

# Identification of Metabolic Signatures Linked to Anti-Inflammatory Effects of *Faecalibacterium prausnitzii*

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**ABSTRACT** *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified on the basis of human clinical data. The mechanisms underlying its beneficial effects are still unknown. Gnotobiotic mice harboring *F. prausnitzii* (A2-165) and *Escherichia coli* (K-12 JM105) were subjected to 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute colitis. The inflammatory colitis scores and a gas chromatography–time of flight (GC/TOF) mass spectrometry-based metabolomic profile were monitored in blood, ileum, cecum, colon, and feces in gnotobiotic mice. The potential anti-inflammatory metabolites were tested *in vitro*. We obtained stable *E. coli* and *F. prausnitzii*-diassociated mice in which *E. coli* primed the gastrointestinal tract (GIT), allowing a durable and stable establishment of *F. prausnitzii*. The disease activity index, histological scores, myeloperoxidase (MPO) activity, and serum cytokine levels were significantly lower in the presence of *F. prausnitzii* after TNBS challenge. The protective effect of *F. prausnitzii* against colitis was correlated to its implantation level and was linked to overrepresented metabolites along the GIT and in serum. Among 983 metabolites in GIT samples and serum, 279 were assigned to known chemical reactions. Some of them, belonging to the ammonia ( $\alpha$ -ketoglutarate), osmoprotective (raffinose), and phenolic (including anti-inflammatory shikimic and salicylic acids) pathways, were associated with a protective effect of *F. prausnitzii*, and the functional link was established *in vitro* for salicylic acid. We show for the first time that *F. prausnitzii* is a highly active commensal bacterium involved in reduction of colitis through *in vivo* modulation of metabolites along the GIT and in the peripheral blood.

**IMPORTANCE** Inflammatory bowel diseases (IBD) are characterized by low proportions of *F. prausnitzii* in the gut microbiome. This commensal bacterium exhibits anti-inflammatory effects through still unknown mechanisms. Stable monoassociated rodents are actually not a reproducible model to decipher *F. prausnitzii* protective effects. We propose a new gnotobiotic rodent model providing mechanistic clues. In this model, *F. prausnitzii* exhibits protective effects against an acute colitis and a protective metabolic profile is linked to its presence along the digestive tract. We identified a molecule, salicylic acid, directly involved in the protective effect of *F. prausnitzii*. Targeting its metabolic pathways could be an attractive therapeutic strategy in IBD.

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The human colonic microbiota is a highly complex microbial ecosystem, and several hundred different species of bacteria are known to colonize the gut (1). The homeostasis established between the host and the resident bacteria plays important functional roles, such as protection against pathogenic organisms, maturation and modulation of the immune system, intestinal maturation, production of short-chain fatty acids (SCFA), mucosal physiology, and production of vitamins, such as vitamin K and biotin (2). Alterations in this homeostasis, named dysbiosis, have a profound impact on human health, as demonstrated in several gastrointestinal diseases, including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), and colorectal cancer (CRC) (3–7). For instance, in genetically predisposed subjects, bacterial dysbiosis, including a decrease of *Firmicutes*, could result

in disordered innate immune reactions and lead to IBD (8). Interestingly, in IBD patients that exhibited endoscopic recurrence, the proportion of the most prevalent species from the *Clostridium leptum* group, *Faecalibacterium prausnitzii*, was lower than in patients without endoscopic recurrence (7). Since then, it has been observed that *F. prausnitzii* decreases with the onset of inflammatory status; the relative abundance of *F. prausnitzii* might be a sensor of intestinal health in adults suffering from Crohn's disease (CD), one of the two major IBD (3). This observation strengthens the paradigm of a mucosal protective role of *F. prausnitzii* through its anti-inflammatory potential.

The mechanisms leading to the beneficial protection of *F. prausnitzii* are still poorly understood. *F. prausnitzii* is predominant in the intestine of many other mammals; such ubiquity sug-

gests that *F. prausnitzii* plays an important physiological role (3, 9). In humans, *F. prausnitzii* accounts for more than 3.5% of the total bacterial population (10) and belongs to the functional metagenomic core (11). Because it is one of the most abundant butyrate-producing bacterial species, its beneficial effect had been first attributed to butyrate, well known for its pleiotropic and beneficial effects in the gastrointestinal tract (GIT) (12, 13). However, we showed that *F. prausnitzii*-mediated butyrate production is not the only host health benefit metabolite linked to this species (7). In fact, in acute or chronic colitis models, the immunomodulation potential of *F. prausnitzii* involves uncharacterized secreted metabolites that directly impact the host immune response (7, 14). Moreover, we have recently proposed that *F. prausnitzii* participates in host epithelial homeostasis (15). The establishment of *F. prausnitzii* along the GIT may result from a combination of environmental factors, such as other commensal bacterial species, redox mediators, oxygen concentration, and mucus layer but also bile salt concentrations, as well as pH (16, 17). In early infancy, the abundance of *F. prausnitzii* is very low and increases after the establishment of primocolonizing bacteria (18). The complexity of conditions required for *F. prausnitzii* colonization might explain why the mechanisms accounting for its protection potential *in vivo* are still poorly understood. Thus, we set up a new model of diassociated mice housing *F. prausnitzii* and *Escherichia coli* (as a primocolonizing bacterial companion) to characterize *F. prausnitzii*'s protective role and metabolic profile during a chemically induced acute colitis.

