] However, underdetection of eosinophils is possible using MBP-based methods,[25] and the correlation between eosinophils and deregulated phosphorylation of OCLN confirmed a link between mucosal inflammation and permeability in FD patients.[6] Interestingly, both the release of MBP from eosinophils and tryptase from mast cells decreased the expression of OCLN and ZO-1 with increased colonic permeability *in vitro*.[26,27] Tryptase cleaves and activates protease-activated receptor 2 (PAR2) on colonocytes, which was similar in the duodenum of FD vs. controls.[20] In this Japanese study, *in vivo* permeability correlated with decreased ZO-1 and increased IL-1β, of which the potential role is discussed below.[20] Nevertheless, mast cell tryptase may play a role in FD pathogenesis,[24] as this was upregulated after duodenal acid infusion and mast cell activation is involved in stress-induced permeability.[10,28] While a detailed analysis of eosinophil- and mast cell-mediators is planned in the near future, associations between mucosal barrier and immune dysfunction do not prove causation as discussed in **section 6.2**.

In my studies, subjects with atopic and autoimmune diseases were excluded as the presence of duodenal eosinophils may partially explain the observed link between FD and allergy.[29,30] Nevertheless, no mucosal Th2-signal has yet been reported, and eosinophils were also identified as crucial effectors of the IL-23-GM-CSF axis, as release of IL-23 by antigen-presenting cells resulted in a pro-inflammatory Th17-phenotype with recruitment of eosinophils via GM-CSF.[31] While a vicious cycle may follow with increased IL-17A production through IL-1β release by eosinophils,[32] homeostatic effects of eosinophil-derived IL-1β with even negative feedback effects on Th17 cells have also been reported in the murine small intestine.[33,34] While this complex interplay has not yet been studied in FD patients, the production of IL-1β by cultured PBMC of FD patients was indeed suggestive of a Th17-response,[35] possibly explaining the overlap with auto-immune diseases.[36] Moreover, increased proportions of effector memory Th2 and Th17 cells were recently found in the duodenum, with the latter also present in the blood of FD patients.[37] The dual adaptive immune signature therefore suggests that multiple triggers may cause FD symptoms.[23,37] As production of IL-1β and intestinal Th17-differentiation as well as eosinophil infiltration depended on the small intestinal microbiome,[25,38] this points to a potential therapeutic role of modulating the gut microbiome in FD, as discussed in **section 6.3**.

Importantly, we showed that symptoms correlated with duodenal eosinophils but no other luminal, mucosal or systemic factors in FD. While the relation with symptoms may be explained by altered duodeno-gastric reflexes due to submucosal neuronal changes with inflammation,[39,40] this has not yet been confirmed. Additional evidence for eosinophil-neuronal interactions from Asia showed increased expression of duodenal glial cell line-derived neurotrophic factor (GDNF), which may play a protective role, as well as nerve fiber density and sprouting in FD patients.[4,41] We and others have also proposed that duodenal low-grade inflammation may lead to systemic immune activation, which was linked with gastric emptying and symptoms.[35] In addition, duodenal hypersensitivity is present in FD.[12] Although duodenal acid induced a functional barrier defect which was independent of mast cell activation in human and mice studies,[10] a recent study showed a cascade of acid-induced neuronal events leading to MC activation and TRPV1 and TRPV4 overexpression, which were at least partly attributable to an impaired release of an endogenous amide in FD patients.[42] Therefore, these and other potential anti-inflammatory and analgesic compounds should be studied, also in relation to TRPV-signaling as in IBS.[43]

Finally, the potential role of food allergens in FD is still unclear. Interestingly, duodenal mucosal food challenge followed by CLE showed positive reactions in 70% of IBS patients who tested negative for classical food allergies but had a 4-fold increased prevalence of personal or family history of atopy.[44] Changes in permeability with increased pore-forming CLDN2- and decreased OCLN-expression, as well as increased intra-epithelial lymphocytes (IEL) and eosinophil degranulation were found, suggestive of an atypical or non-IgE mediated food allergy.[44] Although no changes were found in tryptase, immune profiling was limited and with a short time-span of sampling after challenge.[44] In our study, IEL were similar between and within-groups and recently a role for local IgE-antibodies was studied in IBS with similar numbers of mast cells after mucosal food injection in the recto-sigmoid but a closer localization to nerves and IgE IF-intensity, correlating with abdominal pain.[43] As opposed to colonic mast cells, the role of duodenal eosinophils deserves further study in relation to antigens or other triggers and future studies should include markers of activation and the increasingly recognized heterogeneity of both eosinophils and Th2 cells, which would only be captured using advanced transcriptional profiling.

6.2 Differential effects of PPI

6.2.1 More than acid-suppression

We here provide the first prospective evidence for barrier-protective and anti-eosinophil effects of routine PPI-therapy in FD ('FD-starters'), besides the known acid-suppressive effects (**Chapter 3**). Duodenal pH increased in FD-starters and controls but the magnitude of the pH-increase was not associated with symptom reduction in FD patients. While only a partial improvement of symptoms and duodenal inflammation was found in FD-starters after PPI, the increased baseline permeability was fully reversed but not associated with clinical efficacy of PPI in FD. As noted above, fluctuations in pH and limitations of permeability measures do not exclude a role of luminal effects of PPI, similar to eosinophilic esophagitis (EoE). Historically, EoE was distinguished from PPI-responsive esophageal eosinophilia (REE) by a presumed lack of response to PPI in the former.[45] Besides the acid-suppressive effects, barrier-protective effects of PPI were only confirmed in PPI-REE and not EoE patients,[46] suggesting that the reduction in acid reflux allowed restoration of barrier function with a reduction in inflammation. However, decreased eotaxin-3 and Th2-cytokine expression were also found in PPI-REE, similar to steroid-responsive EoE.[47] Thus, PPI-REE is now considered PPI-responsive EoE as a subset of patients respond to PPI, often for proximal eosinophilia but with persisting distal esophagitis.[47] Indeed, beneficial anti-eosinophil or mucosal effects of PPI may be counteracted by luminal changes as observed for esophageal cells *in vitro*,[48] which could also occur in the duodenum of FD patients after PPI-therapy, especially after long-term use as further discussed below.

