ORIGINAL ARTICLE

Prominence of ileal mucosa-associated microbiota to predict postoperative endoscopic recurrence in Crohn's disease

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ABSTRACT

Objective Following ileal resection for Crohn's disease (CD), recurrence is very frequent. Although several clinical risk factors of recurrence have been identified, predicting relapse remains challenging. Performing an ileocolonoscopy within the first year after surgery is currently recommended to assess endoscopic recurrence and to adjust the treatment. We took advantage of a large prospective multicentric cohort to investigate the role of the ileal mucosa-associated microbiota in postoperative endoscopic recurrence.

Patients and methods Ileal mucosa-associated microbiota was analysed by 16S sequencing at the time of surgery and/or of endoscopic evaluation in 201 patients (288 samples in total) prospectively recruited in France.

Results Ileal mucosa-associated microbiota exhibits profound changes following surgery in CD. Compared with non-recurrence setting, endoscopic recurrence is associated with strong changes in ileal mucosa-associated microbiota that are highly reminiscent of those observed generally in ileal CD compared with healthy subjects with a reduction in alpha diversity, an increase in several members of the Proteobacteria phylum and a decrease in several members of the Lachnospiraceae and the Ruminococcaceae families within the Firmicutes phylum. At the time of surgery, we identified several bacterial taxa associated with endoscopic recurrence and that can better predict relapse than usual clinical risk factors.

Conclusion Surgery has an important impact on ileal mucosa-associated microbiota. Postoperative endoscopic recurrence is associated with changes in microbiota composition and alpha diversity. The gut microbiota has the potential to predict postoperative evolution and recurrence.

Significance of this study

What is already known on this subject?
- Up to 75% of patients with Crohn's disease (CD) will need a surgical bowel resection during their life.
- Surgery is not curative and new lesions will recur within months.
- Clinical risk factors for postoperative endoscopic recurrence have been reported but are far from perfect to predict recurrence.
- Gut microbiota is involved in the pathogenesis of CD.

What are the new findings?
- Profound dysbiosis is seen in patients with CD after ileocaecal resection.
- Endoscopic recurrence is associated with strong changes in ileal mucosa-associated microbiota.
- Gut microbiota at the time of surgery can predict endoscopic recurrence.

How might it impact on clinical practice in the foreseeable future?
- Gut microbiota may help clinicians to better define patients at risk of postoperative relapse.
- Early prophylactic treatment could be initiated based on gut microbiota stratification.
- Gut microbiota represents an important therapeutic target in ileal CD.

INTRODUCTION

About 50%–75% of patients with Crohn's disease (CD) will require a partial bowel resection, at least once, in the course of their disease.[1 2] Following surgery, disease recurrence is very frequent, and CD-related symptoms and complications occur in about 50% of the case at 5 years.[1–3] Several clinical risk factors of postoperative recurrence have been identified, such as active smoking, penetrating behaviour, history of perianal disease, prior intestinal resection, extent of small bowel resection and absence of prophylaxis treatment.[4–6] Inflammation of the proximal resection margin, particularly of the enteric plexus (ie, plexitis), has also been suggested to be a risk factor for postoperative recurrence.[7 8] In order to evaluate the postoperative recurrence risk, it is currently recommended to perform an ileocolonoscopy within the first year after surgery to assess endoscopic recurrence according to the Rutgeerts score in order to adapt the treatment.[6 9] Patients with or without mild endoscopic lesions had a lower risk of clinical recurrence than patients with more severe

endoscopic recurrence. Although ileocolonoscopy remains the gold standard to diagnose recurrence, it occurs several months after surgery and thus precludes early therapeutic intervention that could better prevent disease recurrence as suggested recently.[5 10]

In this context, we built a large prospective multicentric cohort of patients with CD who underwent ileocolonic resection with the aim of identifying predictors of postoperative endoscopic recurrence. We recently described this cohort and showed that male gender, active smoking at surgery and previous intestinal resection were associated with a higher risk of endoscopic postoperative recurrence, while postoperative treatment with an anti-tumor necrosis factor (TNF) agent was associated with a lower risk.[11] In the current study, we took advantage of this cohort to investigate the ileal mucosa-associated microbiota at the time of surgery and at the time of postoperative endoscopic evaluation, as well as its potential to predict postoperative endoscopic recurrence. We showed that the ileal mucosa-associated microbiota underwent major changes following surgery with differences between patients with or without endoscopic recurrence. Moreover, endoscopic recurrence was associated by itself with alteration in the gut microbiota. Finally, using several strategies, we showed that the gut microbiota at the time of surgery might be useful for prediction of postoperative recurrence risk and thus early adjustment of the treatment.

STUDY POPULATION AND METHODS

Study population

The present study was undertaken in parallel with a prospective multicentre study performed by the REMIND study group and aimed to identify predictors of early postoperative endoscopic recurrence.[11] Inclusion criteria were age >18 years, ileal or ileocolonic CD, and an indication of CD-related intestinal surgery (ileocolonic resection) in the absence of intestinal dysplasia or cancer. A postoperative treatment (no treatment/5-aminosalicylic acid, thiopurines or anti-TNF agents) was proposed according to a pre-established algorithm, based on the following risk factors: current smoking, previous bowel resection, penetrating phenotype and active perianal disease. About 6–12 months after surgery, a colonoscopy was performed to assess the endoscopic recurrence according to the Rutgeerts score. Postoperative recurrence was defined by a Rutgeerts score of ≥i2.

Mucosal samples were collected from the surgical specimen in the inflamed portion (M0) of the ileum. All patients underwent endoscopy after surgery and biopsies were sampled from the neoterminal ileum (M6). Biopsies were collected in the ileum and stored at −80°C.

Ileal biopsies collection and DNA extraction

Two biopsies collected on ileal resection specimen or during colonoscopy were frozen at −80°C for further analysis. DNA was extracted as described.[12] Following microbial lysis by both mechanical and chemical methods, nucleic acids were precipitated in isopropanol for 10 min at room temperature, incubated for 15 min on ice and centrifuged for 30 min at 20 000 g and 4°C. Pellets were suspended in 450 µL of phosphate buffer and 50 µL of potassium acetate. After RNase treatment and DNA precipitation, nucleic acids were recovered via centrifugation at 20 000*g* and 4°C for 30 min. The DNA pellet was suspended in 80 mL of trypsin–EDTA buffer. DNA samples were then subjected to 16S sequencing for bacterial microbiota analysis.

