Culturomics to increase the Repertoire of next-generatIon probiotic Strains (CRIS project): The case of *Akkermansia muciniphila*

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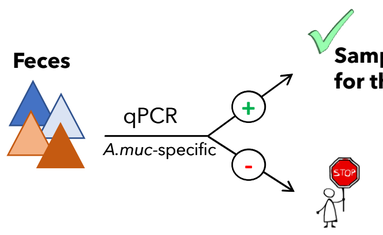
1. Introduction

Research on human gut microbiota has broadened the range of organisms to be used as probiotics. Among these, ***Akkermansia muciniphila* is one of the primary candidates as next-generation probiotic.** This microorganism is a **mucin-degrading bacterium** whose presence in the intestine inversely correlated to several pathologies. The type strain, *A. muciniphila* MucT (= DSM 22959), has been extensively studied and is considered a **beneficial bacterium** due to its **protective effect against metabolic disorders**. However, the critical analysis of the literature is controversial about the safety of this species. Furthermore, the genomic and functional diversity of *A. muciniphila* and that of closely related species has still been poorly studied. **Very few cultured representatives of this species are available, and most studies focus on the type strain.** In this project, new *A. muciniphila* strains were isolated from human fecal samples and their genomes sequenced. In addition, polyclonal antibodies against *A. muciniphila* were used to develop an immuno-flow cytometry (FCM) method for the specific detection of *A. muciniphila* in multispecies cell suspensions. This protocol will be further combined with fluorescence-activated cell sorting (FACS) for improving the isolation of *A. muciniphila* strains.

2. Materials and Methods

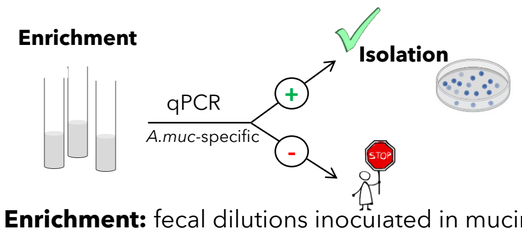
2.1 Enrichment, isolation, and whole genome sequencing

SAMPLING



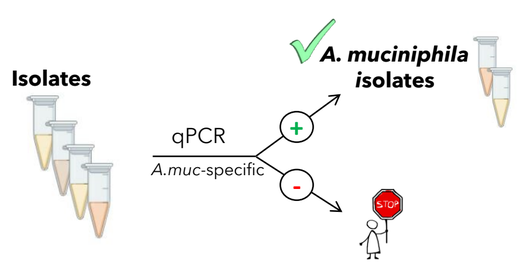
1) Fecal samples collection from 16 healthy adults aged 25 to 60 years
2) Samples selection for the isolation: molecular detection of *A. muciniphila* in feces by species-specific qPCR [1]

ENRICHMENT AND ISOLATION



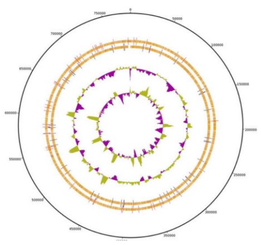
1) Enrichment: fecal dilutions inoculated in mucin-containing medium
2) Molecular assessment of *A. muciniphila* enrichments
3) Spread-plating: enrichment dilutions inoculated on mucin-containing agar plates
4) Strains isolation and purification: Colony picking [2]

IDENTIFICATION



1) *A. muciniphila* isolates identification: species-specific qPCR [1] and partial 16S rRNA gene seq.
2) *A. muciniphila* isolates subtyping: RAPD [3]

GENOME SEQUENCING



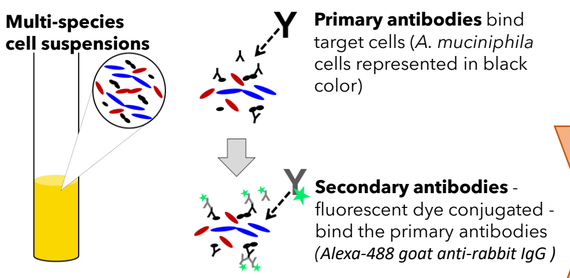
1) Whole genome sequencing of *A. muciniphila* isolated strains (Illumina NovaSeqTM 6000)
2) Draft genomes assembling and annotation (SPAdes; RAST)

2.2 Indirect immunolabeling protocol

PRIMARY ANTIBODIES PRODUCTION

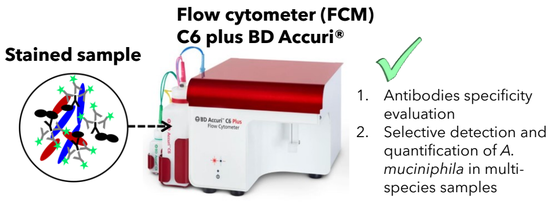
1) **Rabbit immunization**
2) **Rabbit polyclonal antibodies** purified and used as primary antibodies

INDIRECT IMMUNOLABELING



1) Blocking step (PBS pH 7 + 5% v/v goat serum 30 min/RT)
2) Primary antibodies addition 1 µg/ml; 1 h/RT
3) Cells washing
4) Secondary antibodies addition1 µg/ml; 1 h/RT/dark
Note: Optimal antibodies concentration defined by titration experiments

FLOW CYTOMETRY ANALYSIS



1) Stained samples dilution (10^6 cell/ml in PBS pH 7)
2) FCM analysis (excitation: 488 nm; emission: 530 nm)

Note: *To quantify total bacteria in cultures SYBR™ green I (Thermo Fisher) 1X in 1 mL of samples (10^6 cell/ml in PBS pH 7)*

3. Results and Discussion

3.1 Isolation of *A. muciniphila* strains and whole genome sequencing of the isolates

A) *A. muciniphila* was detected in fecal samples from 9 of 16 (56%) volunteers by qPCR analysis and used for the isolation.