Besides the correlations between eosinophils and symptoms, the association of clinical efficacy with changes in eosinophils provide the first prospective data for anti-eosinophil effects of PPI as a potential therapeutic mechanism in FD. In EoE, anti-inflammatory effects of PPI include anti-oxidant properties by binding vacuolar H^+-adenosine triphosphatase (ATPase) and proton receptor GPR65 and inhibition of vascular cell adhesion molecule-1 (VCAM-1) expression, recognized by eosinophil ligands.[49] Inhibition of eotaxin-3 expression through signal transducer and activator of transcription 6 (STAT6) was observed with omeprazole in esophageal cells,[50] but also non-gastric H^+-K^+-ATPases in esophageal, bronchial and nasal epithelial cells.[51,52] Although similar mechanisms may occur in the duodenum of FD patients on-PPI, the partial improvement in symptoms and eosinophilia suggest that higher doses of PPI or more powerful anti-inflammatory therapies may be needed. Recently, a pilot RCT of budesonide (topical steroid) in liquid form showed that changes in duodenal eosinophils were correlated with clinical efficacy in FD, but with no significant reduction compared to placebo.[53] Specific treatments including anti–Siglec-8 antibodies have been trialed in eosinophilic duodenitis, with a decrease in both mucosal eosinophils and mast cells.[54] As not only symptoms but also mast cells decreased in FD-starters with greater than average reductions in duodenal eosinophils after PPI, our findings support the concept of the eosinophil-mast cell axis.[23] In addition, PPI-induced changes in systemic immune activation (high-sensitivity CRP) were present but less pronounced in our FD cohort.

Although the increased awakening cortisol in FD was also reduced after PPI, it was not associated with clinical efficacy of PPI. However, an association was found between changes in eosinophils and cortisol in FD-starters. Interestingly, corticotropin-releasing hormone (CRH) produced by eosinophils acted as a mediator with mast cell activation and increased small intestinal permeability in stressed mice.[55] Moreover, CRH-receptors were present on mucosal eosinophils and mast cells,[56,57] and we previously showed that small intestinal permeability was increased by administration of CRH and blocked by pre-treatment with a mast cell stabilizer in healthy students.[28] Despite the reduction in cortisol and Fd4-passage in FD-starters after PPI, no association was found between both factors. Nevertheless, the eosinophil-mast cell axis is likely involved in the regulation of small intestinal permeability, also during stress with potential effects on the patho-physiology of FD and IBS.[58,59] Although a preclinical study reported that PPI enhanced the stress-induced increase in small intestinal permeability via dysbiosis,[60] these findings cannot be translated to patients as the model lacked similar alterations as FD patients.[61] Changes in gut microbiota on-PPI are however important factors to take into account, also considering the potential exacerbation of NSAID-related small-bowel injury[62,63] and the risk of microscopic colitis,[64] although there is a lack of prospective studies.

6.2.2 Microbiome and dysbiosis

The critical interplay between commensal bacteria and mucosal eosinophils was recently illustrated as changes in small intestinal eosinophil frequency and function in germ-free (GF) mice.[25] We found a lower abundance of duodenal luminal *Neisseria* and *Porphyromonas* and diversity in FD patients vs. controls (**Chapter 4**), which inversely correlated with symptoms and duodenal eosinophils. Similar findings have been reported in patients with functional GI disorders using duodenal aspirates or biopsies.[65,66] While reduced duodenal *Porphyromonas* was possibly linked to increased intestinal permeability in chronic liver disease patients,[67] we found no such correlations. Microbiota-dependent colitis in IL10-deficient mice was driven via increased small intestinal permeability, which could be blocked by a zonulin peptide inhibitor in the small intestine,[68] but eosinophils may also directly affect mucus-resident bacteria and mucosal barrier function.[69] In the absence of approved and effective treatments targeting the mucosal barrier without effects on immune cells, such as PPI, the causality and potential directionality of luminal and mucosal changes in FD remains elusive. Also in this context, an increased mucosal permeability was previously observed in healthy subjects on-PPI and proposed to cause an influx of luminal peptides.[70] However, we found no association between changes in duodenal permeability and eosinophilia in controls after PPI. Moreover, no association

was found between PPI-related changes in pH and either permeability or immune activation. In contrast, hyperpermeability and eosinophilia were associated with increased luminal bile salts in controls after PPI, which differed from FD patients and may indeed be related to microbial effects of PPI.

While PPI-induced changes in the microbiome were mainly studied in stools and the stomach, including an increased abundance of *Streptococcus,* there is a lack of prospective studies and especially for the duodenum, which is highly relevant for FD.[23] Similar to our results on the duodenal microbiome, no major shifts were observed in the fecal microbiome after high dose of PPI for 4 weeks.[71] Nevertheless, changes in specific taxa, including increased *Streptococcus,* and genes involved in bacterial invasion and mucosal barrier function were found.[71] While fecal primary and secondary bile acids were similar after PPI,[71] the increased secondary bile salts in our study were associated with the consistently increased *Streptococcus* (and not vice versa), suggesting microbial effects on the duodenal BS-pool. Moreover, eosinophil infiltration in controls was associated with greater than average increases in luminal *Streptococcus* after PPI, which is in line with the suggested role of gut dysbiosis in allergic Th2-reactions in mechanistic and population-based studies.[72,73] Interestingly, we observed an increased expression of epithelium-derived IL-33 in controls and FD patients after PPI,[74] which was the main driver of microbiota-dependent Th2-inflammation and eosinophilia in preclinical studies.[75] However, both symptom- and eosinophil-reducing effects of PPI-therapy were not associated with either microbial changes or IL-33-expression in our cohort,[74] pointing to stronger anti-eosinophil effects of routine or short-term PPI-therapy in FD patients.