16S DNA sequencing

Bacterial diversity in ileal biopsies was determined by targeting a portion of the ribosomal genes in extracted DNA. A 16S DNA gene fragment comprising the V3 and V4 hypervariable regions (16S sense 5′-TACGGRAGGCAGCAG-3′ and antisense 5′-CTACCNGGGTATCTAAT-3′) was amplified using an optimised and standardised 16S-amplicon library preparation protocol (Metabiote, GenoScreen, Lille, France). Briefly, 16S DNA gene PCR was performed using 5 ng of genomic DNA according to the manufacturer's protocol (Metabiote), 192 bar-coded primers (Metabiote MiSeq Primers) at final concentrations of 0.2 µmol/L and an annealing temperature of 50°C for 30 cycles. The PCR products were purified using an Agencourt AMPure XP-PCR purification system (Beckman Coulter, Brea, California, USA), quantified according to the manufacturer's protocol and multiplexed at equal concentrations. Sequencing was performed using a 300 bp paired-end sequencing protocol on an Illumina MiSeq platform (Illumina, San Diego, California, USA) at GenoScreen, Lille, France. Raw paired-end reads were subjected to the following processes: (1) quality filtering using the PRINSEQ-lite PERL script,[13] by truncating the bases from the 3′ end, which did not exhibit a quality <30, based on the Phred algorithm; (2) paired-end read assembly using fast length adjustment of short reads to improve genome assemblies,[14] with a minimum overlap of 30 bases and a 97% overlap identity and (3) searching for and removing both forward and reverse primer sequences using CutAdapt, with no mismatches allowed in the primer sequences. Assembled sequences, for which perfect forward and reverse primers were not found, were eliminated.

16S sequence analysis

The sequences were demultiplexed and quality filtered using the QIIME V.1.9.1 software package.[15] The sequences were then assigned to operational taxonomic units (OTUs) using the UCLUST algorithm with a 97% pairwise identity threshold and classified taxonomically using the Silva reference database (V.132).[16] Rarefaction was performed (5000 sequences per sample for analysis of M0 samples alone or 3000 sequences per samples in the other cases) and used to compare the relative abundance of OTUs across samples. Alpha diversity was estimated using the Shannon and Chao1 diversity indexes. Beta diversity was measured by a Bray-Curtis distance matrix and was used to build principal coordinate analysis plots. Raw sequence data are accessible in the European Nucleotide Archive (accession number PRJEB31684).

Statistical analyses

Patients' characteristics were expressed as median (range) and compared using Mann-Whitney or χ^2 tests as appropriate. GraphPad Prism V.6.0 (San Diego, California, USA) and R software V.3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) were used for analyses and graph preparation. Statistical analyses were performed using the two-tailed non-parametric Mann-Whitney test or Kruskal-Wallis test with Dunn's multiple comparison test. Statistical significance of sample grouping for beta diversity was performed using Anosim method (9999 permutations). Differential analysis was performed using the linear discriminant analysis effect size (LEfSe) pipeline.[17] The identification of factors associated with the endoscopic recurrence was performed by a logistic regression model using R software V.3.5.2. Random forest analysis was performed using RandomForest R package (10 000 trees, mtry=3) and ROC curves were build using pROC R Package. The p values

Table 1 Patients' characteristics

	All	M0	M6	M0 and M6
	n=201	n=153	n=135	n=87
Men, n (%)	98 (49)	77 (50)	63 (47)	42 (48)
Mean age at surgery (y, SD)	34.6 (12.0)	34.7 (12.1)	34.5 (12.5)	34.6 (12.8)
Indication of surgery, n (%)				
Stenosis	116 (58)	85 (56)	82 (61)	51 (59)
Fistula	71 (35)	56 (37)	46 (34)	31 (36)
Other	14 (7)	12 (7)	7 (5)	5 (5)
Active smoking at surgery, n (%)	66 (33)	44 (29)	49 (36)	27 (31)
Montreal A, n (%)				
A1	28 (14)	19 (12)	18 (13)	9 (10)
A2	151 (75)	116 (76)	100 (74)	65 (75)
A3	22 (11)	18 (12)	17 (13)	13 (15)
Montreal L, n (%)				
L1	119 (59)	84 (55)	84 (62)	49 (56)
L3	82 (41)	69 (45)	51 (38)	38 (44)
Montreal B, n (%)				
B1	31 (15)	25 (16)	21 (16)	15 (17)
B2	105 (52)	74 (48)	70 (52)	39 (45)
B3	65 (33)	54 (36)	44 (32)	33 (38)
Anoperineal lesions, n (%)	47 (23)	37 (24)	31 (23)	21 (24)
Previous intestinal resection, n (%)	39 (19)	30 (20)	27 (20)	18 (21)
Preoperative steroids, n (%)	65 (32)	54 (35)	43 (32)	32 (37)
Preoperative IS, n (%)	63 (31)	48 (31)	42 (31)	27 (31)
Preoperative anti-TNF, n (%)	95 (47)	70 (46)	68 (50)	43 (49)
Antibiotics 1 month before surgery, n (%)	68 (34)	57 (37)	46 (34)	35 (40)
Postoperative IS, n (%)	48 (24)	33 (22)	28 (21)	13 (15)
Postoperative anti-TNF, n (%)	68 (34)	55 (36)	48 (36)	35 (40)
Postoperative antibiotics, n (%)	13 (6)	11 (7)	7 (5)	5 (6)
Mean time between resection and colonoscopy (months, SD)	7.9 (3.5)	7.9 (3.7)	7.9 (3.4)	7.8 (3.6)
Rutgeerts score, n (%)				
i0–i1	106 (53)	81 (53)	67 (50)	42 (48)
i2–i4	95 (47)	72 (47)	68 (50)	45 (52)

IS, immunosupressant; TNF, tumor necrosis factor.

were corrected using the Benjamini and Hochberg procedure to control for the false discovery rate. In all statistical analyses, differences with $p<0.05$ were considered significant.

RESULTS

Characteristics of the study population

Among the 289 patients included between 1 September 2010 and 30 September 2017 and described previously,[8] ileal biopsies at the time of surgery (M0) and/or of endoscopic evaluation (M6) were obtained from 215 patients. After quality control check of 16S sequencing data, 14 samples were excluded and 288 samples from 201 patients remained in the final analysis. Their demographic and clinical characteristics are detailed in table 1.

