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Akkermansia muciniphila Adheres to Enterocytes and Strengthens the Integrity of the Epithelial Cell Layer

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***Akkermansia muciniphila* is a Gram-negative mucin-degrading bacterium that resides in the gastrointestinal tracts of humans and animals. *A. muciniphila* has been linked with intestinal health and improved metabolic status in obese and type 2 diabetic subjects. Specifically, *A. muciniphila* has been shown to reduce high-fat-diet-induced endotoxemia, which develops as a result of an impaired gut barrier. Despite the accumulating evidence of the health-promoting effects of *A. muciniphila*, the mechanisms of interaction of the bacterium with the host have received little attention. In this study, we used several *in vitro* models to investigate the adhesion of *A. muciniphila* to the intestinal epithelium and its interaction with the host mucosa. We found that *A. muciniphila* adheres strongly to the Caco-2 and HT-29 human colonic cell lines but not to human colonic mucus. In addition, *A. muciniphila* showed binding to the extracellular matrix protein laminin but not to collagen I or IV, fibronectin, or fetuin. Importantly, *A. muciniphila* improved enterocyte monolayer integrity, as shown by a significant increase in the transepithelial electrical resistance (TER) of cocultures of Caco-2 cells with the bacterium. Further, *A. muciniphila* induced interleukin 8 (IL-8) production by enterocytes at cell concentrations 100-fold higher than those for *Escherichia coli*, suggesting a very low level of proinflammatory activity in the epithelium. In conclusion, our results demonstrate that *A. muciniphila* adheres to the intestinal epithelium and strengthens enterocyte monolayer integrity *in vitro*, suggesting an ability to fortify an impaired gut barrier. These results support earlier associative *in vivo* studies and provide insights into the interaction of *A. muciniphila* with the host.**

Akkermansia muciniphila is a Gram-negative anaerobe belonging to the *Planctomycetes-Verrucomicrobia-Chlamydiae* superphylum (1). *A. muciniphila* has been found to inhabit the gastrointestinal (GI) tracts of more than 90% of adult subjects analyzed, and it constitutes 1 to 4% of the fecal microbiota (2). *A. muciniphila* is capable of using intestinal mucins, the highly glycosylated proteins of the epithelial mucus layer, as its sole source of carbon and nitrogen (1). Therefore, it is not surprising that this organism has also been detected in high numbers in mucosal biopsy specimens of the human colon (3). The genome of *A. muciniphila* contains a large proportion of genes encoding secreted proteins (567 of the 2,176 open reading frames), 61 of which have been assigned protease, sugar hydrolase, sialidase, or sulfatase activities, suggesting specialization in mucus utilization and adaptation to the gut environment (4). There is growing evidence that *A. muciniphila* is associated with gut health; e.g., fewer *A. muciniphila* cells have been detected in ulcerative colitis (UC) and Crohn's disease (CD) patients, both in clinically active disease and during remission, than in healthy individuals (5, 6). An inverse correlation between *A. muciniphila* levels and the severity of acute appendicitis has also been shown (7). Moreover, fecal counts of *A. muciniphila* cells correlate with the richness of bacterial species and correlate inversely with type 1 diabetes, body weight, and markers of inflammation (8–10). The effects of *A. muciniphila* and its metabolites on mucosal gene expression patterns have been studied using gnotobiotic mice and mouse gut organoids, respectively (11, 12). These studies showed that *A. muciniphila* elicits distinctive changes in the expression of pathways involved in metabolic homeostasis and immune tolerance (11, 12). In addition, *A. muciniphila* has been demonstrated to improve the metabolic profiles of type 2 diabetic mice, to restore mucus layer thickness, and to counteract high-fat-diet-induced lipopolysaccharide (LPS) endotoxemia in obese mice (13).

The ability of intestinal bacteria to adhere to the host epithelium is considered important for efficient colonization and interaction with the host, although experimental data are scarce. In principle, colonizing bacteria can adhere either to the protective mucus gel covering the epithelial cell layer or directly to the enterocytes. In a healthy colon, the epithelial cell layer is fully covered by a thick mucus gel layer, whereas in the small intestine, the mucus layer is thinner and discontinuous (14), allowing for direct contacts between bacteria and host enterocytes. Despite the accumulating evidence for the involvement of *A. muciniphila* in intestinal and metabolic health, the basic mechanisms of interaction with the host have received little attention. In this study, we investigated the adhesion of *A. muciniphila* to human colonic mucus,

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the Caco-2 and HT-29 intestinal epithelial cell lines, and several extracellular matrix (ECM) proteins. In addition, we used *in vitro* models to study the effects of *A. muciniphila* on epithelial integrity and interleukin-8 (IL-8) release by enterocytes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. muciniphila* BAA-835^T (American Type Culture Collection) cells were grown in mucin medium (1), and *Bacteroides fragilis* E-022248^T (= DSM 2151 = ATCC 25285) obtained from the VTT Culture Collection (VTT Technical Research Center of Finland, Espoo, Finland) was cultivated on *Brucella* agar with hemin and vitamin K (Fluka) supplemented with 5% sheep blood. Both strains were grown at 37°C for 2 days in an anaerobic incubator under an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 (Concept Plus anaerobic workstation; Ruskinn Technology Ltd.). *Escherichia coli* K-12-derived TOP10 cells (Invitrogen) were grown with agitation (220 rpm) overnight at 37°C in Luria-Bertani (LB) medium. *Lactobacillus rhamnosus* GG (ATCC 53103) was cultivated overnight in static de Man-Rogosa-Sharpe (MRS) broth (Difco) at 37°C. For the adhesion experiments, bacterial cells were metabolically labeled by supplementing the growth medium with 10 μl ml^{-1} of [6′-^{3}H]thymidine (14.4 Ci/mmol; PerkinElmer).