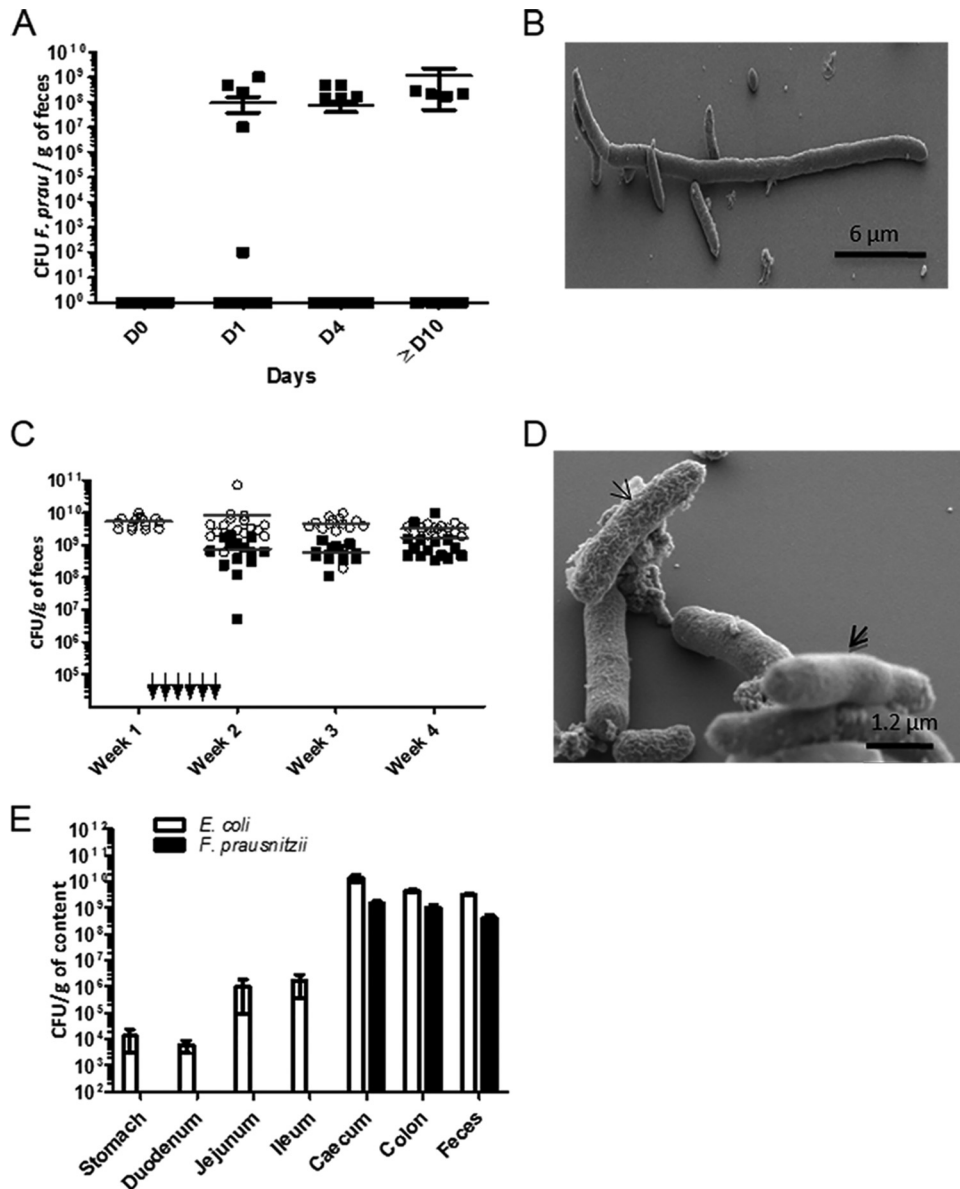
## RESULTS

**Establishment of a stable *E. coli*/*F. prausnitzii*-diassociated mouse model.** We first tried to obtain *F. prausnitzii*-monocolonized mice by intragastric inoculation of  $2 \times 10^8 \pm 1 \times 10^8$  CFU into germfree (GF) mice. After 24 h, no *F. prausnitzii* was detected in the feces, indicating that this species was not able to stably colonize the GIT of GF mice after a single oral inoculation. We then tried to establish *F. prausnitzii* in GF mice by both intragastric and intrarectal inoculations, and as shown in Fig. 1A, we succeeded since the amount of *F. prausnitzii* A2-165 ranged from  $1.5$  to  $5 \times 10^8$  CFU/g of mouse feces. However, this colonization was obtained with a poor yield, with only 5 mice successfully colonized from 18 inoculated. Interestingly, *F. prausnitzii* exhibited an unusual morphology in the cecum of these 5 *F. prausnitzii* mono-associated animals, with cell sizes ranging from 6 to  $25 \mu\text{m}$  (Fig. 1B), which is very rarely seen in *in vitro* cultures.

We thus decided to use *E. coli* as a bacterial companion in order to get stable *F. prausnitzii*-colonized mice (hereafter named the *E. coli*/*F. prausnitzii*-diassociated mouse model). *E. coli* strain JM105 rapidly and stably colonized mice gut during at least 4 weeks after oral inoculation (see Fig. S1 in the supplemental material). By “priming” the GIT of mice with *E. coli* for at least 6 days, a durable and stable implantation of *F. prausnitzii* was systematically obtained in all mice ( $n = 29$ ) (Fig. 1C). The population of *F. prausnitzii* was 10-fold lower ( $3.4 \times 10^8$  to  $2 \times 10^9$  CFU/g of feces) than that of *E. coli* ( $2 \times 10^9$  to  $1.2 \times 10^{10}$  CFU/g of feces). Because of their different morphologies, the two bacterial species *E. coli* and *F. prausnitzii* could easily be distinguished in the cecal content (Fig. 1D). In addition *F. prausnitzii*, as a strict anaerobe species, was only mainly present from the cecum to the rectum, whereas *E. coli* was gradually abundant from the stomach to the colon (Fig. 1E).

**Impact of *F. prausnitzii* on the metabolite profile during inflammation in the gnotobiotic mouse model.** We compared 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in either *E. coli*-monoassociated or *E. coli*/*F. prausnitzii*-diassociated mice in order to establish a metabolite profile under conditions allowing *F. prausnitzii* to display its anti-inflammatory properties (Fig. 2A). To decipher the impact of *F. prausnitzii*, after TNBS induction of colitis, metabolite profiling was performed with ileum, cecum, and colon contents, feces, and serum samples using gas chromatography-time of flight (GC/TOF) mass spectrometry (see Text S1 in the supplemental material) (Fig. 2B). Using a system that combines mass spectrometric and biological metadata, 983 metabolic features were validated. The relational database system BinBase was used for automated metabolites annotation (19, 20), with a criterion of presence in 50% or more of the biological replicates (see Text S1 and Table S1 in the supplemental material). Using a stringent 3-fold-intensity criterion, the number of metabolites distinguishing *E. coli*-monoassociated and *E. coli*/*F. prausnitzii*-diassociated animals greatly varied, depending on the GIT segment (Fig. 2B; see Table S1). In the upper part of the GIT, 86 and 142 metabolites were overrepresented in the ileum and cecum, respectively, in the *E. coli*-monoassociated mice. Conversely, 187 and 482 metabolites were more abundant in the colon and feces from *E. coli*/*F. prausnitzii*-diassociated mice, suggesting a metabolic imprinting correlated to the presence of *F. prausnitzii* (Fig. 1E). A total of 279 metabolic signals were structurally identified and assigned to a compound involved in known chemical reactions (as shown by KEGG number in Table S1). Interestingly, the metabolites identified in the colon mapped onto distinct functional categories (amino acids, energy metabolism, SCFA, lipids, phenolic acid, and sugars), indicating a broad metabolic signature of *F. prausnitzii* (Table 1). Compared to previous metabolomic data (21), our *E. coli*/*F. prausnitzii*-diassociated model displayed many classes of molecules present in conventional animals but absent in axenic animals as energy metabolites, lipids and fatty acids, amino acids, and sugars. Moreover, three metabolites are common: palmitic acid, 3,4-dihydroxyhydrocinnamic acid, and glucuronic acid.