Changes in mucosa-associated microbiota following surgery

The population was largely represented by refractory patients as half of them received anti-TNF agent before surgery and one-fifth had previous intestinal resection. Approximately one-third of the patients received anti-TNF agent in postoperative setting. The study population was well balanced between patients who underwent postoperative endoscopic recurrence (Rutgeerts score i2–i4) or not (Rutgeerts score i0– i1). For 87 patients, samples were available at both M0 and M6.

To assess the changes in ileal mucosa-associated microbiota following surgery, we first focused on the 87 patients for whom samples were available at both M0 and M6. The analysis of beta diversity showed a clustering of samples according to the time point with statistical significance between samples taken at M0 and at M6 (figure 1A). When analysed globally, the alpha diversity, assessed by Shannon and Chao1 indexes, was not significantly different between M0 and M6 (online supplementary figure 1). However, when the patients were separated according to their endoscopic recurrence status at M6, a statistically significant decrease in alpha diversity was observed in patients with endoscopic recurrence (i2–i4) but not in the others (i0– i1) (figure 1B-C).

In accordance with previously published studies, the microbiota was dominated by bacteria from the Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla and from the Ruminococcaceae, Lachnospiraceae, Veillonellaceae, Enterobacteriaceae, Burkholderiaceae and Bifidobacteriaceae families (figure 1D). We then used the LEfSe pipeline to identify bacterial taxa differentially represented between M0 and M6 according to the endoscopic recurrence status at M6 (figure 2). Within the Proteobacteria phylum, several taxa belonging to the Gammaproteobacteria class, including *Klebsiella pneumoniae*, decreased after

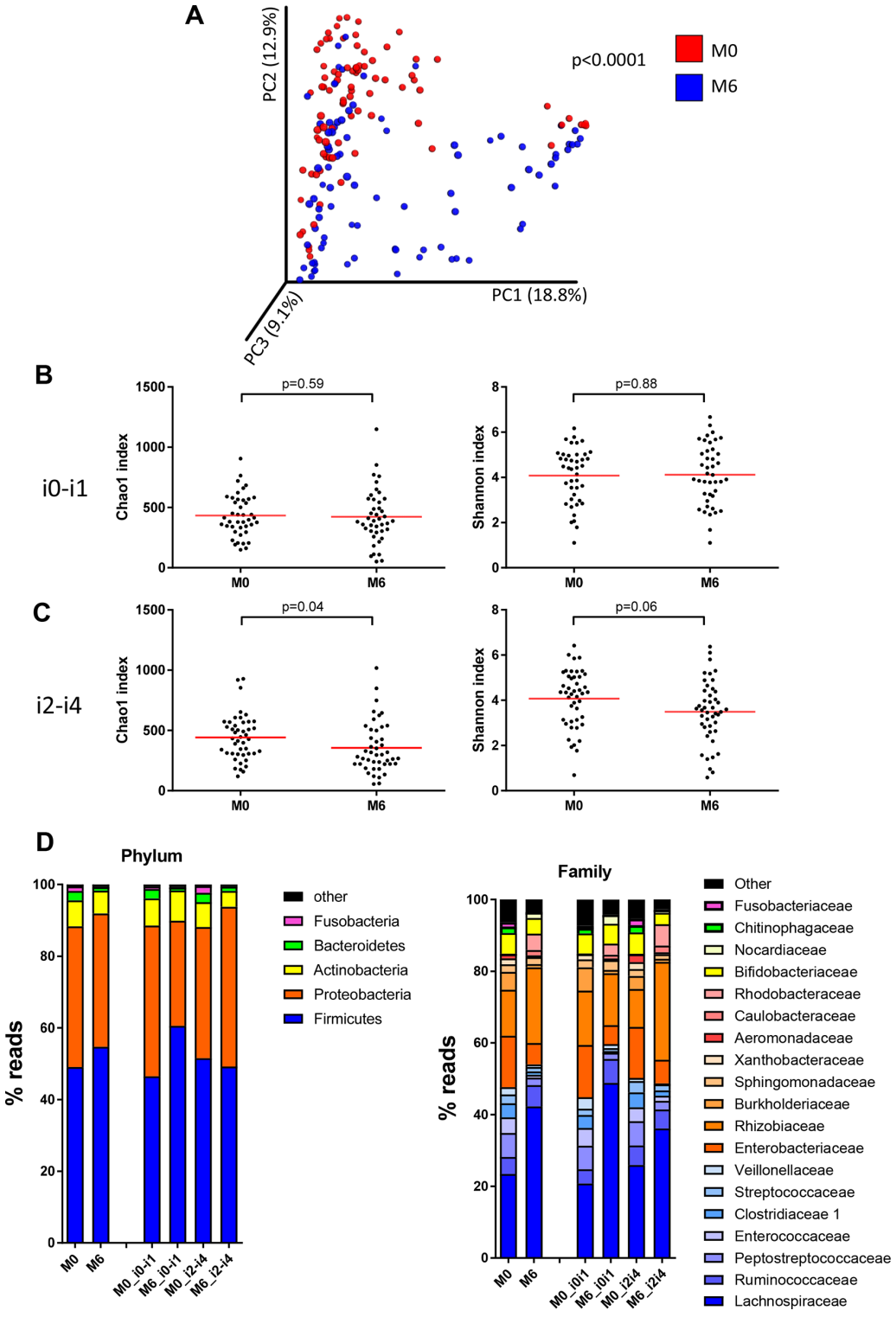

Figure 1 Evolution of mucosa-associated microbiota between M0 and M6. (A) Beta diversity. Principal coordinate analysis of Bray-Curtis distance with each sample coloured according to the disease phenotype. PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using Anosim method. Alpha diversity illustrated by Shannon and Chao1 index in patients without (i0–i1) (B) and with (i2–i4) (C) postoperative endoscopic recurrence (Wilcoxon matched-pairs test). (D) Global composition of bacterial microbiota at the phyla and family level. Patient subgroups are labelled on the x-axis. M0, sample at surgery; M6, sample at endoscopy.