Indeed, one should take the duration of PPI-therapy into account, as we found higher duodenal eosinophil counts in FD patients after long- vs. short-term PPI. While this suggests that beneficial anti-eosinophil effects of PPI may be attenuated over time by counteracting luminal changes, similar to the distal esophagus in EoE,[48] this requires confirmation in a prospective study with a longer duration of follow-up. In addition, increased abundance of luminal *Neisseria* was observed in long-term users with persistently higher *Streptococcus*. The consistent signal of increased *Streptococcus*-abundance on-PPI was also found in the stomach of FD patients and linked to persisting symptoms.[76] Therefore, similar interactions as observed in our controls may explain the (re-)occurrence of duodenal eosinophilia in FD patients with long-term PPI, which were only partially reversed after PPI-withdrawal for 2 months. These findings have important implications for the interpretation of studies with shorter washout periods of PPI, especially in relation to the microbiome. Moreover, baseline symptoms, duodenal eosinophil or mast cell infiltration and permeability were similar between both PPI-naïve (off-PPI) and -refractory FD patients (on-PPI), illustrating why previous cross-sectional studies failed to detect differences in duodenal immune activation and permeability in relation to PPI. Whether this could be prevented with microbiota-directed treatments is unclear, as discussed in the next section.

6.3 Probiotics: hype or hope?

6.3.1 Clinical efficacy

We showed that *Bacillus coagulans* MY01 and *subtilis* MY02 sporebiotics were effective and safe in FD patients in a pilot randomized, double-blind, placebo-controlled trial (**Chapter 5**). Indeed, only one previous study randomized patients with placebo yoghurt as control in uninvestigated dyspepsia.[77] Despite similar overall efficacy, some improvement was found for PDS- but not EPS-like symptoms with LG21.[77] Although patients were off-PPI or other therapies, the authors concluded that add-on therapy to PPI or even prokinetics could be useful.[77] However, PDS-like symptoms improved significantly more with different *Lactobacilli* strains alone vs. the combination of probiotics with PPI, followed by probiotics with prokinetics or antacids in another open-label study.[78] Besides the lack of prior investigations and blinding, the use of validated questionnaires as advised by FDA/EMA is also rare for probiotic trials. We hypothesized that symptoms, measured with a validated daily diary, would be improved by spore-forming probiotics, as these

gastric-acid resistant endospores may outperform traditional probiotic supplements because of improved survival in the small intestine. Importantly, PDS (primary outcome), EPS and key individual symptoms significantly decreased with sporebiotics vs. placebo. Moreover, we extended the clinical efficacy during an open-label phase (total 16 weeks), with symptom-improvement in the original placebo-group. Although subanalyses of spore-forming probiotics as add-on to PPI or monotherapy require confirmation in larger studies, our findings are a first step to establish the position of these probiotics, which were not included in a recent European consensus,[79] in the management of FD patients, also considering their safety and beneficial immune and microbial effects (see below).

Besides a comprehensive clinical evaluation, we studied the potential mechanisms. Previous *in vitro* studies confirmed barrier-protective and anti-inflammatory effects of *B. coagulans* and *subtilis*.[80] Also, barrier-protective effects of probiotics for NSAID-induced enteropathy were mainly found in the duodenum, which is a key region in FD (see **section 6.1**).[81] We found no differences in LBP as a marker of bacterial translocation, which was similar to controls (**Chapter 3**). Similarly, a combination of pre- and probiotics (synbiotics) showed stable LBP but decreased zonulin levels in long-term PPI-users.[82] However, zonulin was not increased in functional GI patients, which can be related to technical limitations.[83] Based on promising animal data of probiotic strains for stress-induced intestinal hyperpermeability, we have also advocated to study barrier-protective effects in humans.[84] However, no effect of *L. plantarum* strains was found on *in vivo* small intestinal hyperpermeability after NSAID.[85] Similarly, we found no protective effect of *L. rhamnosus* CNCM I-3690 on *in vivo* stress-related small intestinal hyper-permeability.[86] Nevertheless, transcriptomic changes in repair processes were noted in the duodenal mucosa and urinary (*in vivo*) markers of permeability may not be sensitive enough,[85] as observed for different combinations of pro- and prebiotics in functional bowel disorders.[87] Despite the absence of significant clinical effects,[87] the combination of *Bacillus*-strains was effective for abdominal discomfort, including uninvestigated dyspeptic symptoms in another study.[88] Therefore, our study is the first to demonstrate clinical efficacy of (spore-forming) probiotics in a randomized and placebo-controlled trial in FD patients diagnosed according to Rome IV criteria.