**Protective effect of *F. prausnitzii* on TNBS-induced colitis is linked to specific metabolic signatures in the colon.** In the *E. coli*/*F. prausnitzii*-diassociated mice, all biological markers indicated a decrease of the inflammation level in the presence of *F. prausnitzii*. After colitis induction, weight loss tended to decrease in the presence of *F. prausnitzii* ( $-3.2\% \pm 1.9\%$  compared to  $-5.2\% \pm 1.5\%$ ). The disease activity index (DAI), Wallace macroscopic scores, and Ameho microscopic scores (architectural derangements, goblet cell depletion, edema/ulceration, and degree of inflammatory cell infiltrates) were significantly ( $P < 0.001$ ) decreased in the colons of *E. coli*/*F. prausnitzii*-diassociated mice (Fig. 3A, B, and C). A severe disruption of the epithelial architecture was observed in *E. coli*-infected, TNBS-treated mice in contrast to *E. coli*/*F. prausnitzii*-diassociated, TNBS-treated mice (Fig. 3D). *E. coli*-infected TNBS-treated mouse colon epithelium was characterized by the absence of crypts and massive immune cell infiltrations. Such tissue erosions were less marked in the presence of *F. prausnitzii*. All of these observations indicated a protective effect of *F. prausnitzii* in our TNBS-induced-colitis model. Moreover, the *F. prausnitzii* population level was inversely correlated to the disease activity markers (weight loss, DAI, and Wallace and Ameho scores), suggesting that its anti-inflammatory effects



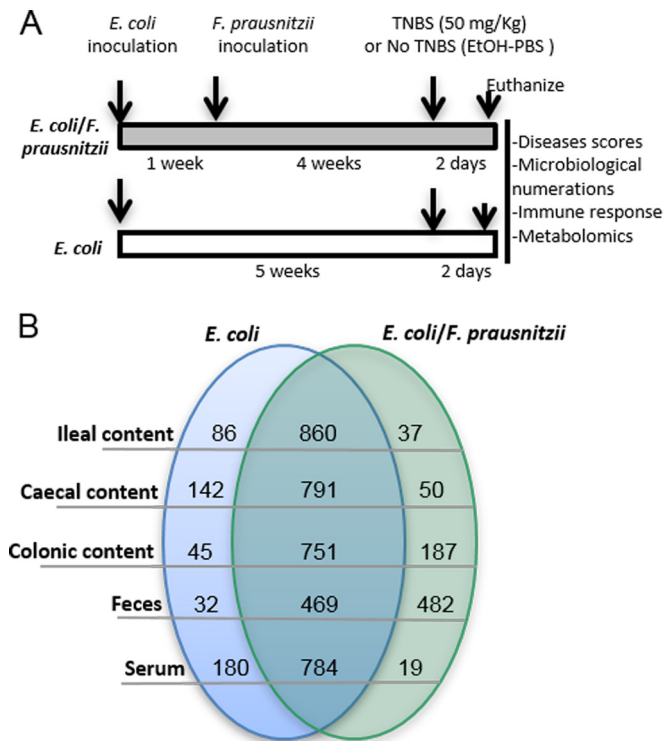
**FIG 1** Setup of the *E. coli*/*F. prausnitzii*-dissociated mouse gnotobiotic model. (A and B) Inoculation of *F. prausnitzii* without *E. coli*. (A) Enumeration in feces of *F. prausnitzii* after gavage in germfree mice ( $n = 18$  inoculations). (B) Scanning electron microscopy images from cecum of *F. prausnitzii*-infected mice. (C, D, and E) Inoculation of *F. prausnitzii* with *E. coli*. (C) Establishment of *E. coli* (open circles) and *F. prausnitzii* (solid squares) in the GIT of mice ( $n = 29$ ) was monitored weekly by enumeration in the feces; arrows represent the various inoculations with *F. prausnitzii* before successful colonization. (D) Scanning electron microscopy images of *E. coli* (thick arrow) and *F. prausnitzii* (thin arrow) in the cecum of *E. coli*/*F. prausnitzii*-dissociated mice. (E) Representation along the GIT of *E. coli* (white bar) and *F. prausnitzii* (black bars) in CFU/g of content in diassociated mice.

could be dose dependent (see Fig. S2 in the supplemental material).

Regarding metabolic profiles, we obtained strong evidence of the magnitude of the effect of an *E. coli*/*F. prausnitzii* microbial context in the TNBS-treated animals. The rarefaction curve showed that 80% of the known metabolites were more represented in the colon content of dixenic mice (see Fig. S3 in the supplemental material). We searched for metabolites segregating the monoxenic and dixenic animals after induction of colitis (*t* test on the mean). For instance, in the colon, butyrate (4-hydroxybutyric acid) ( $P = 0.0006$ ), shikimic acid ( $P = 0.017$ ) related to the salicylic acid pathway ( $P = 0.2055$ ), and indole-3-

lactate ( $P = 0.0035$ ) were overrepresented in the *E. coli*/*F. prausnitzii*-dissociated mice (Table 1 and Fig. 3E). The metabolomic profiles sharply differed according the gut segment (Fig. 2B). For instance, raffinose was more discriminatory in the ileum ( $P = 0.0091$ ) (data not shown) than in the colon ( $P = 0.1703$ ) and was negatively related to *F. prausnitzii* abundance (Fig. 1E).