surgery. However, many taxa belonging to the Alphaproteobacteria class increased at M6 in both patients with and without endoscopic recurrence, but the signal was stronger in patients with recurrence with a global increase in bacteria from the Alphaproteobacteria class. Within the Actinobacteria phylum, bacteria belonging to the Actinomycetales order, including Actinomyces genera, decreased at M6 more strongly in patients without than with endoscopic

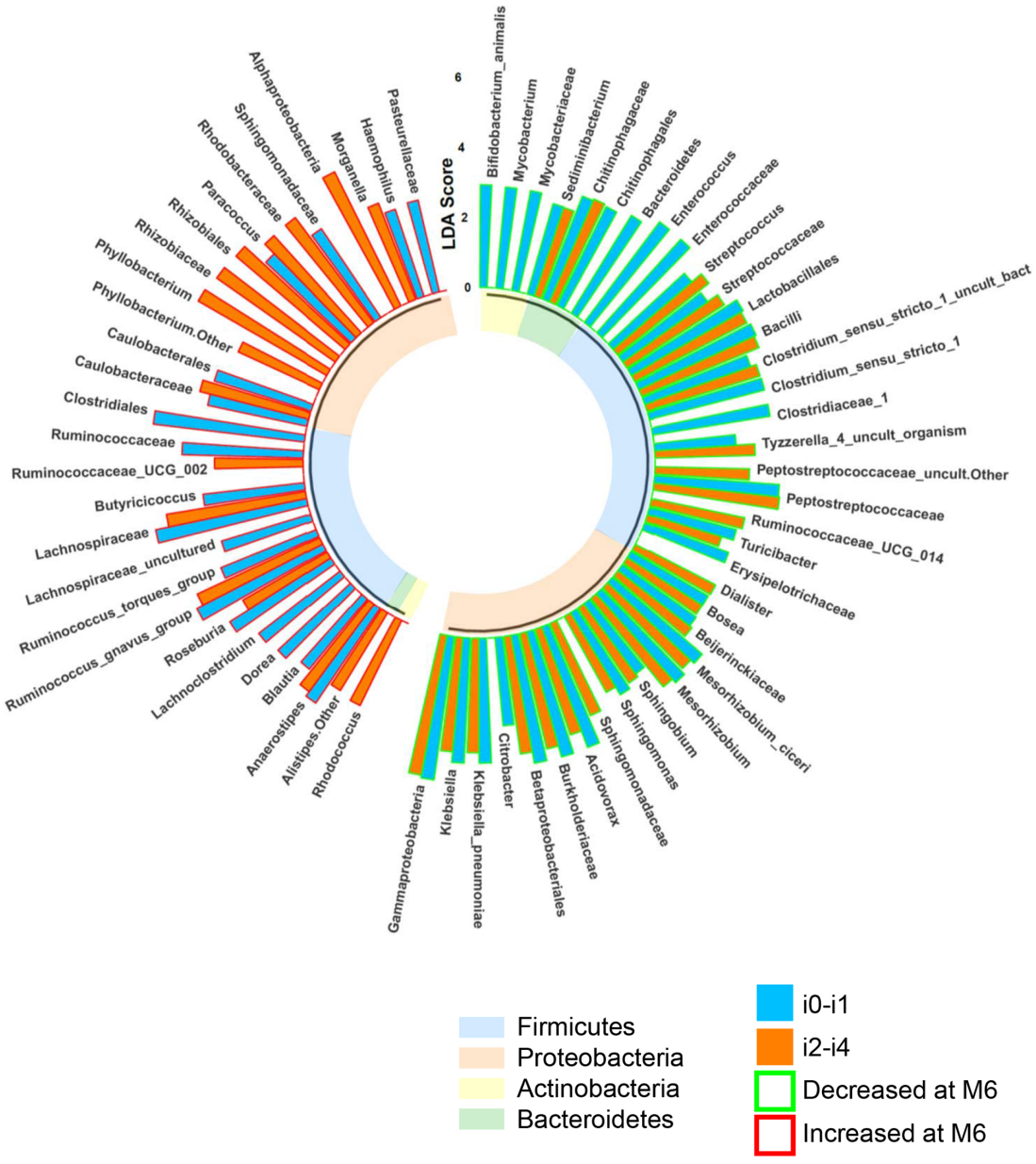

Figure 2 Bacterial taxa differentially represented between M0 and M6 according to postoperative endoscopic recurrence status. Taxa with significant difference (LDA score >2.5) between M0 and M6 are represented. The barplot outline colour indicates if the taxa is increased (red, left part of the figure) or decreased (green, right part of the figure) at M6. The barplot colour indicates if the taxa is modified in patient without (i0–i1, blue) or with (i2–i4, orange) endoscopic recurrence. The inner circle indicates the phylum to which the bacterial taxa belong. M0, sample at surgery; M6, sample at endoscopy. LDA, linear discriminant analysis.

recurrence. Within the Firmicutes phylum, bacteria from the Clostridiales order and notably from the Lachnospiraceae and the Ruminococcaceae families, including *Roseburia*, *Blautia* and *Dorea* genera, increased at M6 more strongly in patients without than with endoscopic recurrence. Conversely, members of the Bacilli class, such as *Streptococcus* and *Enterococcus* genera, increased at M6.

Taken together, these results demonstrate the profound changes in the ileal mucosa-associated microbiota following surgery in CD. These changes tend to reduce the dysbiosis usually associated with ileal CD, such as the bloom of Gammaproteobacteria and the decrease in Lachnospiraceae and the Ruminococcaceae, but are weaker in patients with endoscopic recurrence.