Epithelial cell lines. The Caco-2 (ACC 169) and HT-29 (ACC 299) human colonic epithelial cell lines were purchased from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). The cell lines were grown at 37°C under an oxic atmosphere in an incubator supplemented with 5% CO_2. Caco-2 cells were cultivated in RPMI 1640 (Sigma) containing 20% heat-inactivated (30 min at 56°C) fetal calf serum (FCS; Integro B.V.), 1% nonessential amino acids (Lonza), 15 mM HEPES (Lonza), 2 mM L-glutamine (Lonza), and 100 U ml^{-1} penicillin and streptomycin (PEST; Lonza). HT-29 cells were grown in McCoy 5A medium (Lonza) supplemented with 10% heat-inactivated FCS and with PEST.

Assessment of viability of *Akkermansia muciniphila* under an oxic atmosphere. Anaerobically grown *A. muciniphila* cells were washed once with phosphate-buffered saline (PBS; pH 7.4), which was kept overnight in the anaerobic incubator to remove any oxygen from the buffer. The bacterial cell suspension was diluted to an optical density at 600 nm (OD_{600}) of 0.25. The cell suspension was divided into three 96-well microplates, with 100 μl well^{-1}, and the microplates were incubated for 1 h at 37°C either in the anaerobic incubator, in an incubator with an oxic atmosphere supplemented with 5% CO_2, or under a normal oxic atmosphere. Next, the cells were live-dead stained by adding 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) at final concentrations of 1 μg ml^{-1} and 0.75 μg ml^{-1}, respectively, and incubating for 15 min at room temperature, followed by one wash with PBS. The fluorescence of the stained cell suspensions was measured with a Wallac 1420-012 multilabel counter, using 340-nm and 460-nm (DAPI) and 545-nm and 616-nm (PI) excitation and emission filters, respectively. The stained cell suspensions were then streaked onto microscopic slides and were analyzed by a Leica DM4000 B fluorescence microscope using filter cube A for DAPI and filter cube N2.1 for PI.

Isolation of human intestinal mucus. Human colonic mucus was isolated as described previously (15, 16) from healthy parts of colons from colorectal cancer patients undergoing surgery. The use of human intestinal mucus in the adhesion studies was approved by the ethical committee of the Hospital District of Southwest Finland. All patients who donated intestinal tissue provided written informed consent.

Preparation of a whole-cell antiserum against *Akkermansia muciniphila*. Live *Akkermansia muciniphila* cells were used to produce a polyclonal rabbit antiserum at the Laboratory Animal Centre of the University of Helsinki. Immunization was carried out as described previously (15). Briefly, overnight-grown cells were washed once with PBS and were resuspended in PBS to a final concentration of 10^9 ml^{-1}. This preparation was diluted 1:1 in Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (booster injections). A 200-μl volume of the cell-adjuvant suspension was injected into the rabbit once every 3 weeks, and the animal was sacrificed and blood collected 10 days after the third booster injection. The blood was allowed to clot for 1 h at +37°C, followed by an overnight incubation at +4°C, after which the blood clot was separated from the serum by centrifugation at 5,000 × *g* for 10 min. The serum was divided into aliquots and was stored at −80°C prior to usage.

Immunofluorescence labeling of *A. muciniphila* cells adhering to the Caco-2 and HT-29 cell lines. *A. muciniphila* cells were washed once with PBS, adjusted to an OD_{600} of 0.25, and applied to Caco-2 or HT29 cell monolayers grown for 3 days on 8-well microscope slides. The microscope slides were incubated for 1 h at 37°C either under a normal atmosphere, in an incubator with 5% CO_2, or in the anaerobic incubator. After incubation, the slides were first washed 3 times with PBS and then fixed for 10 min with 4% paraformaldehyde (PFA) in PBS, followed by additional washes with PBS. A 1:100 dilution of the rabbit antiserum raised against whole cells of *A. muciniphila* was applied to the microscope slides (with plain PBS for conjugate control), and the slides were incubated for 1 h at room temperature. Next, the slides were washed 3 times with PBS and were mounted for 1 h at room temperature with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) and DAPI in PBS (each at 1 μg ml^{-1}). Unbound stains were removed by 3 washes with PBS, and the slides were analyzed by a Leica DM4000 B fluorescence microscope with filter cube A for DAPI and filter cube TX2 for Alexa Fluor 594.

Adhesion to extracellular matrix proteins. MaxiSorp 96-well microtiter plates were prepared for adhesion assays by coating the wells overnight at 4°C with 2.5 pmol well^{-1} bovine serum albumin (BSA; Sigma-Aldrich), collagen I (Sigma-Aldrich), collagen IV (Sigma-Aldrich), fetuin (Sigma-Aldrich), fibronectin (Calbiochem), or laminin (Sigma-Aldrich). Coating was carried out in PBS. Next, the wells were washed twice with PBS and were blocked for 1 h at room temperature with 0.5% (wt/vol) BSA in PBS, followed by three additional washes with PBS. The [^{3}H]thymidine-labeled bacteria were washed once with PBS, and the OD_{600} of the bacterial suspension was adjusted to 0.25 before the cells were added to the microtiter wells. *A. muciniphila* cells were allowed to bind to immobilized ECM proteins for 1 h at 37°C, after which the wells were washed three times with PBS. Bound bacteria were lysed by adding 1% SDS–0.1 M NaOH to the wells and incubating the plates for 1 h at 60°C. The radioactivity was determined with a liquid scintillator, and the fraction of bound cells was expressed as the percentage of the radioactivity of the cell suspension initially added to the wells that was retained in the wells after washing.