6.3.2 Microbiota-immune interactions

Importantly, changes in Th17-signaling, including peripheral Th17 cells and IL17A, were associated with clinical efficacy of sporebiotics in FD. Although changes in T cells were more evident after longer-term probiotic treatment, effects on Tregs were found after 8 weeks. Decreased Th17 cells were present within the gut-homing IL17+ subset and additional decreases in Th2, gut-homing and GM-CSF+ T cells as well as increased CD45RA+ Treg cells were found with probiotics in FD patients on-PPI. As both Th17 and Treg cells are regulated by gut commensals,[38,89] the immune effects of sporebiotics may be more pronounced on-PPI due to microbiome-related side effects. Despite the lack of anti-inflammatory effects of the synbiotics mentioned above, the intervention period may have been too short to observe changes in microbial alpha- and beta-diversity.[82] This is also illustrated by fecal microbiota alterations with increased *Faecalibacterium* and *Roseburia* after high adherence to a 1-year Mediterranean diet, which correlated with CRP and IL17.[90] Although both commensal bacteria have anti-inflammatory activity with decreased Th17-signaling,[91,92] only increased *Faecalibacterium* was associated with probiotic efficacy in our study. Both increased *F. prausnitzii* and antigen-stimulated production of IL10 by PBMC were also previously found after intake of *B. coagulans*,[93] confirming potential microbiota-immune interactions of spore-forming probiotics in humans.

Finally, beneficial probiotic effects included a reduction in small intestinal bacterial overgrowth (SIBO), measured with breath tests in FD patients on-PPI after 8 weeks. Due to the limited availability of the substrate and levels of radiation with ^{14}C-glycocholic acid, no comparison was possible with other studies using lactulose or glucose breath tests, which however showed important heterogeneity.[94] While only few have reported PPI-effects on SIBO prevalence in FD, a fourfold increased prevalence was found for methane (CH_4)

but not hydrogen (H_2) positive SIBO in PPI-users, confirming the improved detection of non-H_2 producing bacteria.[95] Thus, our baseline results using glycocholic acid more closely resembled H_2- and/or CH_4-positive SIBO and even duodenal aspirate cultures in FD.[96] Indeed, increased bacterial cell counts in gastric fluid and duodenal brushings were found with PPI, even more so after long-term (> 1y) intake, which were decreased by a combination of *Lactobacillus* strains.[97,98] Although another study confirmed a reduction of highly prevalent intestinal genera in the gastric fluid with LG21 in FD patients off-PPI,[13] we did not perform breath tests off-PPI as the role of SIBO and beneficial probiotic effects are more likely on-PPI. Therefore, add-on of sporebiotics to PPI may be indicated in case PPI-withdrawal is not feasible, as this could reverse inadvertent luminal effects, which may cause duodenal alterations in long-term users and with persistent changes after PPI-withdrawal for 8 weeks (**Chapters 3 & 4**). The main findings are graphically summarized in the **Figure 6.1**.

6.4 Future perspectives

Emerging data, including the results from my doctoral thesis, increasingly point towards the duodenum as a key integrator in FD. While baseline correlations and associations between PPI-induced changes in duodenal eosinophils and symptoms strengthen the link, more selective treatments are needed to determine their potential causal role. As most available treatments for FD have not significantly evolved in the last 20 years, this would open new perspectives and hopefully improve outcomes of this difficult to treat condition. Indeed, targeting gastric and central dysfunction has not resulted in efficacious and/or safe treatment options, also due to the fact that these changes may be secondary to duodenal alterations. However, the exact mechanisms through which duodenal immune activation induces neuronal hyperexcitability and symptoms is incompletely understood, especially in relation to potential eosinophil-reducing therapies, including PPI.

Differential effects of PPI also point to the role of luminal changes, including the microbiome, in determining mucosal inflammation, which may be of critical importance after long-term use of PPI. While this opens perspectives for microbiota-directed treatments in FD patients with progressive or refractory symptoms on first-line therapy, the effects of probiotics on duodenal alterations in PPI-naïve patients deserve special attention. Besides the potential role for duodenal eosinophils in the diagnostic work-up and treatment algorithm, systemic immune activation with gut-homing and/or Th17 cells may also become a non-invasive test for FD diagnosis and follow-up. Future studies should include immune- and microbiota-analyses in FD patients to discover new potential biomarkers and therapeutic targets, allowing the identification of novel subgroups and development of targeted treatments, which is an unmet medical need in daily clinical practice.