***F. prausnitzii* is a metabolically active species in vivo.** The beneficial mitigation effects, observed locally in the colon in the presence of *F. prausnitzii*, were confirmed in the serum, where systemic inflammation markers were reduced. First, the degree of infiltration by polymorphonuclear neutrophils (estimated by tissue myeloperoxidase [MPO] activity) was significantly decreased



**FIG 2** Experimental design of TNBS-induced colitis and metabolomic profiling. (A) Protocol of TNBS induction of colitis in *E. coli*-monoassociated and *E. coli/F. prausnitzii*-dissociated BALB/c mice. Different readouts are indicated in the box. (B) Numbers and comparison, using a stringent 3-fold-intensity criterion, of metabolites in the GIT segments and in the serum of *E. coli*-monoassociated and *E. coli/F. prausnitzii*-dissociated mice after a TNBS-induced colitis, identified by mass spectrometric and biological meta-data.

during TNBS-induced colitis in the presence of *F. prausnitzii* ( $P < 0.001$ ) (Fig. 4A). In the presence of *F. prausnitzii* after TNBS induction of colitis, the serum cytokine response was globally similar to the one measured without TNBS (see Table S2 in the supplemental material). Interestingly, in the serum, the levels of proinflammatory cytokines, such as interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-2, and IL-5, were statistically decreased to a level corresponding to a noninflammatory status (Fig. 4B).

The high number of metabolites detected in the serum samples was noteworthy (Fig. 2B; see Table S1 in the supplemental material). Nineteen metabolites were overrepresented in the serum of *E. coli/F. prausnitzii*-dissociated mice. Seven were only found in the serum, and 11 were also found in the colon, where *F. prausnitzii* was most prevalent. Four metabolites were present in ileum, cecum, colon, and feces, but they are so far unknown (BinBase no. ID436364, -269147, -200610, and -310063) (Fig. 4C). The serum metabolome shared 5 entities with both colon and feces (uric acid, elaidic acid, lyxose, and unknown compounds BinBase ID231544 and ID459916). As observed in the colon content, the presence of *F. prausnitzii* is correlated to high levels of salicylic acid ( $P = 0.367$ ) and shikimic acid ( $P = 0.4328$ ) (Fig. 4D). Strikingly,  $\alpha$ -ketoglutaric (AKG) acid was 10-fold more abundant in the serum of *E. coli/F. prausnitzii*-dissociated mice ( $P = 0.028$ ), together with the linked citric acid and uric acid pathway ( $P = 0.085$  and  $P = 0.0733$ , respectively). AKG acid is important in ammonia

recycling and is reported to be depleted during gastrointestinal dysbiosis (22). Its involvement in cell proliferation and differentiation through the activity of the AKG-acid-dependent enzymes has recently been reviewed (23). The concomitant analysis of salicylic, shikimic, and AKG acids as standard in the GC mix validated their identification.

**The four metabolites specifically associated with the presence of *F. prausnitzii* possess anti-inflammatory capacities *in vitro*.** We tested the immunomodulatory properties of four candidate molecules in a human colon adenocarcinoma cell line model (HT-29) by using *F. prausnitzii* supernatant, butyrate, and the 5-aminosalicylic acid (5-ASA) as controls. As shown in Fig. 5, salicylic acid (10  $\mu$ M) blocked IL-8 production, as did butyrate (10 mM), *F. prausnitzii* supernatant, and 5-ASA (20 mM). Shikimic acid, raffinose, and AKG acid (100  $\mu$ M) did not block IL-8 production, and a mixture of the different molecules did not show any additional effect (data not shown).

## DISCUSSION

In this study, we show for the first time that *F. prausnitzii* is a highly active commensal bacterium involved in the reduction of colitis through *in vivo* modulation of metabolites along the GIT and in the peripheral blood. This novel approach clearly confirms the anti-inflammatory potential of *F. prausnitzii* and provides new clues about its ecological lifestyle and mechanisms in intestinal physiopathology.

We showed that efficient *F. prausnitzii* implantation requires the GIT preparation by *E. coli*, a primocolonizer of the sterile human newborn gut. Such an implantation kinetic is concordant with previous reports (15, 24) and with the late presence of *F. prausnitzii* in the human gut 7 months after birth (18). Thus, the time needed for *F. prausnitzii* colonization could correspond to the decrease of the redox potential ( $E_h$ ), leading to more reduced conditions in the lower GIT (15, 25). However, other ecological factors should not be excluded, such as physiological modification of nutrient sources by the other species leading to the onset of an efficient trophic chain. Interestingly, sugars and energy metabolisms were deeply modified in the presence of *F. prausnitzii* (Table 1), suggesting a modification of their availability. While *E. coli* abundance gradually increased from stomach to colon, *F. prausnitzii* localization seemed to depend upon anaerobic conditions only found in the lower gut. These observations confirm that gradients in the physicochemical conditions along the GIT influence the biogeography of the colonic mucosal microbiome (26). Thus, our gnotobiotic rodents can be considered a relevant model to provide ecological information about GIT colonization kinetics by extremely oxygen-sensitive species. Moreover, we showed that the colonization pattern of *F. prausnitzii* in the GIT reflects its metabolic imprinting. *F. prausnitzii*, abundant in the lower GIT (cecum and colon) (Fig. 1E), had a gradually metabolic impact along the GIT (Fig. 2B). Even if *F. prausnitzii* was absent in the ileum, metabolic changes were observed in the ileum and at a distance in the serum (Fig. 4C and D). By using ileal content, we counted the *F. prausnitzii* population, and we cannot exclude that bacteria were present under the detection threshold or in a viable-but-not-culturable state. We detected 86 metabolites specific for *E. coli* in the ileum and 37 that are linked to the presence of *E. coli* and *F. prausnitzii* (Fig. 2B). Twenty-eight are unknown, and 9 could be identified (see Table S1 in the supplemental material). Among them are sugars, an antioxidant (*trans*-sinapinic acid),