Binding to human intestinal epithelial cell lines and mucus. Thymidine-labeled *A. muciniphila* and *L. rhamnosus* GG cells were collected by centrifugation and were washed once with RPMI 1640 or McCoy 5A medium without supplements, or with PBS, for the assay of adhesion to Caco-2 cells, HT-29 cells, or mucus, respectively. After washing, the OD_{600} of the bacterial suspension was adjusted to 0.25 in the respective medium or PBS. Caco-2 and HT-29 cells were grown for 3, 8, and 21 days and were washed twice with culture medium before the addition of bacteria. Mucus-coated wells were prepared as described for ECM protein-coated wells above, by incubating the wells with 50 μg well^{-1} human mucus in PBS, followed by blocking with 0.5% BSA in PBS. The OD-adjusted bacterial suspensions were added to the wells, which were then incubated for 1 h at 37°C. The epithelial cells with bacteria were incubated in the CO_2 incubator. The wells were then washed three times with PBS, and the bound bacteria were lysed and quantified as described above.

TER assay. Caco-2 cells (5×10^4/insert) were seeded in Millicell cell culture inserts (pore size, 3 μm; Millipore) and were grown for 8 days. Bacterial cells were washed once with RPMI 1640 and were applied to the inserts at an OD_{600} of 0.25 in RPMI 1640. Transepithelial electrical resistance (TER) was determined with a Millicell ERS-2 TER meter (Millipore) from cell cultures at 0 h, 24 h, and 48 h after the addition of bacterial cells.

Induction of IL-8 production in HT-29 cells. Ten thousand HT-29 cells per well were seeded onto 96-well microplates and were grown for 8 days in McCoy 5A medium with supplements. *A. muciniphila*, *B. fragilis*, and *E. coli* cells were washed, and the OD_{600} was adjusted as described

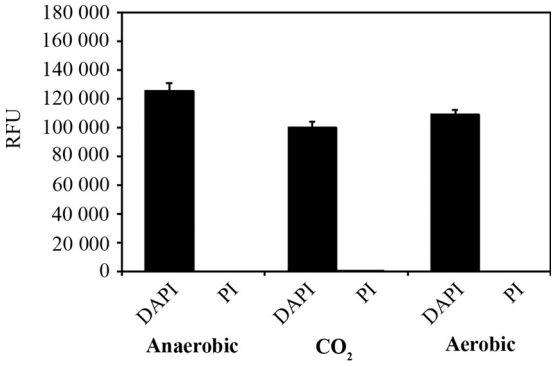

FIG 1 Live-dead fluorescence staining of *A. muciniphila* cells exposed to different atmospheres. *A. muciniphila* cells were incubated for 1 h under an aerobic, 5% CO_2, or anaerobic atmosphere. Relative fluorescence units (RFU) were measured after staining of the cells with DAPI or propidium iodide (PI), which stains only dead or seriously impaired cells. Background fluorescence from nonstained cells has been subtracted from the RFU values obtained. The results shown are means and standard deviations for four parallel samples.

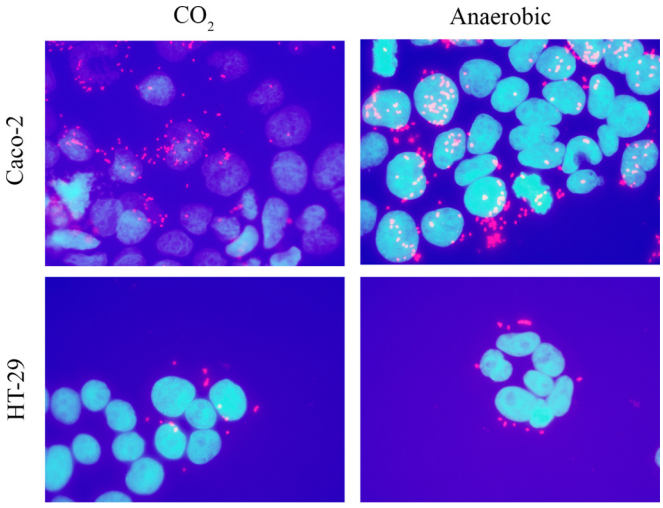

FIG 2 Adhesion of *A. muciniphila* cells to the Caco-2 and HT29 human epithelial cell lines. Adhesion was carried out under an anaerobic or 5% CO_2 atmosphere and was assessed by immunofluorescence microscopy. Enterocyte nuclei were strained with DAPI (blue), and *A. muciniphila* cells were stained with a polyclonal rabbit antiserum raised against whole *A. muciniphila* cells and a secondary antibody conjugated with Alexa Fluor 594 (red).

above in McCoy 5A medium. Bacteria were serially diluted in McCoy 5A medium to 1:100, 1:1,000, and 1:10,000, which corresponded to 10^6, 10^5, and 10^4 cells/ml, respectively. Two hundred microliters of each dilution was added to HT-29 wells, and the cocultures were kept for 3 h at 37°C in the CO_2 incubator. For positive-control wells, 1 ng/ml of *E. coli* lipopolysaccharide (LPS; Sigma) was added to the culture medium, whereas plain culture medium served as a negative control. After the 3 h of incubation, the concentration of interleukin-8 (IL-8) in the culture supernatant was measured with an OptEIA Human IL-8 ELISA set (BD Biosciences) according to the manufacturer's instructions.