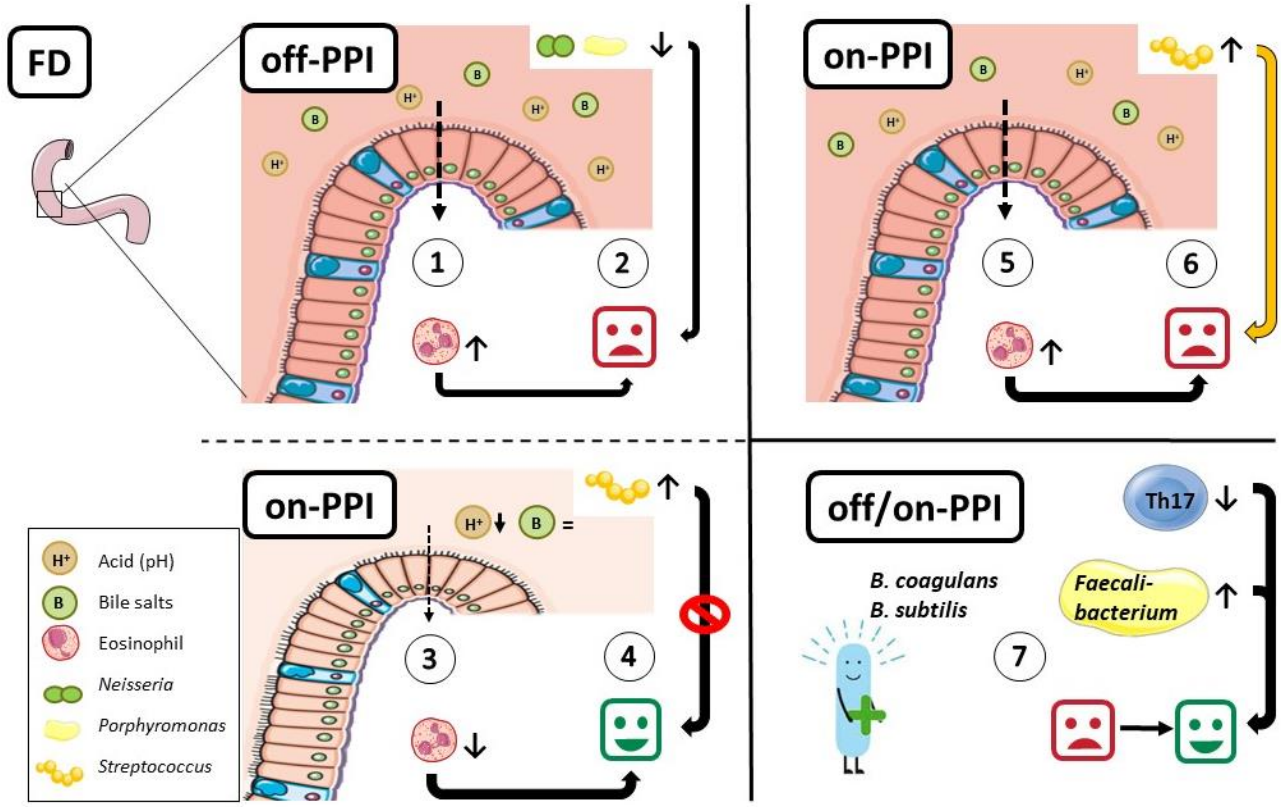

Figure 6.1: Graphical summary of the main findings. In PPI-naïve FD patients (off-PPI), increased duodenal mucosal permeability (dashed arrow) and eosinophil infiltration were observed compared to controls, but only eosinophilia correlated with symptoms (1). Moreover, the lower abundances of luminal *Neisseria* and *Porphyromonas* correlated with symptoms (2). Acid-suppressive, barrier-protective and eosinophil-reducing effects of short-term PPI were observed (on-PPI), but only the latter was associated with clinical efficacy (3). In contrast, microbial changes including increased luminal *Streptococcus* were not associated with PPI-effects (4). In FD patients on long-term acid suppression, duodenal mucosal hyperpermeability and eosinophilia were also observed (5). Interestingly, the persistently higher abundance of luminal *Streptococcus* suggested a role for inadvertent microbiota changes, similar to our observations of luminal and mucosal PPI-effects in healthy controls (6). Finally, treatment with spore-forming probiotics (combination of *B. coagulans* and *subtilis*) was clinically effective in FD patients (off- or on-PPI) and associated with changes in immune activation (Th17-signaling) and microbiota (*Faecalibactrium*) (7). FD, functional dyspepsia; PPI, proton pump inhibitor.

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CHAPTER 7
SUMMARY

7 SUMMARY

7.1 Summary

Patients with functional dyspepsia (FD) complain of epigastric symptoms with no identifiable cause. Although current first-line therapy with acid suppression (proton pump inhibitors or PPI) is effective for many patients, the mechanism of action remains unknown. In this doctoral thesis, a prospective study was set up in which 28 FD patients ('starters') took a daily PPI for 4 weeks. Through biopsies of the duodenum, collected during endoscopy, the number of immune cells (eosinophils and mast cells) were counted and mucosal permeability was determined before and after acid suppression.

Treatment with PPI resulted in a reduction of symptoms, decreased inflammation and a normalization of mucosal hyperpermeability. The decrease in the number of duodenal eosinophils was strongly associated with symptom-reduction, which was now demonstrated for the first time. The presence of inflammation can thus be seen as a new biomarker, which may improve diagnosis. At the same time these results call for the development of targeted therapies, as the overall efficacy of PPI is still limited and long-term acid suppression may lead to adverse events.

These potentially inadvertent effects were found in a parallel prospective study of 30 healthy volunteers, with increased immune cell infiltration and mucosal permeability after intake of the same PPI for 4 weeks. Moreover, similar changes were found in a second group of 19 FD patients ('stoppers'), which were followed after withdrawal of long-term acid suppression. In order to study a potential association with the microbiota, mucosal brushing of the duodenum was performed besides the routine biopsies during endoscopy to determine the bacterial community composition.

Although a link between specific bacteria and symptoms or eosinophils was found, bacterial changes in the group of 'starters' were not associated with beneficial effects of (short term) PPI. A role for the microbiome was confirmed in healthy controls, with an increased abundance of *Streptococcus,* which was associated with inadvertent effects of PPI. The increased *Streptococcus* abundance was also found and persisted after withdrawal of PPI for 8 weeks in the group of 'stoppers'. Thus, a disturbed bacterial composition of the duodenum is a possible side effect of long-term use of PPI.

Finally, a novel treatment with probiotics was investigated in this doctoral thesis. A pilot randomized, double-blind and placebo-controlled trial was conducted with the spore-forming *Bacillus coagulans* MY01 and *Bacillus subtilis* MY02 in 68 FD patients with or without concomitant intake of PPI. The efficacy and safety of these probiotics was demonstrated, with a potential mechanism via immunological and microbial changes. A reduction in bacterial overgrowth, which is a frequently investigated side effect of long-term PPI use, was also shown in FD patients with concomitant intake of PPI.

The novel findings of this doctoral thesis will now be used to investigate targeted therapies for the underlying duodenal inflammation on the one hand, and to confirm the positive findings with spore-forming probiotics on the other hand. Hopefully this will lead to a better use of existing and future treatments for this common but difficult to manage disorder.