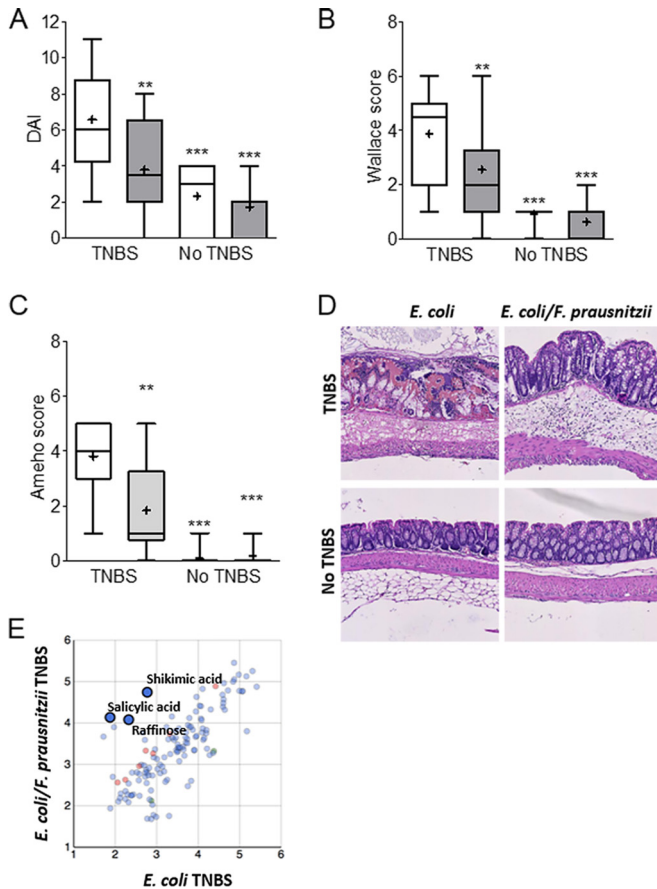
TABLE 1 Known metabolites statistically overrepresented in the colon of *E. coli*/*F. prausnitzii*-dissociated mice compared to *E. coli*-monoassociated mice after TNBS-induced colitis

Class	Metabolite	KEGG/PubChem ID no.	Dixenic/monoxenic intensity ratio	SEM	P value
Amino acids	Spermidine minor	C00315	7.675	0.69	0.009
	<i>N</i> -Acetylaspartic acid	SID 136743417	5.472	0.29	0.000
	Quinic acid	C00296	3.682	0.30	0.019
	<i>O</i> -Acetylserine	C00979	2.821	0.12	0.0005
	Isothreonic acid	SID 103172351	2.220	0.17	0.047
	Threonic acid	C01620	2.120	0.11	0.012
	Homocystine	C01817	0.490	0.06	0.031
	2-Ketoisocaproic acid	C00233	0.402	0.05	0.003
Energy metabolites	Phosphoric acid	C00009	5.334	0.39	0.003
	Glucose-6-phosphate	C00092	2.354	0.11	0.005
	Fructose-6-phosphate	C00085	2.0364	0.10	0.015
	Glycerol	C00116	2.0206	0.07	0.003
	$\beta$ -Sitosterol	C01753	0.0449	0.13	0.020
Lipids and fatty acids	4-Hydroxybutyric acid	C00989	3.277	0.16	0.000
	Palmitic acid	C00249	2.765	0.17	0.014
	Caprylic acid	SID 11528290	2.637	0.12	0.001
	Ethanolamine	C00189	4.854	0.22	0.000
	Phytol	C01389	2.4277	0.15	0.015
	Squalene	C00751	2.004	0.13	0.046
Phenolic acids	Idonic acid	SID 135170473	3.779	0.40	0.049
	<i>trans</i> -Sinapinic acid	C00482	2.618	0.14	0.003
	Syringic acid	C10833	2.180	0.11	0.008
	Malonic acid	C04025	2.131	0.14	0.037
	3-Phenyllactic acid	C05607	2.080	0.10	0.011
	3,4-Dihydroxyhydrocinnamic acid	SID 111677818	0.269	0.06	0.001
Pyrimidine	Pseudouridine	C02067	2.556	0.17	0.019
	Orotic acid	C00295	0.279	0.09	0.011
Sugars	Fructose	C00095	5.023	0.29	0.001
	Mannose	C00159	2.896	0.23	0.025
	Glyceric acid	C00258	2.644	0.10	0.000
	<i>N</i> -Acetyl-D-hexosamine	C03136	2.591	0.19	0.036
	1,5-Anhydroglucitol	C07326	2.484	0.18	0.027
	$\beta$ -Gentiobiose	C08240	2.423	0.11	0.003
	Arabinose	C00259	2.356	0.11	0.005
	Tagatose	SID 163300922	2.240	0.16	0.037
	Ribonic acid	C01685	2.187	0.11	0.013
	2-Deoxyerythritol	SID 108236	2.002	0.11	0.023
	Glucuronic acid	C00191	0.321	0.02	0.000
	Glycero-guloheptose	SID 117585658	0.085	0.07	0.001
	Raffinose	C00492	0.024	0.15	0.027
	Inulotriose	SID 4849	0.004	0.03	0.000
Other	Indole-3-lactate	C02043	5.427	0.39	0.003
	Shikimic acid	C00493	2.220	0.13	0.017
	Putrescine	C00134	0.487	0.05	0.015

and neurotransmitter precursors (5-amino valeric acid lactone, a homologue of  $\gamma$ -aminobutyric acid [GABA] [27], and *N*-acetyl aspartic acid [28]). In the literature, an impact of *F. prausnitzii* on ileal physiology and particularly permeability has been already reported (29). These results, and similar findings reported by Velagalpuladi et al. (21), suggest that a direct or indirect effect of *F. prausnitzii* can be observed at a systemic level and not only where its population level is high.

During dysbiosis, the site-specific colonization and the domi-

nance of *F. prausnitzii* in the colon are affected, which might contribute to the etiology of diseases of the large intestine (24). We have previously shown that *F. prausnitzii* presents anti-inflammatory properties in acute and chronic TNBS-induced-colitis models in conventional mice (7, 30), and this result was also observed in simplified gnotobiotic model. Moreover, our model allowed us to compare the population levels of *E. coli* and *F. prausnitzii* in feces to assess the impact of TNBS induction of colitis on bacterial populations (see Fig. S2 in the supplemental material). A



**FIG 3** Analysis of colonic responses to a TNBS-induced colitis. (A) Disease activity index (DAI), (B) Wallace macroscopic score, (C) Ameho histologic score, (D) representative hematoxylin-eosin-safran staining of colonic tissue sections from *E. coli*-monoassociated (white bars) ( $n = 16$  and  $12$ ) and *E. coli/F. prausnitzii*-dissociated (gray bars) ( $n = 18$  and  $11$ ) mice with or without colitis, respectively. (E) Metabolomic analysis of *E. coli*-monoassociated ( $n = 8$ ) compared to *E. coli/F. prausnitzii*-dissociated ( $n = 9$ ) mouse colon content after TNBS treatment. The values are expressed as box-and-whisker plots with interquartile range, with the top portion of the box representing the 75th percentile and the bottom portion representing the 25th percentile. The horizontal bar within the box represents the median, with the mean shown as “+.” Asterisks indicate significant differences: \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ . Metabolomics results are expressed as log intensities of metabolites with KEGG ID ( $t$  test, red and green,  $P \leq 0.05$ ).