Western blotting. *A. muciniphila* and *E. coli* cells were suspended in loading buffer and were boiled for 5 min, followed by SDS-PAGE under denaturing conditions on 4-to-15% gradient gels (Bio-Rad) and electroblotting onto polyvinylidene difluoride (PVDF) Immobilon P membranes (Millipore). The membrane was first probed with a rabbit polyclonal antiserum against *E. coli* LPS (Bioss Inc.) and then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad) diluted 1:100,000. The blot was visualized using the Amersham ECL Advance Western blotting detection kit (GE Healthcare Bio-Sciences) according to the manufacturer's instructions.

Preparation of immunogold-labeled thin sections and TEM. Lowicryl HM20-embedded thin sections of *A. muciniphila* and *E. coli* cells were prepared as described previously (17). Briefly, the cells were washed once with phosphate buffer (0.1 M Na phosphate, pH 7.4) and were fixed for 4 h at room temperature in 4% PFA and 0.1% glutaraldehyde in phosphate buffer. After fixation, the cells were collected and were resuspended in 2% PFA in phosphate buffer, followed by embedding in Lowicryl HM20 resin by freeze substitution. Ultrathin sections cut from polymerized Lowicryl were placed on nickel grids and were blocked for 20 min in 1% BSA, 0.5% fish skin gelatinase (FSG), and 1% FCS in phosphate buffer, after which they were incubated for 1 h with antisera against *E. coli* LPS (Bioss Inc.) or lipid A (Glycobiotech) in 2% BSA, 0.1% Tween 20, and 0.1% FSG in phosphate buffer. The grids were then washed several times with pAg (colloidal gold particles conjugated to protein A) buffer (0.2% BSA, 0.01% Tween 20, and 0.01% FSG in phosphate buffer) and were incubated for 20 min with 10-nm pAg diluted 1:55 in pAg buffer. The grids were washed several times with phosphate buffer and were washed extensively with water. Prior to analysis by transmission electron microscopy (TEM), the grids were poststained with uranyl acetate and lead citrate. The grids were analyzed with a JEOL 1400 transmission electron microscope.

Statistical analysis. Three to five parallel wells (i.e., technical replicates) were used in each experiment, and all experiments were repeated two to six times. A pairwise Student *t* test was used to determine significant differences ($P < 0.05$) between the control and samples or between two different experimental conditions. In the figures, mean values ± standard deviations for technical replicates (parallel wells) of representative experiments are shown.

RESULTS

Oxygen sensitivity of *A. muciniphila*. In order to assess the viability of *A. muciniphila* under the experimental conditions used in our *in vitro* assays, we first analyzed the effects of different incubation atmospheres on *A. muciniphila* cells by using live-dead fluorescence staining. Importantly, *A. muciniphila* cells that were kept for 1 h under oxic, 5% CO_2, or anoxic conditions showed similar staining patterns, and more than 90% of the cells stained live, i.e., stained only with DAPI (Fig. 1; see also Fig. S1 in the supplemental material), indicating that *A. muciniphila* can tolerate oxygen. Since *A. muciniphila* cells were not severely compromised by the use of oxic incubation conditions, the different incubation conditions were also compared in an adhesion experiment. We incubated the bacterium with Caco-2 or HT29 cell monolayers under an anaerobic or 5% CO_2 atmosphere, followed by immunofluorescence microscopy with antibodies raised against whole *A. muciniphila* cells. We could not see any difference between the levels of binding under the two different atmospheres (Fig. 2). Similarly, binding to ECM proteins was not affected by the presence of oxygen (data not shown). On the basis of these results, and for practical reasons, we performed all subsequent experiments with enterocytes under a 5% CO_2 atmosphere and other experiments under a normal atmosphere.

Adhesion of *A. muciniphila* to extracellular matrix proteins. The adhesion of *A. muciniphila* to human ECM proteins (collagens I and IV, fibronectin, and laminin) was studied by determining the binding of radiolabeled *A. muciniphila* cells to immobilized ligands. Bovine serum albumin was used as a negative control, and fetuin was used as a representative highly glycosylated protein. In comparison with background-level binding to BSA, *A. muciniphila* bound significantly only to laminin (Fig. 3). Binding

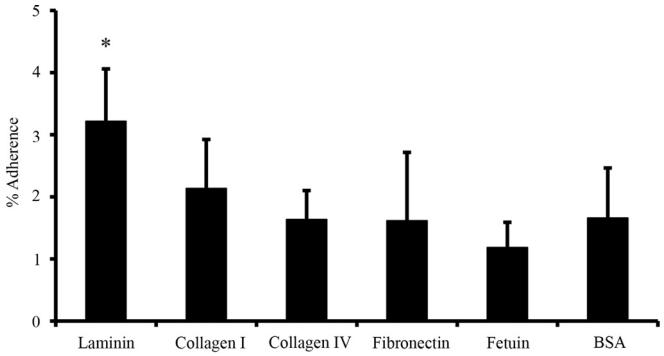

FIG 3 Binding of *A. muciniphila* to ECM proteins. Metabolically labeled *A. muciniphila* cells were allowed to bind to different human extracellular matrix proteins. The results shown are means and standard deviations for five parallel wells. The asterisk indicates a level of binding significantly different ($P < 0.05$) from background binding (to BSA).