7.2 Samenvatting

Patiënten met functionele dyspepsie (FD) hebben last van maagklachten zonder duidelijke oorzaak. Hoewel de huidige eerstelijnsbehandeling met zuurremmers (protonpompinhibitoren of PPI) effectief is bij vele patiënten, blijft het mechanisme waarop ze werken onbekend. In dit doctoraatsonderzoek werd een prospectieve studie opgezet waarin 28 FD-patiënten ('starters') dagelijks een PPI innamen gedurende 4 weken. Via biopsies van de twaalfvingerige darm, genomen tijdens een endoscopie, werd het aantal aanwezige ontstekingscellen (eosinofielen en mestcellen) geteld en de doorlaatbaarheid van de darm bestudeerd voor en na zuurremmers.

De behandeling met PPI zorgde voor een vermindering van klachten, een afname van ontsteking en een normalisatie van de doorlaatbaarheid van de darm. De afname van het aantal eosinofielen in de twaalfvingerige darm was sterk geassocieerd met de vermindering van klachten, wat nu voor de eerste keer werd aangetoond. De mate van ontsteking kan dus beschouwd worden als een nieuwe biomerker, die kan helpen om de juiste diagnose te stellen. Tegelijk vormt het een belangrijk startpunt voor het ontwikkelen van gerichte behandelingen, gezien zuurremmers niet bij alle patiënten werken en langdurig gebruik nadelige gevolgen kan hebben.

Deze mogelijks nadelige effecten werden in een parallelle prospectieve studie opgemerkt bij 30 gezonde proefpersonen, met een toename van ontstekingscellen en doorlaatbaarheid van de darm na inname van dezelfde PPI gedurende 4 weken. Bovendien werden gelijkaardige veranderingen gezien in een tweede groep van 19 FD-patiënten ('stoppers'), waarbij het stopzetten van een langdurige inname van zuurremmers bestudeerd werd. Om een verband met de darmflora te onderzoeken, werden naast routine biopten ook oppervlakkige schraapsels van het slijmvlies van de twaalfvingerige darm genomen tijdens de endoscopie om de bacteriële samenstelling te bepalen.

Hoewel een link tussen bepaalde bacteriën met symptomen en eosinofielen werd aangetoond, waren bacteriële veranderingen in de groep van 'starters' niet geassocieerd aan de gunstige effecten van (kortdurende) PPI. Een rol van de bacteriële darmflora werd bevestigd in de gezonde proefpersonen, met een toename van *Streptococcus*, welke aan de nadelige effecten van PPI geassocieerd was. Bovendien persisteerde de toename van *Streptococcus* zelfs na stoppen van PPI gedurende 8 weken in de groep van 'stoppers'. De verstoorde bacteriële samenstelling van de twaalfvingerige darm is dus een mogelijks nadelig effect van het lange termijn gebruik van PPI.

Tot slot werd in dit doctoraatsonderzoek een nieuwe behandeling met probiotica onderzocht. Hiervoor werd een gerandomiseerde, dubbelblinde en placebo-gecontroleerde studie met de spore-vormende *Bacillus coagulans* MY01 en *Bacillus subtilis* MY02 opgezet in 68 FD patiënten met of zonder gelijktijdige inname van PPI. De werkzaamheid en veiligheid van deze probiotica werden aangetoond met een mogelijk werkingsmechanisme via immunologische en microbiële veranderingen. Een daling in bacteriële overgroei, hetgeen een veel onderzochte nevenwerking is van langdurig PPI-gebruik, werd ook aangetoond in FD-patiënten met inname van zuurremmers.

De nieuwe inzichten van dit doctoraatsonderzoek zullen nu gebruikt worden om enerzijds gericht op zoek te gaan naar nieuwe middelen om de onderliggende ontsteking van de twaalfvingerige darm te onderdrukken en anderzijds de positieve bevindingen met spore-vormende probiotica te bevestigen. Hopelijk zal dit leiden tot een beter gebruik van bestaande en toekomstige geneesmiddelen voor deze frequente maar moeilijk te behandelen aandoening.

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Personal contributions

The author of this doctoral thesis performed all of the work (including study design, sample collection and processing, statistical analysis and interpretation of the data, and drafting of all manuscripts) with the following exceptions:

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Conflicts of interest

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Patents

Lucas Wauters, Lukas Van Oudenhove, Jan Tack and Tim Vanuytsel are co-inventors on “Composition for amelioration of anxiety and/or stress”, WO/2020/250040 and PCT/IB2020/000530.

Lucas Wauters and Tim Vanuytsel are co-inventors on “Bacillus coagulans and Bacillus subtilis for the prevention and treatment of functional gastro-intestinal disorders”, WO/2020/201153 and PCT/EP2020/058850

Currriculum vitae

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University:

2012-2015: KU Leuven, Leuven, Belgium. Master in Medicine (MD); with highest honor
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2002-2008: Sint-Albertuscollege, Haasrode, Belgium (Greek-Mathematics)

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07/2021: Poster of distinction. Congress of Intestinal Rehabilitation & Transplant Association (virtual)
10/2019: Poster Champ Award. UEG Week, Barcelona, Spain
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Educational Experience:

10/2021: Case-presenter, UEG Week postgraduate course, "Diagnosis and treatment of malnutrition"
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07/2019: Lead author, UEG online course on Functional Dyspepsia (EACCME-accredited)
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Professional Service:

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2020-2021: Maarten Lambaerts (Masterthesis Medicine, KU Leuven)
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List of publications and presentations

Publications (peer-reviewed):

Wauters L, Ceulemans M, Vanuytsel T. Duodenum at a crossroads: key integrator of overlapping and psychological symptoms in functional dyspepsia? *Neurogastroenterology and Motility* 2021*, in press.*

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Wauters L, Vanuytsel T. Applications of peptide hormone ligands for the treatment of dumping and short bowel syndrome. *Current Opinion in Pharmacology* 2018;43:118-123.