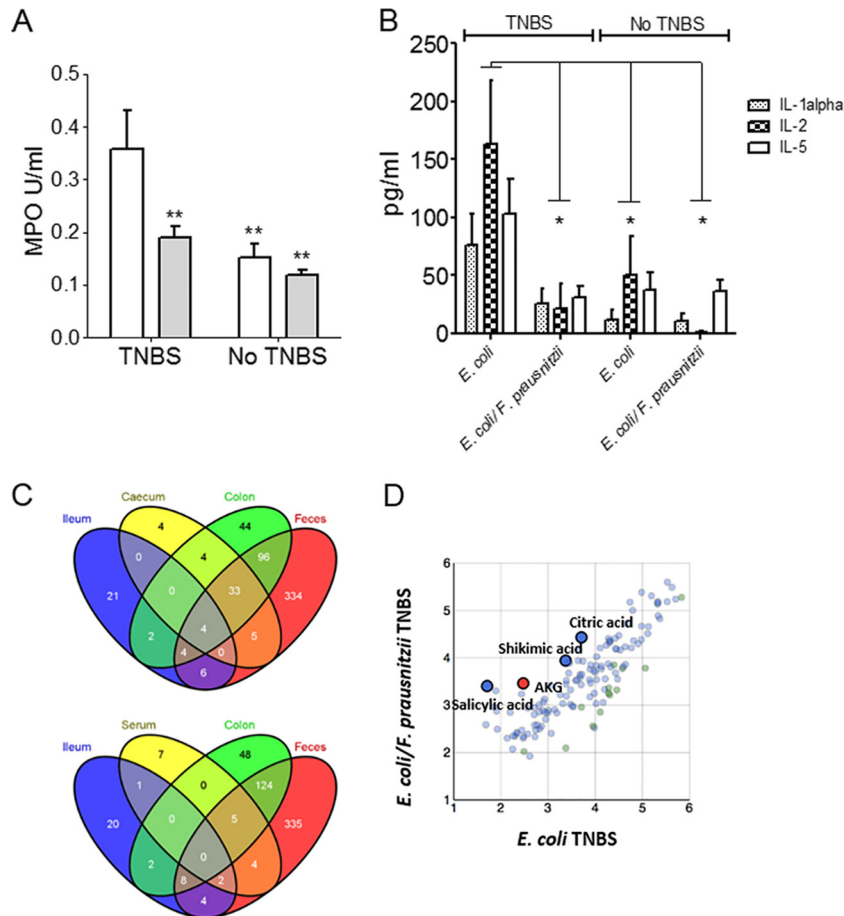
TNBS challenge decreased the *F. prausnitzii* population, and the remaining level of *F. prausnitzii* clustered the mice into two groups (threshold of  $10^8$  *F. prausnitzii* cells/g of feces). Inflammation markers were decreased when the *F. prausnitzii* population was high. Because the level of colitis mitigation was directly correlated to its GIT abundance, our results confirmed that *F. prausnitzii* might be both a biomarker and a major factor of human health in adults (3). A review suggested that an increase in oxygen tension in the gut, related to the oxidative burst due to inflammatory reaction itself, could be the cause of the dysbiosis in IBD patients (25). Our results allowed us to hypothesize that the decreased *F. prausnitzii* level observed in IBD patients is a consequence of inflammation but also participates in the inflammation process, leading to the disease’s vicious circle (31).

In our simplified microbiota, we identified metabolites that can be produced either by the host or by the bacteria. Moreover,

most of those metabolites are not assigned to any functions. This reflects the paucity of knowledge about reciprocal functional adaptation between host and microbes and the high percentage of microbial genes lacking annotation in metagenomic studies (1, 32). Li et al. had identified *F. prausnitzii* as an active bacterial species in humans, modifying butyrate-derived metabolites in urine (11). Using our metabolomic approach, we confirmed that the amount of 4-hydroxybutyric acid was increased in feces, colon, and cecum, where *F. prausnitzii* was established (see Table S1 in the supplemental material). Butyrate plays a major role in gut homeostasis, particularly through its anti-inflammatory activities in the colon mucosa (33, 34). This impact was confirmed *in vitro* using concentrations similar to those found in *F. prausnitzii* culture supernatants (10 mM) (Fig. 5). However, its role remains controversial since its effects seem to be dose and time dependent but also depend on the cellular model used (14, 35). In our study, butyrate specifically produced by *F. prausnitzii* (see Fig. S4A in the supplemental material) was not directly linked to health parameters (see Fig. S4B).

Based on our metabolomic approach, we identified metabolites specifically associated with the presence of *F. prausnitzii*. These findings support the concept that beneficial effects from commensals are linked to their large metabolic impact on host physiology. In fact, salicylic acid, at a concentration close to the one detected in the colon, decreased IL-8 production *in vitro* (Fig. 5). Interestingly, 40% of *Fusobacterium prausnitzii* (former nomenclature for *F. prausnitzii*) strains were able to ferment salicin, an alcoholic  $\beta$ -glucoside that can be converted into salicylic acid (36). In the pharmaceutical industry, salicylic acid is the precursor of 5-ASA, a drug used in the treatment of IBD (37). We showed that salicylic acid was as effective as 20 mM 5-ASA *in vitro* (38). Furthermore, we identified shikimic acid, a precursor for the biosynthesis aromatic compounds, including salicylic acid through the achorismate synthase pathway (39). Shikimic acid, upstream in the pathway of 5-ASA production, did not have the same anti-inflammatory effect *in vitro*. However, *in vivo*, it is demonstrated that 3,4-oxo-eisopropylideneshikimic acid (ISA), a derivative of shikimic acid, protects against TNBS-induced colonic damage in rats (40). We also identified raffinose, an oligosaccharide only fermented by the gut microbiota since monogastric animals do not express pancreatic  $\alpha$ -galactosidase (41). Its uptake pathways are affected in celiac disease, whereas only transcellular uptake is affected in CD patients. Mucosal permeability has been found to be an important factor for raffinose permeation (42). Velagapudi et al. detected this metabolite in much higher abundance in GF than in conventional animals (21). *F. prausnitzii* impacts the host inflammation pattern but could also play a key role in maintaining gut permeability (29, 43), intestinal epithelial homeostasis (15), and energy balance, particularly through sugar pathway (44) (Table 1).

