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Intestinal Integrity and *Akkermansia muciniphila*, a Mucin-Degrading Member of the Intestinal Microbiota Present in Infants, Adults, and the Elderly[▽]

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Fluorescence in situ hybridization and real-time PCR analysis targeting the 16S rRNA gene of *Akkermansia muciniphila* were performed to determine its presence in the human intestinal tract. These techniques revealed that an *A. muciniphila*-like bacterium is a common member of the human intestinal tract and that its colonization starts in early life and develops within a year to a level close to that observed in adults (10^8 cells/g) but decreases ($P < 0.05$) in the elderly.

The human gastrointestinal (GI) tract is colonized by a complex and diverse microbiota that shows different compositions and activities depending on environmental and genetic factors (8, 14, 16, 18, 19). The intestinal microbiota starts to establish at birth and continues to develop and change during life and also provides a barrier against colonization of pathogens and harmful substances, protecting intestinal integrity, and stimulates the development of the immune system (10, 14).

A mucus layer covering the GI tract has been reported to serve as a source of nutrients for bacterial growth (6). Thus, its presence influences intestinal colonization by attracting bacteria that have the ability to survive and multiply within the mucus layer. The mucus layer also contributes to host defense by preventing bacterial adhesion or invasion and toxin binding to the mucosal surface (11). Nevertheless, the association of the microbiota with the mucus is not well understood and requires further investigation.

A novel mucin-degrading bacterium designated *Akkermansia muciniphila* has been isolated and characterized from a fecal sample from a healthy adult (6). Subsequent studies based on 16S rRNA gene cloning and sequencing have demonstrated that *A. muciniphila* is present in distinct parts of the human mucosa as well as in fecal samples (7, 9, 17). The relation between mucin and bacteria varies depending on the microbiota, and several studies have reported a potential involvement of mucin-degrading bacteria in pathogenesis of intestinal diseases (3). Mucosal surfaces, microbiota, and mucus secretion may be altered due to environmental factors, including age. As a consequence, the composition and thickness of the mucus layer may be modified (4). Such changes may alter the contact between intestinal microbiota and mucosal dendritic cells. As the mucus quality and quantity alter during ageing, we hypothesized that the levels of intestinal *A. muciniphila* could be different during human life, and detection of the presence of this bacterium requires detailed investigation.

Fecal samples from healthy infant subjects 1 month old (n = 50), 6 months old (n = 50), and 12 months old (n = 50); healthy adults from 25 to 35 years old (n = 54); and elderly subjects from 80 to 82 years old (n = 45) were collected and analyzed. Two grams of fresh fecal samples was used and suspended in 20 ml of phosphate-buffered saline. The suspension was vortexed thoroughly with 3-μm-diameter glass beads and centrifuged at 800 × g for 1 min to remove debris. Different aliquots from the pure culture and fecal samples were used to extract DNA and to fix cells for fluorescence in situ hybridization (FISH) analysis. One volume of the supernatant was transferred into 3 volumes of fresh 4% paraformaldehyde and fixed at 4°C overnight. The bacteria were stored in 50% ethanol–phosphate-buffered saline at −20°C until analysis. One volume was used for DNA extraction by using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Two specific primers were designed from the variable regions of the 16S rRNA gene sequence of *A. muciniphila*. The GenBank program from NCBI (BLAST) was used to verify that both primers were specific to the target organism only. The primers selected for detection of *A. muciniphila* were named on the basis of nomenclature from Alm et al. (1). These primers were S-St-Muc-1129-a-a-20 (AM1), with the sequence 5′CAG CAC GTG AAG GTG GGG AC, and S-St-Muc-1437-a-A-20 (AM2), with the sequence 5′CCT TGC GGT TGG CTT CAG AT (Table 1). All primers were purchased from MWG (Ebersgerg, Germany). To check the specificity of the amplification, standard PCR amplification was performed, using as the template DNA from 20 intestinal isolates (Table 2); 96 cloned 16S rRNA genes from uncultured bacteria belonging to the most dominant groups found in the GI tract, including *Bacteroidetes*, *Clostridium* cluster XIVa, and *Clostridium* cluster IV; and other clones belonging to disparate clusters, including *Bifidobacterium*, *Lactobacillus*, and *Atopobium* (5, 15, 18). The PCR primers were specific for *A. muciniphila* at 60°C, with amplification of a product of the expected size (327 bp)

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TABLE 1. Primer sequences used in this study

Primer	Sequence	Nomenclature	Annealing temp (°C)	Reference
AM1	CAGCACGTGAA GGTGGGGAC	S-St-Muc-1129-a-a-20	50	5
AM2	CCTTGCGGTTG GCTTCAGAT	S-St-Muc-1437-a-A-20	50	5

(data not shown). All other nontargeted strains tested and the cloned 16S rRNA genes from uncultured GI tract bacteria showed no amplification during PCR (data not shown).