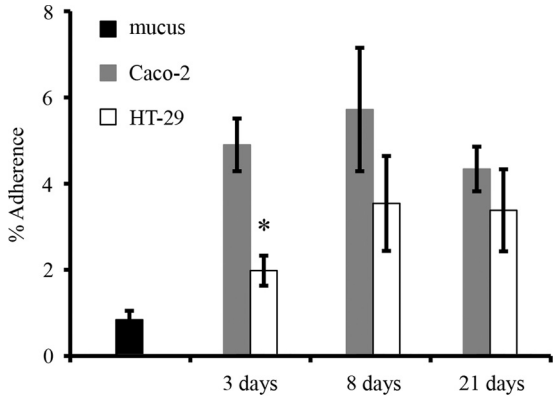

FIG 5 Adherence of *A. muciniphila* to Caco-2 and HT-29 cells at different growth stages. Levels of binding to mucus are shown for comparison. Data are means and standard deviations for five parallel wells. The asterisk indicates a significant difference ($P < 0.05$) in adhesion to the different cell lines at the same growth stage.

to collagens I and IV, fibronectin, and fetuin was at the background level.

Binding to human intestinal mucus and epithelial cells. Next, we studied the adhesion of *A. muciniphila* to the Caco-2 and HT-29 cell lines and to mucus. *L. rhamnosus* GG was included in the experiments as a positive-control strain, since its ability to bind to human mucus and enterocytes has been well established (18, 19). Surprisingly, *A. muciniphila* did not bind human colonic mucus—the level of adhesion was less than 1%, which can be considered nonspecific, background-level binding—while approximately 20% of the added *L. rhamnosus* GG cells were found to be mucus bound (Fig. 4). In contrast, the level of adhesion of *A. muciniphila* to human enterocytes was comparable to that of *L. rhamnosus* GG (Fig. 4). Next, the binding of *A. muciniphila* to enterocytes grown for different periods (3 days, 8 days, and 21 days) was studied. The differentiation of Caco-2 cells starts within 3 to 4 days after confluence (20, 21), and we used undifferentiated cells with confluent growth (3 days) and cells at two differentiation stages, grown for 8 and 21 days (i.e., 5 and 18 days after confluence, respectively). The HT-29 cell line does not differentiate, but we used the same growth times for comparison. In general, *A. muciniphila* adhered equally well to both enterocyte lines at all growth states, except for 3-day-old HT-29 cells, to which it adhered at a lower level (Fig. 5).

Effect of *A. muciniphila* on Caco-2 monolayer integrity. The impact of *A. muciniphila* on the integrity of the epithelial cell layer was assessed by determining the development of the transepithelial electrical resistance (TER) of a Caco-2 monolayer, which was cocultured with *A. muciniphila*. TER is a measure of ion passage across tissue or a cultured enterocyte monolayer (22), and therefore, the epithelial barrier function is commonly assessed by determining the TER. *E. coli* was chosen as a representative bacterium that has adverse effects on epithelial cell monolayer integrity (23), and *B. fragilis* was included in the assay for comparison. The bacteria were administered to 8-day-old Caco-2 cells, and TER was measured at 24-h intervals. After 24 h of cocultivation, both *A. muciniphila* and *B. fragilis* had significantly increased the TER, whereas the TER of Caco-2 cocultures with *E. coli* had decreased significantly, relative to that of Caco-2 cultures without added bacteria (Fig. 6). At this time point, the OD_{600} values for *A. muciniphila* and *B. fragilis* cell suspensions showed essentially no growth, whereas the number of *E. coli* cells had increased slightly

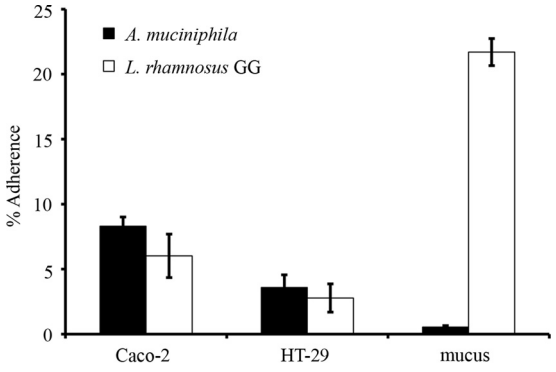

FIG 4 Adherence of *A. muciniphila* to the Caco-2 and HT-29 cell lines and to human intestinal mucus. *A. muciniphila* and *L. rhamnosus* GG (positive-control strain) cells were allowed to bind to human enterocytes or immobilized intestinal mucus. Means and standard deviations for five parallel wells are shown.

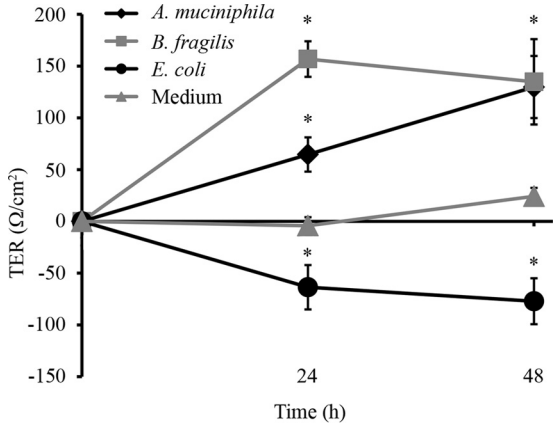

FIG 6 Impact of *A. muciniphila*, *B. fragilis*, or *E. coli* on the development of the TER of a Caco-2 monolayer. Means and standard deviations for three parallel wells are shown. Asterisks indicate TER values significantly different ($P < 0.05$) from that of the control (growth medium without bacteria).