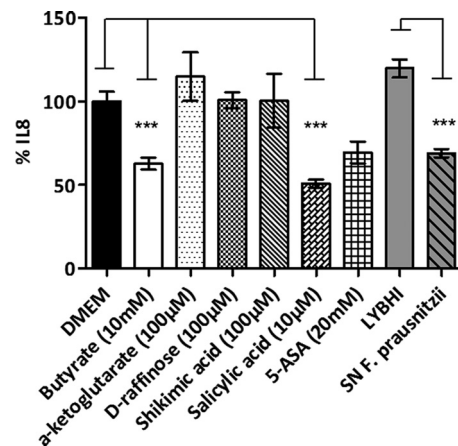
In contrast to the metabolites overrepresented in the presence of *F. prausnitzii*, underrepresented ones could be involved in anti-inflammatory process (Table 1). For instance, a significant decrease in homocystine was observed in the presence of *F. prausnitzii*. This amino acid results from the oxidation of homocysteine, which has been reported to be proinflammatory (45). Also, a decrease in putrescine together with an increase in spermidine in the dixenic mice could inhibit the secretion of inflammatory cytokines (46). Our approach provided a lot of hypotheses



**FIG 4** Analysis of serum immune response after TNBS-induced colitis. (A) MPO activity from *E. coli*-monoassociated (white bars) ( $n = 16$  and  $12$ ) and *E. coli/F. prausnitzii*-diassociated (gray bars) ( $n = 18$  and  $11$ ) mice with or without colitis, respectively. (B) Blood cytokine levels (IL-1 $\alpha$ , IL-2, and IL-5) from *E. coli*-monoassociated ( $n = 9$  and  $8$ ) and *E. coli/F. prausnitzii*-diassociated ( $n = 10$  and  $8$ ) mice with or without colitis, respectively. (C) Venn diagram depicting common metabolites between gut contents (cecum, colon, ileum, and feces) and serum (serum, colon, ileum, and feces). (D) Serum metabolites from *E. coli*-monoassociated ( $n = 8$ ) compared to *E. coli/F. prausnitzii*-diassociated ( $n = 10$ ) mice after TNBS treatment. Values are expressed as the mean  $\pm$  SEM. Asterisks indicate significant differences: \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ . Metabolomic data are expressed as log intensities of metabolites with KEGG ID ( $t$  test, red and green,  $P \leq 0.05$ ).

about the role of *F. prausnitzii* in intestinal physiopathology for future investigations.

The serum is a biofluid providing an excellent opportunity to assess the extent of interplay between bacterial metabolic and systemic human pathways (47). *F. prausnitzii* modulated the metabolome of the host blood by decreasing IL-1 $\alpha$ , IL-2, and IL-5 concentrations. For instance,  $\alpha$ -ketoglutaric (AKG) acid was statistically detected in the serum of mice colonized with *F. prausnitzii* and *E. coli* (Fig. 4). This organic acid, important for the proper metabolism of all essential amino acids and the transfer of cellular energy in the citric acid cycle, is a glutamic acid precursor involved in protein synthesis and the regulation of blood glucose levels (48). AKG did not show any significant effect on IL-8 production by HT-29 in our *in vitro* model (Fig. 5). This may mean that our conditions are not appropriate or that, as reported by Cairns and Mak (23), AKG acid may need a specific transporter/biochemical transformation before being active. Individuals with high protein intake, bacterial infections, or gastrointestinal dysbiosis may benefit from supplemental AKG acid to help metabolic health. Personalized health care investigations could be routinely



**FIG 5** Immunomodulation capacities of *F. prausnitzii* supernatant and identified molecules *in vitro*. IL-8 production in TNF- $\alpha$ -stimulated HT-29 cells is shown. Experiments were done in triplicate. Results are expressed as picograms of IL-8 per milligram of protein and have been normalized with the IL-8 produced after the coincubation with PBS or LYBHI medium. SN, *F. prausnitzii* supernatant (20%). \*\*\*,  $P \leq 0.001$ .

set up to target inflammation biomarkers or check *F. prausnitzii* mitigation through salicylic, shikimic, and AKG acid levels in feces or serum.

To conclude, *F. prausnitzii* is a highly metabolic bacterium that impacts the host via different pathways to preserve its homeostasis. We have identified metabolites, particularly salicylic acid, involved in its anti-inflammatory capacities but also highlighted the complex and dynamic cross talk with the host under inflammation conditions.

## MATERIALS AND METHODS

**Bacterial culture.** *F. prausnitzii* A2-165 (DSM 17677), isolated from human fecal stool (49), was grown at 37°C in YBHI medium (brain-heart infusion medium supplemented with 0.5% yeast extract [Difco]) supplemented with cellobiose (1 mg/ml [Sigma]), maltose (1 mg/ml [Sigma]), and cysteine (0.5 mg/ml [Sigma]) in an anaerobic chamber filled with 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% H<sub>2</sub>. *Escherichia coli* JM105 was grown on LB medium (Difco) at 37°C.

**Animals and experimental design.** Germfree 7- to 8-week-old BALB/c mice (male) were obtained from the germfree rodent breeding facilities of Anaxem-Micalis (INRA, Jouy-en-Josas, France). Animals were kept in flexible-film isolators (Getinge-La Calhène, Vendôme, France) in standard cages (5 mice/cage) with sterile wood shavings as animal bedding. Mice were given free access to autoclaved tap water and to a standard diet, R03-40 (Scientific Animal food and Engineering, Augy, France), sterilized by gamma irradiation at 45 kGy (IBA Mediris, Fleurus, Belgium). Isolators were maintained under controlled conditions of light (0730 to 1930 h), temperature (20 to 22°C), and humidity (45 to 55%). All procedures were carried out according to European Community Rules of Animal Care and with authorization 12-092 from the French Veterinary Services.

**Inoculation of germfree mice with *F. prausnitzii*.** To obtain *F. prausnitzii*-monoassociated mice, germfree animals receive both intragastric and intrarectal inoculations of a 100× concentrated culture of *F. prausnitzii* A2-165 (10<sup>9</sup> CFU/inoculation). For intragastric gavages, mice were first treated with 0.1 ml of sodium bicarbonate (0.2 M) and then received 0.5 ml of the bacterial suspension 10 min later. For intrarectal inoculation, mice were first anesthetized by an intraperitoneal injection of a volume (1% of the body weight) of 10% ketamine hydrochloride (Imalgem 500 [Meril]) and 6% xylazine (Rompun [Bayer]) in 0.9% NaCl and then treated with 0.2 ml of the bacterial suspension.