Quantitative PCR amplification and detection were performed with optical-grade 96-well plates with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, United Kingdom). Each reaction mixture of 25 μl was composed of SYBR green PCR master mix (Applied Biosystems, United Kingdom), 1 μl of each of the specific primers at a final concentration of 0.25 μM, and 1 μl of template DNA. The PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the nontargeted PCR product. Standard curves were created using serial 10-fold dilutions of *A. muciniphila* pure culture DNA corresponding to 10^2 to 10^{10} cells as determined by flow cytometry counts. The bacterial concentration of each sample was calculated by comparing the threshold cycle (C_T) values obtained from the standard curve. All samples were analyzed in duplicate.

In addition, FISH coupled with flow cytometry (FCM-FISH) was carried out by following the procedure described in reference 5, using a specific oligonucleotide probe, MUC-1437 (Table 3), targeting one region of the 16S rRNA gene of *A. muciniphila*, to compare and validate the method. Total bacterial cell numbers were enumerated, using fluorescein-labeled probe EUB-338 (2, 13) as a positive control and probe Non-EUB (2, 13) as a negative control, to monitor the nonspecific hybridizations (Table 3). Absolute bacterial cell counts were determined by addition of Flow-Count fluorospheres, following the supplier's instructions (Beckman Coulter). Flow-cytometric analyses were performed using a BD LSR II flow cytometer (Becton Dickinson) equipped with a 488-nm laser at 15 mW. Data were stored as list mode files and analyzed offline using BD FACSDiva version 4.1.1 software (Becton Dickinson).

A linear relationship was observed between the cell counts and C_T values ($r^2 = 0.9906$) when the number of cells per reaction mixture was between 10^2 and 10^9. Reliable quantification of cells was not possible at concentrations below 10 cells per reaction mixture. This corresponds to a detection limit of 5×10^2 cells/g feces. Melting curve analysis of the amplicons obtained by real-time PCR generated a specific peak at 85°C in the 16S rRNA gene of *A. muciniphila*. The inter-PCR reproducibility of the quantification was found to be very high based on the C_T values obtained for two DNA extracts (a pure culture and a fecal specimen harboring approximately 10^8 to 10^9 *A. muciniphila*-like bacteria/g) in five replicate runs. The average C_T values obtained for those samples were 11.0 (range, 10.9 to 11.1) and 11.2 (range, 11.0 to 11.4). Similar results were observed with other samples and in other dilutions (average and standard deviation, 11.1 ± 0.5). By analysis of duplicate DNA extracts from fecal samples, the interextract variability was found to be less than 10% (data not shown).

Application of the real-time-PCR approach to fecal samples of infants (up to 1 year old), young adults (25 to 35 years old), and elderly subjects (80 to 82 years old) showed that *A. muciniphila*-like bacteria appeared in early life and increased significantly in number from early life to adult age (Fig. 1 and

TABLE 2. Species and origins of reference strains used for validation of the *Akkermansia muciniphila* primers (AM1 and AM2) and probe (MUC-1437)

Species	Origin[b]
Akkermansia muciniphila	ATCC BAA-835
Atopobium minutum[a]	DSM 20586
Bacteroides distasonis	DSM 20701
Bacteroides fragilis[a]	DSM 2151
Bacteroides pyogenes	CCUG 15419
Bacteroides tectus	CCUG 25929
Bacteroides thetaiotaomicron[a]	RRI
Bacteroides uniformis[a]	DSM 6597
Bifidobacterium lactis[a]	DSM 10140
Bifidobacterium bifidum[a]	DSM 20082
Bifidobacterium adolescentis[a]	DSM 20083
Bifidobacterium breve[a]	DSM 20091
Bifidobacterium longum[a]	DSM 20090
Clostridium aminophilum	ATCC 49906
Clostridium sticklandii	ATCC 12662
Clostridium perfringens[a]	DSM 756
Clostridium sporogenes	DSM 1664
Colinsella aerofaciens[a]	DSM 3979
Coprococcus sp.[a]	RRI
Enterococcus faecalis	RRI
Escherichia coli[a]	NCTC 12900
Eubacterium biforme	DSM 3989
Eubacterium cylindroides	RRI
Eubacterium rectale	RRI
Eubacterium ruminantium	RRI
Faecalibacterium prausnitzii	RRI
Lachnospira multiparus	RRI
Lactobacillus acidophilus[a]	ATCC 4356
Lactobacillus amylovorus[a]	DSM 20531
Lactobacillus rhamnosus[a]	ATCC 53103
Lactobacillus plantarum[a]	NCIMB 8826
Lactobacillus reuteri[a]	DSM 20016
Megasphaera elsdenii	NCIMB 8927
Mitsuokella multiacidus	RRI
Peptostreptococcus anaerobius	ATCC 27337
Pepstostreptococcus micros	DSM 20468
Prevotella albensis	DSM 11370
Prevotella brevis	ATCC 19188
Prevotella bryantii[a]	DSM 11371
Prevotella ruminocola[a]	ATCC 19189
Roseburia intestinalis	RRI
Ruminococcus albus	RRI
Ruminococcus bromii	RRI
Ruminococcus flavefasciens	RRI
Ruminococcus hansenii	DSM 20583
Ruminococcus productus	DSM 2950
Streptococcus bovis	RRI
Veilonella parvula	RRI
Victivallis vadensis[a]	DSM 14823