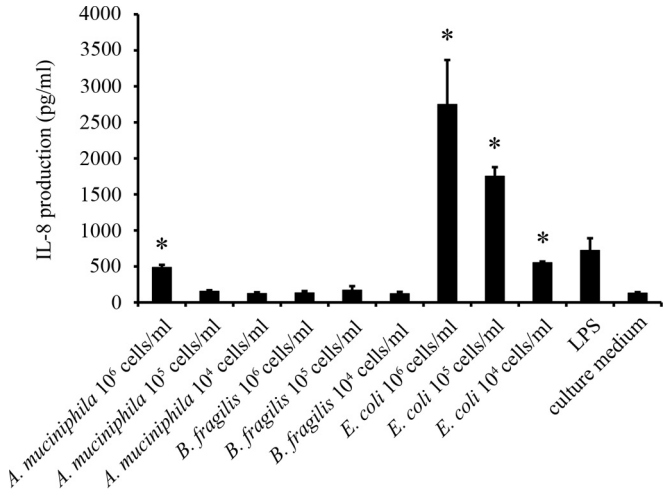

FIG 7 Induction of IL-8 production in HT-29 cells by *A. muciniphila*, *B. fragilis*, and *E. coli*. Means and standard deviations for three parallel wells are shown. Asterisks indicate IL-8 production levels significantly ($P < 0.05$) above the background level (growth medium without bacteria or LPS). LPS (1 ng ml^{-1}) from *E. coli* was included as a positive control.

(from an OD_{600} of 0.25 to an OD_{600} of 0.38) (see Fig. S2 in the supplemental material). At 24 h, the positive impact on cell monolayer integrity was more profound with *B. fragilis* than with *A. muciniphila*, whereas *E. coli* with an essentially similar cellular density clearly affected TER development negatively. At 48 h after the establishment of cocultures, the TER of a Caco-2 cell layer cultured with *E. coli* had decreased even more, while the TER of Caco-2 cocultures with *A. muciniphila* had risen to the same level as that of *B. fragilis* cocultures (Fig. 6). The cell densities of *B. fragilis* and *A. muciniphila* did not change during the 48 h of incubation in the Caco-2 medium, and the bacterial cells did not seem to be severely compromised either, as evidenced by the live-dead staining results (see Fig. S2). The *E. coli* cell suspension, on the other hand, showed an increase in the OD_{600} from 0.25 to 0.5, i.e., one cell division occurred during 48 h, which is likely to have affected the further decrease of TER in *E. coli* cocultures.

Effect of *A. muciniphila* on interleukin-8 production in HT-29 cells. The proinflammatory capacity of *A. muciniphila* was assayed by measuring its effect on IL-8 production by HT-29 cells. The HT-29 cells were incubated with different numbers of *A. muciniphila*, *B. fragilis*, or *E. coli* cells, while pure culture medium served as a background control and pure LPS as a positive control. *E. coli* was used in this assay because it has been shown to trigger IL-8 secretion *in vitro*, whereas *B. fragilis* was included because its LPS has been demonstrated to differ markedly from that of *E. coli* (24, 25). As expected, *E. coli* elicited a strong, dose-dependent IL-8 response in HT-29 epithelial cells, whereas no IL-8 production was induced when the cells were exposed to *B. fragilis* (Fig. 7). While lower doses of *A. muciniphila* failed to show any effect on IL-8 production, an increase in IL-8 release was observed with the largest number of *A. muciniphila* cells (1:100 dilution; 10^6 bacteria/ml^{-1}) (Fig. 7). However, a similar level of IL-8 release was achieved with only 10^4 *E. coli* cells ml^{-1}, suggesting that the proinflammatory effect of *A. muciniphila* on enterocytes is minor compared to that of *E. coli* (Fig. 7).

Presence of LPS in *A. muciniphila* cells. Since *A. muciniphila* showed only minor proinflammatory activity on HT-29 cells, we investigated whether *A. muciniphila* produces LPS and, if so, whether *A. muciniphila* LPS is distinct from that of *E. coli*. For that purpose, we first analyzed whole-cell preparations of *A. muciniphila* and *E. coli* by Western blotting. The antiserum against *E. coli* LPS reacted with *E. coli*, whereas only a weak signal, if any, could be detected with an *A. muciniphila* lysate (Fig. 8A). Next, we analyzed thin sections of *A. muciniphila* cells immunolabeled with antibodies against lipid A from *E. coli* by TEM. As shown in Fig. 8B, anti-lipid A antibodies reacted with the envelopes of both *A. muciniphila* and *E. coli* cells.