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Omari T, **Wauters L**, Rommel N, Kritas S, Myers JC. Esophageal pressure-flow metrics in relation to bolus volume, bolus consistency and bolus perception. *United European Gastroenterology Journal* 2013;1:249-58.

Omari T, Papathanasopoulos A, Dejaeger E, **Wauters L**, Scarpellini E, Vos R, Slootmaekers S, Seghers V, Cornelissen L, Goeleven A, Tack J, Rommel N. Reproducibility and agreement of pharyngeal automated impedance manometry with videofluoroscopy. *Clinical Gastroenterology and Hepatology* 2011;9:862-7.

Conference abstracts (first author):

Wauters L, Tito R, Ceulemans M, *et al.* Duodenal dysbiosis and efficacy of proton pump inhibitors in functional dyspepsia: cause or consequence? Oral presentation at the United European Gastroenterology Week (UEGW), October 3-5, 2021 (virtual). Poster presentation at the European Society for Neurogastroenterology and Motility (ESNM) meeting, September 2-4, 2021 (virtual).

Wauters L, Demolder T, Ceulemans M, *et al*. Nutrient intake is not associated with duodenal pathology in functional dyspepsia. Poster presentation at the European Society for Clinical Nutrition and Metabolism (ESPEN) Congress, September 9-14, 2021 (virtual).

Wauters L, Tito R, Ceulemans M, *et al*. The small intestinal microbiota in Crohn's disease is characterised by increased luminal diversity but stable mucosa-associated communities. Poster presentation at the congress of European Crohn's and Colitis Organisation (ECCO), July 8-10, 2021 (virtual).

Wauters L, Clarysse M, Jochmans I, *et al*. Reversal of treatment-refractory malabsorption after resection of small bowel diverticulosis: a case series. Poster of distinction at the congress of Intestinal Rehabilitation & Transplant Association (CIRTA), June 30-July 2, 2021 (virtual).

Wauters L, Ceulemans M, Geboers K, *et al.* Efficacy and safety of spore-forming probiotics in functional dyspepsia: a randomized placebo-controlled trial. Oral presentations at Belgian Week of Gastroenterology (BWGE), March 3-5, 2021 and Digestive Disease Week (DDW), May 21-23, 2021 (virtual).

Wauters L, Ceulemans M, Lambaerts M, *et al.* Effect of proton pump inhibitors on duodenal pH, bile salts and mucosal permeability in healthy volunteers. Poster presentation at the Functional Neurogastro-enterology and Motility (FNM) meeting, March 25-28, 2021 (virtual).

Wauters L, Ceulemans M, Lambaerts M, *et al.* Duodenal bile salts and mucosal changes are linked with gastric emptying and symptoms in functional dyspepsia patients. Oral presentation at BWGE, March 4-6, 2020 in Antwerp. Poster presentations at DDW, May 2-5, 2020 and FNM, March 25-28, 2021 (virtual).

Wauters L, Cools L, Ceulemans M, *et al.* Proton pump inhibitors increase cortisol reactivity in functional dyspepsia patients, independent of duodenal barrier and immune function. Oral presentation at UEGW, October 11-13, 2020 (virtual).

Wauters L, Ceulemans M, Frings D, *et al.* Proton pump inhibitors reduce duodenal eosinophilia and symptoms in functional dyspepsia patients by anti-inflammatory rather than acid-suppressive effects. Oral presentation at DDW, May 2-5, 2020 (virtual).

Wauters L, Ceulemans M, Lambaerts M, *et al.* Proton pump inhibitors reduce duodenal hyperpermeability duodenal eosinophilia and symptoms in functional dyspepsia patients. Oral presentation at the ESNM meeting, September 5-7, 2019 in Lisbon (Young Investigator award). Poster presentations at DDW, May 18-21, 2019 in San Diego and UEGW, October 19-23, 2019 in Barcelona (poster champ).

Wauters L, Lambaerts M, Frings D, *et al.* Duodenal hyperpermeability and markers of inflammation are linked with gastric emptying and symptoms in functional dyspepsia patients. Poster presentations at the ESNM meeting, September 5-7, 2019 in Lisbon and UEGW, October 19-23, 2019 in Barcelona.

Wauters L, Smokvina T, Geboers K, *et al.* Effect of *Lactobacillus rhamnosus* strain on stress-related intestinal permeability in healthy adults (ProSPer): A randomized double-blind placebo-controlled trial. Oral presentations at the International Probiotic Conference (IPC), June 17-20, 2019 in Prague and ESNM meeting, September 5-7, 2019 in Lisbon (Trainee Award). Poster presentation at UEGW, October 19-23, 2019 in Barcelona.

Wauters L, Arts J, Caenepeel P, *et al.* Efficacy and safety of lanreotide in postoperative dumping syndrome: A phase III double-blind placebo-controlled cross-over study. Oral presentations at the FNM meeting, March 25-28, 2018 in Amsterdam and UEGW, October 22-34, 2018 in Vienna (National Scholar Award).

Wauters L, Van der Voort V, Dobbels P, *et al.* Antithrombotics Do Not Impact the Performance of Immunochemical Fecal Occult Blood Testing for Colorectal Cancer Screening. Oral presentations at BWGE, February 9-11, 2017 in Antwerp and DDW, May 6-9, 2017 in Chicago.

Wauters L, Peeters K, Van Hootegem A, *et al.* Meckel's enterolith: a rare cause of small bowel obstruction. Oral presentation at BWGE, February 9-11, 2017 in Antwerp.

Wauters L, Smets F, De Greef E, *et al.* Characteristics of children with Crohn's disease failing sustained remission despite anti-TNF exposure. Oral presentation at UEGW, October 15-19, 2016 in Vienna. Poster presentation at the World Congress of Pediatric Gastroenterology, Hepatology and Nutrition (WCPGHAN), October 5-8, 2016 in Montreal.