[a] The strains of these species were tested using the AM1 and AM2 primers.
[b] RRI, Rowett Research Institute (Aberdeen, United Kingdom).

TABLE 3. Probe sequences and hybridization conditions used in this study

Probe	Sequence	Target	Hybridization conditions		Reference(s)
			Temp (°C)	% Formamide	
EUB-338	GCTGCCTCCC GTAGGAGT	Most bacteria	50	0	2, 13
NON-EUB	ACATCCTACG GGAGGC	None	50	0	2, 13
MUC-1437	CCTTGCGGTT GGCTTC AGAT	*Akkermansia*	50	20	5

Table 4). The presence of *A. muciniphila*-like bacteria was detected in 8 of the 50 fecal samples of 1-month-old infants (16% of the samples) at levels from 2.05 to 4.36 log cells/g. Bacteria related to *A. muciniphila* were detected in 36 out of 50 children aged 6 months (72% of the samples) and 45 of 50 children at 12 months of age (90% of the samples). The values ranged from 2.50 to 7.30 log cells/g and 2.80 to 9.50 log cells/g in samples from 6- and 12-month-old infants, respectively. The number of bacteria related to *A. muciniphila* increased significantly ($P < 0.05$) from early life to adult age and reached levels ranging from 5.00 to 8.80 log cells/g in all samples from adults. These data indicate that *A. muciniphila*-like bacteria are colonizing the intestinal tract in early life and develop within a year to a level close to that observed in adults. Remarkably, the concentrations of *A. muciniphila* cells in fecal samples from elderly subjects were significantly decreased (>1 logarithmic unit, to 6.00 log cells/g; $P < 0.05$) compared to those in samples from adults.

In addition, all fecal samples tested were analyzed also by

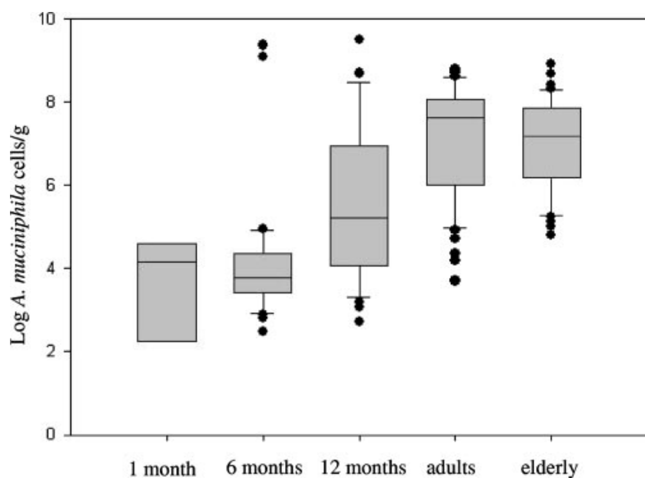

FIG. 1. *A. muciniphila* levels in human fecal samples as determined by real-time PCR over a life span. Data represent the positive samples. The line in the box represents the median (50th percentile), with the lower line the 25% border (25th percentile) and the upper line the 75% border (75th percentile). The end of the upper vertical line represents the maximum data value, outliers not considered. The end of the lower vertical line represents the lowest value, outliers not considered. The separate dots indicate outliers.