To obtain dioxenic *E. coli*/*F. prausnitzii*-dissociated mice, germfree mice were orally inoculated with a fresh culture of *E. coli* JM105 (10<sup>8</sup> to 10<sup>9</sup> CFU/ml). Twenty-four hours later, when *E. coli* was established in the GIT, 10<sup>9</sup> CFU of *F. prausnitzii* at 0.2 ml was administered to a donor mouse by intragastric gavage as described above. As soon as *F. prausnitzii* was established in the donor mouse, its feces were homogenized in water inside the isolator and immediately administered by intragastric gavage to other mice pretreated with sodium bicarbonate.

Viable bacteria in the inoculum were enumerated before and after gavage by plating of appropriate dilutions on supplemented YBHI agar in the anaerobic chamber. To enumerate the A2-165 strain in feces, fresh samples were introduced into the anaerobic chamber, serially 10-fold diluted, and plated on supplemented YBHI agar. In order to selectively enumerate A2-165 in the feces of dioxenic mice by eliminating *E. coli* cells, fecal samples were treated with sodium azide (3%) for 5 min at 37°C and then plated on sodium azide (0.02%) YBHI agar.

**TNBS-induced colitis.** Mice were weighed before TNBS administration and anesthetized by an intraperitoneal injection as described above. TNBS (Sigma-Aldrich) was dissolved in 0.9% NaCl-ethanol (50:50 vol/vol). Fifty microliters of TNBS (50 mg/kg body weight) was administered intrarectally (using a 3.5 French catheter [Solomon Scientific]) to mice maintained for 30 s in a vertical position. Control mice received 50 μl of 0.9% NaCl-ethanol solution intrarectally. Inflammation was monitored 48 h after TNBS administration.

**Scanning electron microscopy.** Scanning electron microscopy analyses were performed at the MIMA2 platform (INRA, Massy, France) from 0.2 g of cecal samples as previously described (50).

**Tissue preparation for histological analysis.** Flushed colons were opened longitudinally, cut into 2-cm sections, and rolled according to the Swiss roll procedure (51). The samples were fixed in 4% paraformaldehyde (4 h, room temperature), dehydrated, and embedded in paraffin according to standard histological protocols. Four-micrometer sections of distal colon were mounted on SuperFrost Plus slides. To assess TNBS-induced colitis, the sections were stained with hematoxylin-eosin and examined blindly according to the Ameho criteria (52).

**Inflammation score assessment of TNBS-induced colitis.** The colon was removed without mesenteric fat and mesentery, carefully opened, and cleaned with phosphate-buffered saline (PBS). Colonic damage and inflammation were assessed blindly according to the Wallace score (53).

**MPO activity.** MPO activity was measured using the method of Bradley et al. (54), modified as follows. Tissue samples were homogenized (50 mg/ml) in ice-cold 50-mM potassium phosphate buffer (pH 6) containing 5% hexadecyl trimethyl ammonium bromide (Sigma-Aldrich) and hydrogen peroxide. MPO is expressed in units per milligram of wet tissue.

**Cytokine analysis.** Colonic samples were homogenized in 400 μl of Tris-HCl buffer containing protease inhibitors (Sigma-Aldrich) in a tissue lysers. Samples were centrifuged for 20 min, and the supernatant was frozen at -80°C until further analysis. Before sacrifice, blood samples were obtained from the retro-orbital venous plexus and centrifuged, and sera were stored at -80°C. Cytokine levels were determined by flow cytometry using Cytometric bead array analysis (mouse inflammation kit) (BD, NJ).

**Metabolite extraction and analysis.** Fifty microliters of serum and feces, intestinal content, cecal content, and colonic content were lyophilized and subjected to 10 s of bead beating. Metabolite extraction was performed as previously described (20) with 700 μl of a precooled methanol-isopropanol-water (3:3:2) mixture in an ultrasonic water bath (5 min). After centrifugation, 650 μl of the supernatant was collected (5 min, 15,000 rpm) and dried before derivatization. Metabolite analysis was performed according to Fiehn et al. (55).

**In vitro experiments with the HT-29 cell line.** The protocols used in this study were adapted from Kechaou et al. (56). The human colon carcinoma cell line HT-29 was cultured and stimulated with recombinant human tumor necrosis factor alpha (TNF-α) (5 ng/ml; PeproTech, NJ) as described previously. Cell incubations were done in the presence or not of different metabolites tested at the indicated concentration: butyric acid sodium salt, reference 303410, Sigma-Aldrich; shikimic acid, reference S5375, Sigma-Aldrich; salicylic acid, reference 84210, Sigma-Aldrich; D-(+)-raffinose pentahydrate, reference R0514, Sigma-Aldrich; α-ketoglutaric acid disodium salt dihydrate, reference 75892, Sigma-Aldrich; 5-aminosalicylic acid, reference 18858, Sigma-Aldrich. All samples were analyzed in triplicate. After incubation, cell supernatants were collected and mixed with an antiprotease cocktail according to the manufacturer's instructions (Complete EDTA-free protease inhibitor; Roche Applied Bioscience), and frozen at -80°C until further analysis of interleukin-8 (IL-8) concentrations by an enzyme-linked immunosorbent assay (ELISA) (BioLegend, San Diego, CA, and Mabtech, Sweden, respectively).

**Statistical analysis.** Data are reported as means ± standard errors of the means (SEM) and for figures using box-and-whisker plots with the interquartile range, where the top portion of the box represents the 75th percentile and the lower portion represents the 25th percentile. The horizontal bar within the box represents the median, with the mean shown as "+." The upper horizontal bar outside the box represents the 95th percentile, and the lower horizontal bar outside the box represents the 15th percentile. Statistical analysis was performed using one-way analysis of variance followed by Student-Newman-Keuls multiple comparison *post hoc* analysis, and with a *P* value of <0.05 considered significant. For metabolomic analysis, the resulting data sets were imported into the Mat-



Lab software for univariate and multivariate statistical analyses. Univariate statistics evaluated the number and percentage of features that are either unique or vary significantly between *E. coli*-monoassociated and *E. coli/F. prausnitzii*-dissociated animals. Venn diagrams were constructed as described in reference 57.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00300-15/-/DCSupplemental>.

Text S1, DOC file, 0.04 MB.  
Figure S1, TIF file, 0.02 MB.  
Figure S2, TIF file, 0.1 MB.  
Figure S3, TIF file, 0.1 MB.  
Figure S4, TIF file, 0.1 MB.  
Table S1, XLS file, 0.1 MB.  
Table S2, DOC file, 0.1 MB.

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