Wauters L, Smets F, De Greef E, *et al.* Accelerated step-up approach and long-term disease outcomes in pediatric patients with Crohn's disease. Oral presentations at UEGW, October 15-19, 2016 in Vienna. Poster presentation at the WCPGHAN, October 5-8, 2016 in Montreal (Young Investigator award).

Wauters L, Smets F, De Greef E, *et al.* Tailored step-up approach results in beneficial long-term disease outcome in the prospective Belgian pediatric Crohn's disease registry (BELCRO). Oral presentations at BWGE, February 18-20, 2016 in Brussels (Best Oral Presentation), the meeting of the Belgian Society of Pediatrics, March 10-11, 2016 in Brussels and the congress of ECCO, March 17-19, 2016 in Amsterdam.

Wauters L, Smets F, De Greef E, *et al.* Type of treating physician is associated with long-term disease outcome in the prospective Belgian registry of pediatric Crohn's disease (BELCRO). Oral presentation at BWGE, February 18-20, 2016 in Brussels. Poster presentations at the meeting of the Belgian Society of Pediatrics, March 10-11, 2016 in Brussels and the congress of ECCO, March 17-19, 2016 in Amsterdam.

Wauters L, Nightingale S, Sulaiman B, *et al.* Functional dyspepsia is associated with duodenal eosinophilia in an Australian pediatric cohort. Oral presentation at the European Pediatric GI Motility Meeting, October 1-3, 2015 in Sorrento. Poster presentation at UEGW, October 26-28, 2015 in Barcelona.

Wauters L, Harris P, Serrano C, *et al.* Childhood recurrent abdominal pain is associated with duodenal eosinophilia regardless of H. Pylori infection. Poster presentation at the European Pediatric GI Motility Meeting, October 1-3, 2015 in Sorrento.

Wauters L, Joniau S, Verschueren P, *et al.* Anti-TNF treatment and renal cell carcinoma in patients with inflammatory bowel disease, rheumatoid arthritis and spondyloarthritis: trigger or cure? Oral presentation at BWGE, February 25-28, 2015 in Brussels. Poster presentation at the congress of ECCO, February 18-21, 2015 in Barcelona.

Wauters L, Brown T, Venter C, *et al.* Impact of regional and national milk allergy in primary care guidelines and training program on recognition and treatment of cow's milk allergy. Oral poster at the Food Allergy and Anaphylaxis Meeting, October 9-11, 2014 in Dublin.

Wauters L, Selleslagh M, Van Oudenhove L, *et al.* Balloon dilation of the esophago-gastric junction alters lower as well as upper esophageal sphincter function in patients with achalasia. Poster presentation at the World Congress of the International Society for Diseases of the Esophagus, October 15-17, 2012 in Venice.

Personal acknowledgements

Looking back at the past 4 years, I'm confidently reassured in my decision of a doctoral challenge, both on a personal and professional level. I'm grateful for the lessons learned and, more importantly, for the people who guided and joined me on this long journey.

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During these past 4 years, I've had the privilege of working more closely with a small but rapidly expanding 'group Tim'. Thank you Alison for the French touch and babysitting (not only on the rats), I hope you find a new brood. While I cannot claim the same for myself, Matthias is a prime example of the pupil surpassing the master in research and I'm confident that the many optimizations will soon make way for exciting results! In any case, you have now even more proven to be the right candidate for this challenging follow-up study. Jolien, I'm happy you are involved and trust that your projects will also bring us important answers.

I've also been lucky to expand my comfort zone and research interests through other fellow-PhD's. Astrid, you are the right person for this complex project and know that I will help and support you in succeeding, also from abroad! Inge, thank you for technical insights and taking our common interest to the next level. Nele, thank you for introducing me to highly relevant topics, in what I hope is the beginning of a further collaboration. In the Raes-lab, I've had the pleasure to learn from the studies of Astrid, Erik and Tanine and I'm really looking forward to all of your results!

If not for the expert committee, I've also been challenged in my own work by many students. Thanks to all of you, especially Dennis and Maarten for sticking around, and good luck in your careers. While not the same via email and zoom, I hope we can meet again in person and continue the international collaboration Grace! In Leuven, many (more) analyses were done thanks to the friendly help of Anja, Greet, Jetty and Raf. I'm also grateful for the training received from Leen and Kim from the groups of Prof. Niels Hellings (UHasselt) and Tom Van de Wiele (UGent) during fruitful collaborations. Besides the trial support of MyHealth, I thank UEG, Biocodex and Danone for making research possible.

Finally, I've received tremendous support from my friends and family. Although moving out of Leuven and lockdown hampered social contacts, we are lucky to have great friends living and working nearby. Thanks to Jef, Renee, Jan and Ines for being role models in family life, I hope we can spend many more weekends (if not weeks) together. Thanks to Jan, Dries, Koen, Simon and partners for positive feedback and reinforcement. Both friends and family have also supported me from abroad, and I'm grateful for all the opportunities and international experiences through to my parents. The PhD-burden was shared through the experiences of many family members but the human aspect and love for gastroenterology with my mother.

The one person dealing with every emotion, but unfortunately also with the lack of presence at times, was my wife An. While we may feel this PhD is both mine and yours, you have convincingly decided to pursue your own PhD and I'm thrilled to support you in this ambition. Perhaps the biggest lesson (and not challenge) for us will be to put all of this in perspective, as our biggest goal is yet to come. I can't wait to be constantly busy and occupied with a new family member, much like you have devoted plenty of time to Billie. Even if this will not always be possible next year, we are confident to find a way as the will is great. Thank you for your flexibility and unconditional support. I love you and dedicate this work to you.

Lucas,

Leuven, October 2021