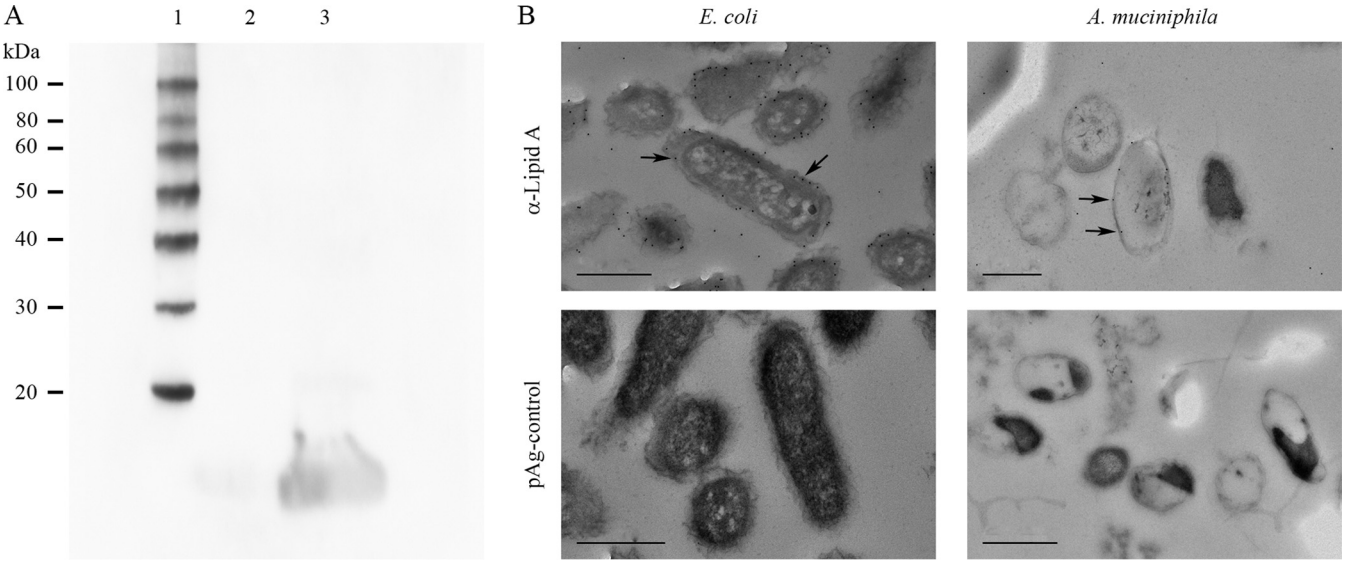

FIG 8 Analysis of LPS and lipid A contents of *A. muciniphila*. (A) Western blotting of whole-cell lysates of *A. muciniphila* (lane 2) and *E. coli* (positive control) (lane 3) using an antiserum against *E. coli* LPS. Lane 1, molecular mass standard. (B) Electron micrographs of thin-sectioned and immunostained *A. muciniphila* and *E. coli* bacteria. The bacteria were immunostained using an antiserum against *E. coli* lipid A and 10-nm colloidal gold particles conjugated to protein A (pAg). Arrows indicate 10-nm gold particles. Bars, 500 nm.

DISCUSSION

Akkermansia muciniphila is an anaerobic bacterium isolated from human feces. Our results show that *A. muciniphila* is an aerotolerant anaerobic bacterium rather than a strict anaerobe, in contrast to many other members of the human intestinal microbiota. Our preliminary plate count data indicate that 80% of *A. muciniphila* cells exposed to atmospheric oxygen levels for 1 h survive (data not shown). Currently, we cultivate *A. muciniphila* successfully by inoculating the cultures in a laminar hood under a normal oxic atmosphere and incubating them in an anaerobic jar with a chemically created oxygen-free CO_2 atmosphere (Anaerocult; Merck) instead of the anaerobic cabinet. Thus, there is no need to treat the organism as a highly oxygen sensitive anaerobe.

The ability to bind to the epithelial surface is thought to enhance the colonization of the digestive tract by a bacterial strain. The bacterium might, in principle, adhere to intact epithelium through binding to enterocytes, different components of the mucus gel, or other bacteria inhabiting the epithelial surface. In the event of disruption of the mucosal surface, epithelial binding could also be achieved by binding to various components of the extracellular matrix. In this study, we analyzed the adhesion of the human commensal *A. muciniphila* to various components of the human GI tract epithelium. Remarkably, *A. muciniphila* showed no binding to human colonic mucin, even though it lives in an intimate relationship with the intestinal mucosa and also utilizes it as a nutrient. We have shown previously, by using the same mucus binding assay, that the human intestinal isolates *L. rhamnosus* GG and *Bifidobacterium bifidum* DSM20456 and MIMBb75 bind strongly to human colonic mucus (15, 18). However, *L. rhamnosus* and *B. bifidum* are nonmucolytic bacteria, whereas *A. muciniphila* is a mucin-degrading bacterium. Therefore, the result may reflect the mucinolytic nature of *A. muciniphila*; i.e., we cannot presently rule out the possibility that *A. muciniphila* cells might be detached from the immobilized mucus as a result of their mucin-degrading enzymatic activity. The binding assay incubations were carried out under aerobic conditions, which did not severely damage *A. muciniphila* cells. Presumably, the main mucolytic enzymes of *A. muciniphila* may stay active under these conditions. On the other hand, since *A. muciniphila* adhered to cultured enterocytes, it is possible that epithelial enterocytes could serve as bona fide intestinal docking sites for adherent *A. muciniphila* cells. Therefore, in future work, we shall investigate whether this counterintuitive observation, i.e., the lack of mucus-binding ability of *A. muciniphila*, is due to the experimental conditions used in the mucus binding assay. Nevertheless, since *A. muciniphila* is found to reside on the colonic mucus, the question of how this organism manages to stably occupy this constantly renewing ecological niche remains to be answered.