TABLE 4. Numbers of *A. muciphila*-like bacteria per gram of feces in fecal samples analyzed by real-time PCR and FCM-FISH

Sample group	No. of infected samples/total no. of samples	Log no. of cells/g feces[a] as determined by:	
		Quantitative PCR	FCM-FISH
Infant (1 mo old)	8/50	3.90 (2.25–4.32)[c]	4.56 (5.06–4.03)[c]
Infant (6 mo old)	36/50	3.90 (3.52–4.36)	6.50 (5.90–7.40)[b]
Infant (12 mo old)	45/50	5.20 (4.08–7.00)	7.33 (7.05–7.94)[b]
Adult	54/54	7.61 (6.02–8.05)	8.10 (7.90–8.32)[b]
Elderly	43/45	6.00 (4.22–7.66)	7.28 (6.63–7.96)[b]

[a] Total bacteria in samples ranged from 9.0 to 10 log cells/g of fecal samples. Due to nonnormal distribution, microbial data are expressed as medians, with interquartile ranges in parentheses. The Mann-Whitney U test was applied in comparisons among groups.

[b] Significant differences ($P < 0.05$) were determined by a Mann-Whitney test comparing qPCR and FCM-FISH data.

[c] All data from different age groups were significantly different ($P < 0.05$), with only one exception: data from 1-month-old and 6-month-old infants.

FCM-FISH for comparison to the results obtained by quantitative real-time PCR. A good correlation between the results obtained with the two methods was found (Table 4). Nevertheless, in fecal samples harboring small numbers of *A. muciniphila*-like bacteria, the FCM-FISH approach estimated their numbers to be about 1 logarithmic unit higher than the real-time PCR method, probably due to background and/or unspecific signals (Table 4).

The results of the quantitative real-time PCR and FISH-FCM results showed a good correlation, but differences were found between these methods in samples with low numbers ($<10^4$ cells/g) of bacteria related to *A. muciniphila*. Similarly, FCM-FISH and real-time PCR results showed different levels of *A. muciniphila*-like bacteria when suspensions of pure cultures with concentrations ranging from 4 to 2 log cells/g were tested. FCM-FISH tended to detect more *A. muciniphila*-like bacteria than real-time PCR. In some cases, FCM-FISH could lead to an overestimation of the number of *A. muciniphila*-like bacteria in fecal samples with low content, due to the formation of unspecific hybridizations in complex matrices of fecal samples or to undescribed *Akkermansia* species that do not provide an amplification product with the developed primers targeted to *A. muciniphila*. In addition, cell hybridizations with fluorescent probes provide background levels that a flow cytometer may detect as cells, increasing the percentage of cells detected compared to that of cells obtained by PCR. Thus, the advantage of this specific PCR technique is that the method is approximately 10 to 100 times more sensitive and specific than the culture and FISH methods.

The presence of *A. muciniphila*-like bacteria was detected in fecal samples from infants 1 month old, but the number increased rapidly with the age (6- and 12-month-old infants and adults). The numbers of bacteria related to *A. muciniphila* almost doubled in the age period ranging from 6 to 12 months. These data are in agreement with earlier observations (12) which reported the establishment of mucin-degrading microbiota in children from birth to the age of 2 years, based on agar gel electrophoresis of their fecal samples. The establishment of mucin-degrading bacteria was reported during the first months of life and is completed when the children are around 2 years old (12). These data indicate that *A. muciniphila* is colonizing

the intestinal tract in early life and develops within a year to a level close to that observed in adults (Fig. 1). *Akkermansia* was detected in all samples from adults at levels ranging from 5.00 to 8.80 log cells/g, and this is in agreement with early data (5). Our results suggest that the possible rise in concentrations of bacteria is related to *A. muciniphila* along with normal mucosa development. This rise may be associated with the integrity of the developing healthy intestinal tract with normal mucus production. However, the numbers of *A. muciniphila*-like bacteria in elderly subjects (80 to 82 years old) were significantly decreased (1 logarithmic unit; $P < 0.05$) compared to those in young and middle-aged adults. Although it cannot be ruled out that these bacteria do not show a dependency on mucin as a carbon and nitrogen source similar to that of *A. muciniphila*, it is likely that this reflects the coevolution of specific mucin-degrading bacteria and mucus production.

Taken together, the techniques described here are accurate, rapid, and easy methods for quantification of *A. muciniphila* in human feces. These methods will facilitate rapid and reliable counting of large numbers of samples, contributing to the efficient use of intestinal bacterial assays in research as well as in the assessment of dietary management of diseases. We also demonstrate for the first time the validation and development of a quantitative real-time PCR for detection and quantification of bacteria related to the mucin-degrading bacterium *A. muciniphila*. Our results demonstrate that *A. muciniphila* is present and colonizes the intestinal tract in early life and develops within a year to a level close to that observed in healthy adults. Further studies are needed to clarify the role of *A. muciniphila* in microbiota development and immune development in early childhood and old age.

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