Altered ileal mucosa-associated microbiota in patients with postoperative endoscopic recurrence

We then focused on the 135 samples obtained during postoperative endoscopic evaluation (M6) to investigate the bacterial taxa associated with endoscopic recurrence. The analysis of beta diversity showed a statistically significant difference between samples from patients with (i2-i4) and without (i0–i1) endoscopic recurrence (figure 3A) but not between patients with (Harvey-Bradshaw index , HBI ≥4) or without (HBI <4) clinical activity (online supplementary figure 2A). Similarly, the alpha diversity, assessed by Shannon index, was lower in patients with than without endoscopic recurrence (figure 3B), while no difference was observed between patients with and without clinical activity (online supplementary figure 2B). Alteration in

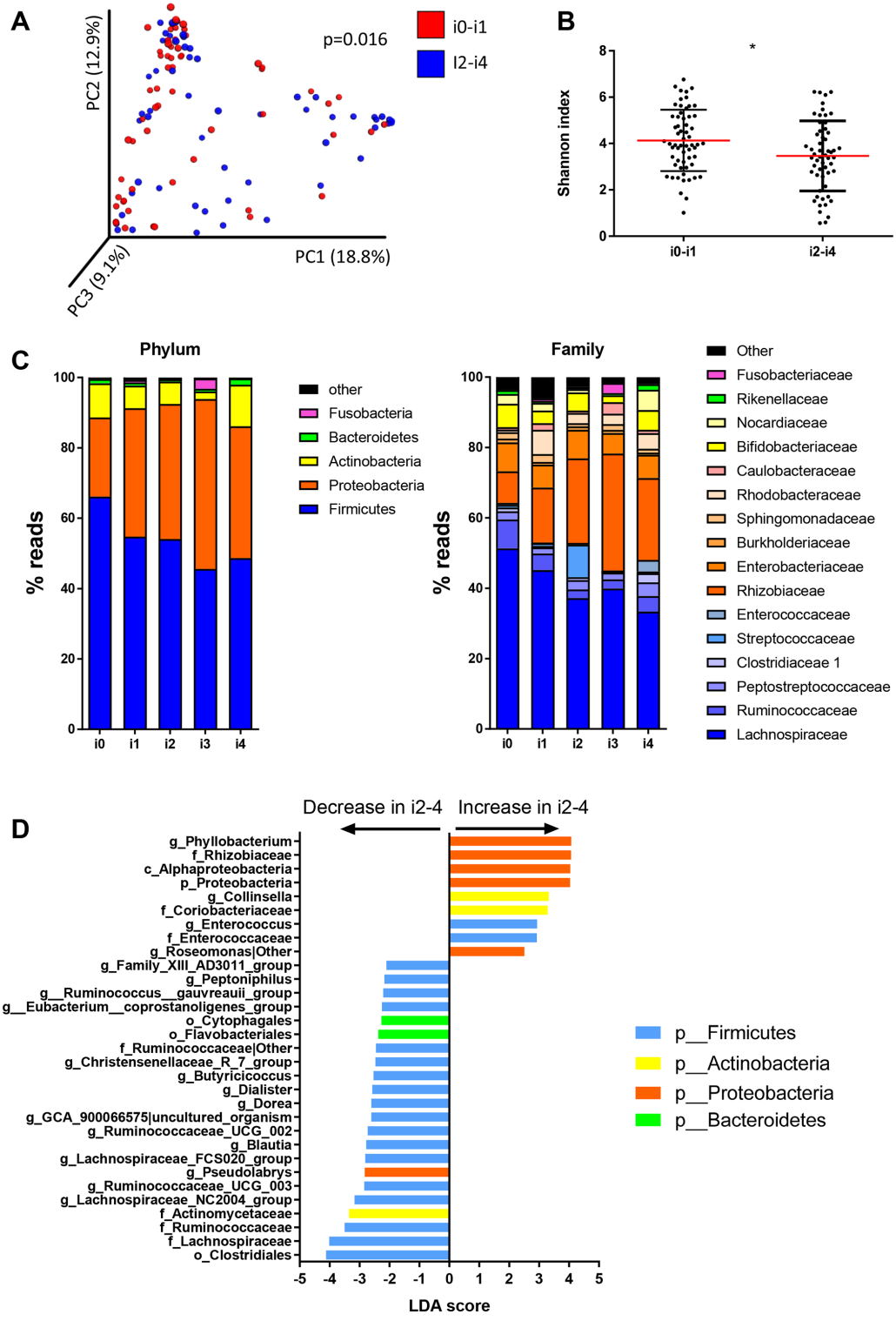

Figure 3 Altered bacterial gut microbiota in patients with postoperative endoscopic recurrence. (A) Beta diversity. Principal coordinate analysis of Bray-Curtis distance with each sample coloured according to the disease phenotype. PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using the Anosim method. (B) Shannon index of alpha diversity in the indicated groups (Mann-Whitney test). (C) Global composition of bacterial microbiota at the phyla and family levels. Patient subgroups are labelled on the x-axis and are expressed as the relative operational taxonomic unit abundance for each group. (D) Bacterial taxa differentially represented in patients with (i2–i4) or without (i0–i1) postoperative endoscopic recurrence, with a statistical level of significance according to LDA (LDA score >2). LDA, linear discriminant analysis; M0, sample at surgery; M6, sample at endoscopy.

microbiota composition increased in parallel with the Rutgeerts score with the expansion of the bacteria from the Proteobacteria phylum and the contraction of the bacteria from the Firmicutes phylum (figure 3C). We then used the LEfSe pipeline to identify bacterial taxa differentially represented between patients with and without endoscopic recurrence at M6 (figure 3D). Increases