In contrast to its lack of binding to mucus, *A. muciniphila* showed firm binding to the cultured colonic epithelial cell lines Caco-2 and HT-29. Although the highest *A. muciniphila* cell counts are found in the colon, the small intestine is also colonized by substantial numbers of *A. muciniphila* cells (5). In the small intestine, the mucus layer is permeable to bacteria, allowing direct contacts between bacteria and enterocyte surfaces (14). Therefore, *A. muciniphila* may use direct binding to enterocytes as a colonization strategy in the small intestine. The epithelial cell layer in the GI tract is renewed approximately once every 4 to 5 days in a process in which undifferentiated enterocyte progenitors derived from stem cells move from the intestinal crypts toward the tips of villi (26). The immature enterocytes differentiate *en route* to the villus tip, where they replace the old enterocytes, which are shed into the intestinal lumen (27). Interestingly, the enterocyte binding strength of *A. muciniphila* was independent of the growth state of Caco-2 cells, indicating that the host-binding site utilized by the bacterium is expressed on the cell surface irrespective of the state of cell differentiation. It is well known that the expression of surface molecules is influenced by the differentiation stage in epithelial cells (28, 29) and that bacterial adhesion to enterocytes is affected by the profile of surface-associated molecules in the enterocytes (30). Based on our findings, it seems possible that *A. muciniphila* is able to bind enterocytes at various differentiation stages *in vivo*.

The intestinal epithelium is subject to mechanical stress during the digestion of food, and as a result, the epithelium frequently suffers minor breaks in its integrity (26, 31). These wounds expose the subepithelium, along with its ECM network, to the intestinal lumen, allowing intestinal bacteria temporary access to the ECM components. ECM components, being natural constituents of the network of macromolecules, are also found in the mucus (32). Since we found that *A. muciniphila* binds laminin and undifferentiated Caco-2 cells, it is tempting to speculate that *A. muciniphila* might participate in the competitive exclusion of pathobionts from the sites of injury and fortify the *de novo*-established enterocyte monolayer after an epithelial insult. In favor of this view is our observation that *A. muciniphila* significantly increased the TER in coculture with Caco-2 cells, whereas *E. coli* decreased the TER under the same culture conditions. The strengthening of epithelial barrier function could also explain several *in vivo* observations linking *A. muciniphila* not only to gut health but also to systemic health. The impaired integrity of intestinal epithelium leads to the accumulation of LPS from Gram-negative gut inhabitants in the serum, resulting in metabolic endotoxemia with concomitant inflammation (33, 34). Since diabetes and obesity have been linked with increased gut permeability and low-grade inflammation (35, 36), LPS-induced endotoxemia has been suggested as one of the causative agents of obesity and its related metabolic disorders (33, 37, 38). Our *in vitro* observation that *A. muciniphila* fortifies epithelial barrier function could provide a working hypothesis for attempts to rationalize the *in vivo* findings connecting decreased fecal *A. muciniphila* levels with diabetes and obesity (8, 9) and could reveal one possible mechanism behind the protective effect of the bacterium against high-fat-diet-induced LPS endotoxemia in obese mice (13).

IL-8 is a mediator of inflammation, causing immune cells to migrate to the site of infection and inducing phagocytosis, and it plays an important role in host defense against pathogens (39). However, the stimulation of massive IL-8 production in a healthy, intact epithelium would lead to unnecessary inflammation and disturbance of mucosal homeostasis. We found that *A. muciniphila* induced IL-8 production in enterocytes at cell concentrations 100-fold higher than those for *E. coli*. Thus, *A. muciniphila* does not seem to be able to provoke a strong inflammatory cascade in the epithelium. The finding is in line with the reported *in vivo* investigations linking *A. muciniphila* with noninflamed rather than inflamed mucosa (5–7). On the other hand, the low-level proinflammatory stimulation of enterocytes by *A. muciniphila* may keep the mucosa-associated immune system alerted at an appropriate level. The *A. muciniphila* genome sequence contains

the genetic elements necessary for the production of LPS (4). In contrast to *E. coli* or its LPS, *A. muciniphila* did not induce strong IL-8 release from HT-29 cells. Thus, *A. muciniphila* LPS does not seem to be a powerful activator of host Toll-like receptor 4 (TLR4) and likely differs structurally from *E. coli* LPS. Recently, *B. fragilis* LPS has been shown to signal through TLR2, not through the well-recognized LPS receptor TLR4 (24). *B. fragilis* LPS is structurally atypical, differing from classical LPS by the length of the O-antigen polysaccharide and the phosphorylation of lipid A diglucosamine (24, 25). We attempted to detect LPS in *A. muciniphila* by Western blotting using polyclonal antisera raised against *E. coli* LPS. Since this approach was unsuccessful with *A. muciniphila*, we performed immunoelectron microscopic analysis of thin-sectioned *A. muciniphila* and *E. coli* cells. By this method, we were able to immunostain thin sections of *A. muciniphila* cells with anti-lipid A antiserum, indicating that the bacterium produces lipid A and therefore, most likely, also LPS. We propose that there are structural and antigenic disparities between the LPS polysaccharide structures of *A. muciniphila* and *E. coli*, as evidenced by the reactivity of anti-lipid A antibodies but the nonreactivity of antibodies raised against *E. coli* LPS. These findings warrant future exploration of the precise immunosignaling properties of *A. muciniphila*, including the identification of the host side receptors involved therein.

In this study, by using *in vitro* methods, we have revealed several interactions of *A. muciniphila* with intestinal epithelium. The somewhat unexpected binding preference of *A. muciniphila* for epithelial cells and laminin over colonic mucus raises questions about the possible physical niches utilized by this organism to stably colonize the human GI tract. Our findings that *A. muciniphila* is capable of adhering to both nondifferentiated and mature enterocytes and that upon this interaction, the bacterium does not provoke a proinflammatory reaction but instead elicits the strengthening of epithelial integrity open multiple exciting paths for further study of the putative beneficial interactions of *A. muciniphila* with the host.

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