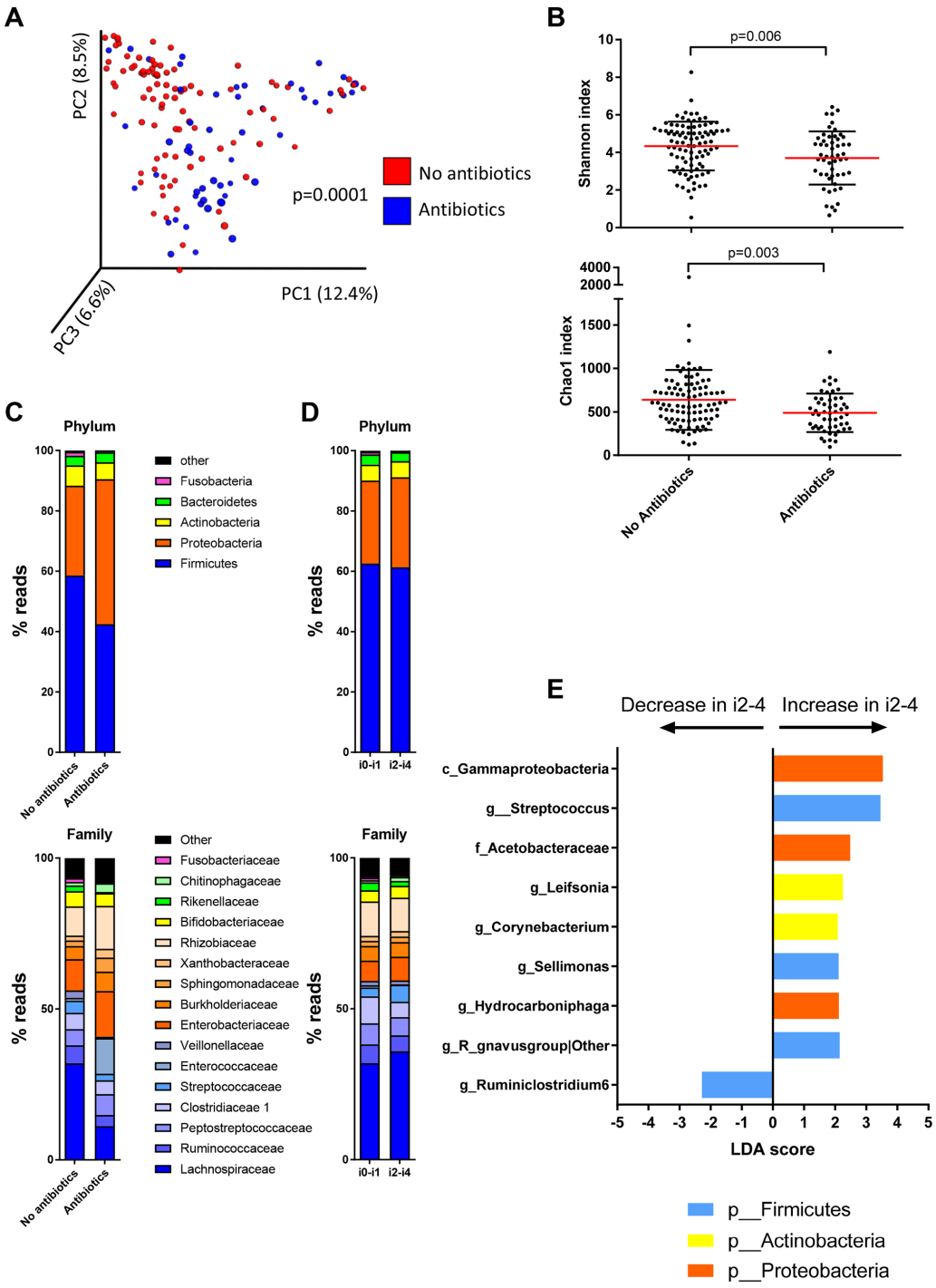

Figure 4 Bacterial gut microbiota at M0 according postoperative endoscopic recurrence status. (A) Beta diversity. Principal coordinate analysis of Bray-Curtis distance with each sample coloured according to the antibiotics intake in the last month before surgery. PC1, PC2 and PC3 represent the top three principal coordinates that captured most of the diversity. The fraction of diversity captured by the coordinate is given as a percentage. Groups were compared using the Anosim method. (B) Shannon and Chao1 indexes of alpha diversity in the indicated groups (Mann-Whitney test). Global composition of bacterial microbiota at the phyla and family levels in patients with or without antibiotics intake in the last month before surgery (C) and according postoperative endoscopic recurrence status (D). Patient subgroups are labelled on the x-axis and expressed as the relative operational taxonomic unit abundance for each group. (E) Bacterial taxa differentially represented at M0 in patients who will (i2–i4) or will not (i0–i1) undergo postoperative endoscopic recurrence, with a statistical level of significance according to LDA (LDA score >2). For (D) and (E), patients who received antibiotics in the month before surgery, as well as those who received an anti-TNF agent as postoperative treatment, were excluded. LDA, linear discriminant analysis; M0, sample at surgery; M6, sample at endoscopy.

in Proteobacteria and particularly Alphaproteobacteria, as well as in Coribacteriaceae family and in *Enterococcus* genera, were observed in patients with recurrence. Similarly, a decrease in several members of the Firmicutes phylum and particularly the Lachnospiraceae and the Ruminococcaceae family, including *Eubacterium*, *Ruminococcus*, *Butyricoccus*, *Dorea*, and *Blautia* genera, was observed in patients with recurrence compared with those without recurrence.

Table 2 Multivariable analysis: microbiota predictors of endoscopic recurrence

	Excluding patients with postoperative anti-TNF			Including patients with postoperative anti-TNF		
	OR	95% CI	P value	OR	95% CI	P value
Gammaproteobacteria+	16.7	2.3 to 263.9	0.015*	2.2	0.7 to 7.1	0.173
Corynebacterium+	14.4	0.8 to 831.7	0.13	9.8	1.6 to 94.9	0.025*
Sellimonas+	4.4	0.6 to 39.4	0.15	2.3	0.7 to 8.3	0.173
Ruminiclostridium6+	0.02	0.0008 to 0.25	0.009**	0.17	0.04 to 0.6	0.01**
Hydrocarboniphaga+	6.5	0.2 to 755.3	0.355	2.2	0.5 to 9.9	0.29
Ruminococcus_gnavus group.Other+	21.8	2.9 to 324.9	0.008**	2.9	0.95 to 9.7	0.065

+Above median; * p<0.05; ** p<0.01

Taken together, these results demonstrate that patients with endoscopic recurrence exhibit strong changes in their ileal mucosa-associated microbiota compared with patients without recurrence. These changes are highly reminiscent of those observed generally in ileal CD compared with healthy subjects with a reduction in alpha diversity, an increase in several members of the Proteobacteria phylum and a decrease in several members of the Lachnospiraceae and the Ruminococcaceae families within the Firmicutes phylum.

Ileal mucosa-associated microbiota at surgery as a predictor of postoperative endoscopic recurrence

We then looked for a role of the gut microbiota at surgery as a predictor of postoperative endoscopic recurrence. For this purpose, we took into account the 153 samples taken at surgery (M0). If the gut microbiota has a predictive role, it is very likely that antibiotics given in a preoperative setting can mask it. Indeed, we confirmed that antibiotics given during the month before surgery had a dramatic impact on the microbiota with highly significant changes in beta diversity (figure 4A), a decrease in alpha diversity as assessed by Shannon and Chao1 indexes (figure 4B), and an increase of Proteobacteria at the expense of Firmicutes (figure 4C, online supplementary figure 3). We thus excluded the patients who received antibiotics in the month before surgery for further analysis. As anti-TNF given as a postoperative treatment is effective in preventing recurrence and was also identified in this cohort to be associated with maintenance of remission,[11] we took into account this parameter in the process of discovering bacterial taxa predicting endoscopic recurrence. Alpha diversity at M0 was not able to discriminate patients with and without endoscopic recurrence at M6 (online supplementary figure 4). We thus used the LEfSe pipeline on a refined population excluding patients with postoperative anti-TNF treatment to identify bacterial taxa differentially represented at M0 between patients with and without endoscopic recurrence at M6 (figure 4D,E). Among the identified taxa, the abundance of Acetobacteriaceae, *Leifsonia* and *Streptococcus* was highly correlated with the abundance of *Corynebacterium*, and they were thus excluded for further analysis. We then performed a logistic regression, taking into account the clinical factors we previously identified as predictors of endoscopic recurrence, that is, male gender, active smoking and previous intestinal resection.[11] As indicated in table 2, Gammaproteobacteria, *Ruminiclostridium 6* and *Ruminococcus gnavus* group.Other remained significantly associated with endoscopic recurrence.

Finally, we re-run the analysis including patients who received postoperative anti-TNF agents and adding in the model the postoperative treatment by anti-TNF as a new parameter. The trend persisted for Gammaproteobacteria and *R. gnavus* group but was not statistically significant anymore. The protective effect of the high abundance of *Ruminiclostridium 6* was confirmed, and the deleterious effects of high abundance of *Corynebacterium* appeared (table 2). In the same population, we then built a score, taking into account these two significant taxa and each of the previously identified clinical risk factors (active smoking at surgery, male gender and previous intestinal resection). In the patients who did not receive postoperative anti-TNF agents, the score correlated well with the endoscopic recurrence rate with 14%, 53% and 86% of Rutgeerts score ≥i2 in patients respectively with 0–1, 2 and >2 risk factors (figure 5A). In patients who received postoperative anti-TNF agents, the score was much less predictive of endoscopic recurrence (online supplementary figure 5A), suggesting that taking postoperative anti-TNF agents impacts the natural disease course. We then included the patients who received postoperative anti-TNF agents in the analysis and considered this treatment as a protective factor in the score. In this setting, the correlation with endoscopic recurrence was good with 18%, 30%, 53% and 81% of Rutgeerts ≥i2 in patients, respectively, with 0–1, 2, 3 and >3 risk factors (figure 5B).

Finally, we used random forest method taking into account the gut microbiota (taxa identified in figure 4E) with or without the previously identified clinical risk factors to predict endoscopic recurrence. For this purpose, the population of interest was split in a training set (70% of the population of interest, randomly chosen) and a validation set (remaining 30% of the population). ROC curves showed interesting area under the curve (AUC) of 81.0% (60.8%–100%)%) (figure 5C). In the random forest model, the three most informative taxa were *Streptococcus*, *R._gnavus* group.Other and Gammaproteobacteria. Adding in the model the clinical factors to gut microbiota taxa of interest did not improve it with an AUC in the validation set of 78.6% (56.9%–100%)%) (figure 5D). In accordance with the previous results, these models were inefficient in patients who received postoperative anti-TNF agents (online supplementary figure 5B, -C. Taken together, these results suggest that gut microbiota at the time of surgery might be a predictive factor of endoscopic recurrence and that it might be used to guide treatment. Moreover, postoperative anti-TNF agents seem to modify natural disease course and endoscopic recurrence risk.

DISCUSSION

In this study, we showed that the ileal mucosa-associated microbiota exhibits profound changes following surgery in CD and that endoscopic recurrence is associated with strong differences in ileal mucosa-associated microbiota compared with a non-recurrence setting. Using two different approaches, we showed that gut microbiota at the time of surgery might be a predictive factor of endoscopic recurrence.

Although previous studies looked at the effect of surgery on the gut microbiota in CD,[18] the current study is, by far, the

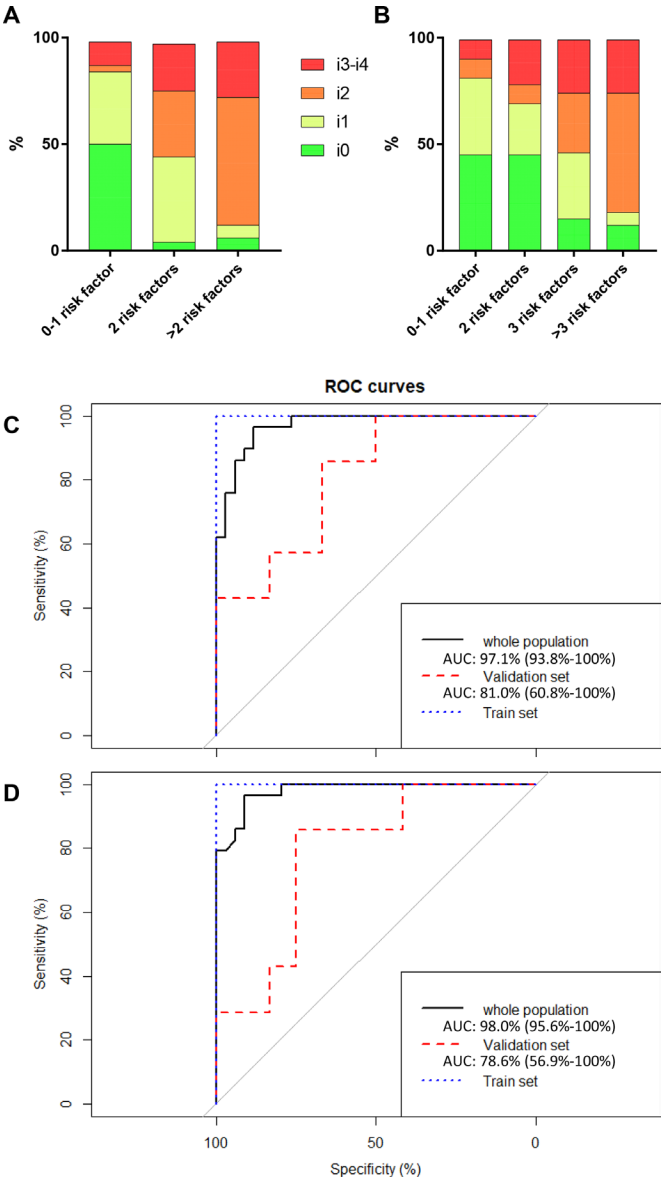

Figure 5 Bacterial gut microbiota at M0 as predictor of postoperative endoscopic recurrence Rugeerts score according to the number of risk factors (A) in patients who did not receive postoperative anti-tumor necrosis factor (TNF) agents and (B) in patients who received or did not receive postoperative anti-TNF agents. Risk factors taken into account were active smoking at surgery, male gender, previous intestinal resection, *Ruminiclostridium 6* abundance>median; *Corynebacterium* abundance>median (and not receiving postoperative anti-TNF agent for (B). Area under the curve for random Forest model, taking into account microbiota factors without (C) or with (D) clinical factors. Results shown for training set (70% of the population of interest), validation set (30% of the remaining population) and whole population. Patients who receive antibiotics in the last month before surgery were excluded from the analysis. AUC, area under the curve; ROC, receiver operating characteristic.

largest one and the only one that studied ileal mucosa-associated microbiota instead of faeces. Between M0 and M6 time points, we observed a shift in the microbiota composition. Besides the classical changes in the relative proportion of Firmicutes and Proteobacteria observed in CD between flare and remission, we observed shifts inside each phylum. Within Proteobacteria, the Gammaproteobacteria class decreased between M0 and M6, while the opposite was seen for the Alphaproteobacteria class. Within the Firmicutes, the Bacilli class decreased between M0 and M6, while the Clostridiales order increased. Interestingly, the increase in Clostridiales and notably in bacteria from the Lachnospiraceae and the Ruminococcaceae families (including *Roseburia*, *Blautia* and *Dorea* genera), which are considered as associated with intestinal health, was much clearer in patients without than with endoscopic recurrence. Similarly, a decrease in alpha diversity was observed between M0 and M6 in patients with endoscopic recurrence but not in the non-recurrence setting. In the same way, at M6, compared with patients without endoscopic recurrence, those with endoscopic recurrence exhibited a lower alpha diversity, suggesting a stronger alteration in the gut microbiota ecosystem. Moreover, the ileal mucosa-associated microbiota at M6 was different in composition according to the endoscopic recurrence status. Interestingly, the microbiota alpha and beta diversity was not different in patients with (HBI ≥4) or without (HBI <4) clinical activity at M6, suggesting that the gut microbiota does not correlate well with clinical symptoms but does with endoscopic activity, indicating a potential as a biomarker for mucosal healing. The main alterations observed in patients with endoscopic recurrence were reminiscent of the changes associated with ileal CD when compared with healthy subjects with an increase in several taxa from the Proteobacteria phylum and a decrease in many taxa from the Firmicutes phylum, and notably from the Lachnospiraceae and the Ruminococcaceae families. We specifically looked at *Faecalibacterium prausnitzii* as we previously showed that its abundance at M0 was predictive of postoperative endoscopic recurrence at M6.[19] Moreover, other groups have also observed that a low level of *F. prausnitzii* might be associated with postoperative endoscopic recurrence.[20 21] Indeed, in our cohort, the level of *F. prausnitzii* was very low and detected by sequencing only in less than 10% of the patients at M0 and at M6. We thus used real-time qPCR to get a higher sensibility. Using this method, we did observe that *F. prausnitzii* abundance at M6 was moderately able to discriminate the patients with (Rutgeerts ≥i1) from those without (Rutgeerts ≥i0) endoscopic recurrence (AUC for delta CT: 63.9% (52.8%–74.9%), data not shown). The almost undetectable level of *F. prausnitzii* in the studied population prevented to have it identified as a prognostic marker. The refractoriness of the population of patients included in this study, recruited in tertiary centres, might be an explanation for the very low level of *F. prausnitzii*. Similarly to a previously published study by Mondot and colleagues,[20] a high amount of *Enterococcus* genera and a low amount of *Dorea* genera were associated with endoscopic recurrence. Unlike the POCER study and colleagues, we did not identify the *Proteus* genera as associated with endoscopic recurrence.[21] This might be related to differences in environmental factors (the current study was performed in France, while the POCER study was performed in Australia) or in postoperative treatment as all the patients of the POCER study received metronidazole for 3 months following surgery.[21]

In the two approaches we used, the gut microbiota at the time of surgery was predictive of postoperative endoscopic recurrence. In the random forest strategy, adding clinical factors did not even improve the model. Among the most discriminative features, the high abundance of bacteria from the Gammaproteobacteria, the *R. gnavus* group and *Corynebacterium* genera were predictive of recurrence. The high abundance of Gammaproteobacteria and of *R. gnavus* have been shown in many studies to be associated with ileal CD.[22–28] *Corynebacterium* has been associated with IBD and with primary sclerosing cholangitis with or without IBD,[29–31] as well as with idiopathic inflammatory bowel disease in dogs.[32] These data

suggest that these taxa might be involved in the disease pathogenesis. Interestingly, in patients who received anti-TNF agents as postoperative treatment, the ability of these microbiota parameters to predict recurrence was lost, confirming that these treatments have some efficacy to prevent recurrence and suggesting they can modify natural disease course.

Our results confirm that the reduction of microbial diversity is associated with the intestinal inflammatory process. This microbiota alteration could be associated or even impact other biological parameters, including host immune responses. We recently analysed the T cell repertoire in 57 patients of our cohort.[33] Clonal T cell expansions that result in a reduction of T cell diversity at the time of surgery was associated with postoperative endoscopic recurrence. These clonal expansions could also correlate with microbial alterations.

This study has several limitations. First, the studied population is heterogeneous and is mostly representative of tertiary centres recruitment. Second, although the study was prospective, no central reading was performed for colonoscopy. Third, all the results are based on ileal mucosa-associated microbiota and no data from faeces were available. Regarding the research for predictive factors of recurrence, although we split the population between a training and a validation set for the random forest approach, a strictly independent cohort would be required to confirm the present findings. This cohort is ongoing, and we plan to validate these results in a second cohort in the following years.

In conclusion, this study demonstrates that surgery has an important impact on ileal-mucosa-associated microbiota and that postoperative endoscopic recurrence is associated with changes in its composition and alpha diversity. The gut microbiota has the potential to predict postoperative evolution and recurrence but is limited by the relatively frequent use of antibiotics before surgery in CD. Independent studies are needed to confirm these results.

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