B) We obtained a pure culture of new strains only from 5 fecal samples, instead only enrichments for the other 4. **Only 10 isolates out of 200 (5%) were identified as *A. muciniphila*,** confirming that isolation and cultivation of *A. muciniphila* is a challenge.

C) The RAPD profiles allowed differentiating the *A. muciniphila* isolates into 5 subtypes, different from the type strain *A. muciniphila* DSM 22959. Each subtype was isolated from a different donor, indicating that different subjects are colonized from different strains.

D) One isolate for each subtype was selected for **whole genome sequencing (Tab. 1)**.

Table 1. General genomic features of the fecal isolated *A. muciniphila* genomes

Collection strain (*A. muciniphila*)	Genome size (Mb)	GC (%)	N° Contigs	N50 (bp)	L50	N° CDSs
DSM22959	2.70	55.8	162	1443811	1	2675
SAP1	3.08	55.2	880	300745	4	3532
AMaP1	3.84	55.0	3172	84513	15	6015
VTP7	3.60	55.0	1898	268952	4	4705
RCP22	3.48	54.9	2068	208857	5	4364
AMuP9	3.32	56.0	1431	107009	8	4220

CDSs, CoDing Sequences

Ongoing activities

1. **Comparative genomic analysis**
2. **Safety evaluation** – antimicrobial susceptibility, mucin degradation capability
3. **Impact on immune system** – *in vitro* study

3.2 Immuno-FCM method to specific detect and quantify *A. muciniphila* in complex cell suspensions

A) Polyclonal **antibodies were specific at species level** being able to: i) detect *A. muciniphila* DSM 22959 and new isolated strains of *A. muciniphila* **(Fig.1A)**; ii) not bind other intestinal species **(Fig.1B)**

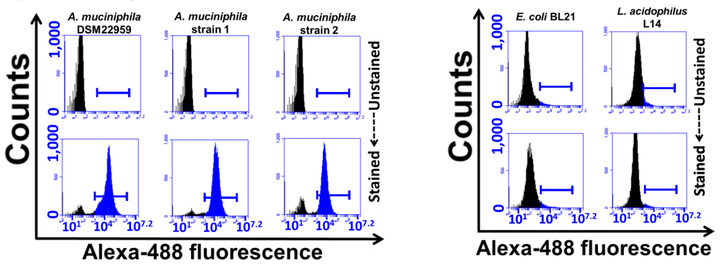


Figure 1. Examples of target cells **(A)** and not target cells **(B)** are reported as histograms before (unstained) and after (stained) the antibody labeling. Antibody-stained cells were identified in the blue gate.

B) The immune-FCM method was applied to detect and quantify *A. muciniphila* in multi-species samples, containing *A. muciniphila* DSM 22959 and *Escherichia coli* BL21 or *Lactobacillus acidophilus* L14 at known density. Pure culture of each species was quantified separately by SYBR™ green I staining, before being used for assembling the mixed cultures. Results show: **i) *A. muciniphila* was specifically detected in multi-species cell suspensions (Fig. 2)**;

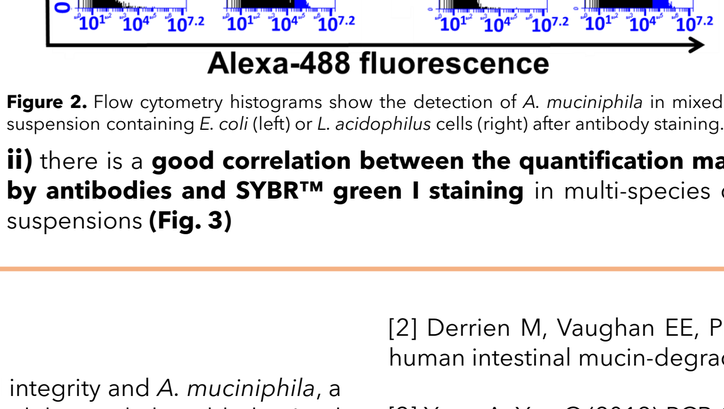


Figure 2. Flow cytometry histograms show the detection of *A. muciniphila* in mixed cell suspension containing *E. coli* (left) or *L. acidophilus* cells (right) after antibody staining.

ii) there is a **good correlation between the quantification made by antibodies and SYBR™ green I staining** in multi-species cell suspensions **(Fig. 3)**

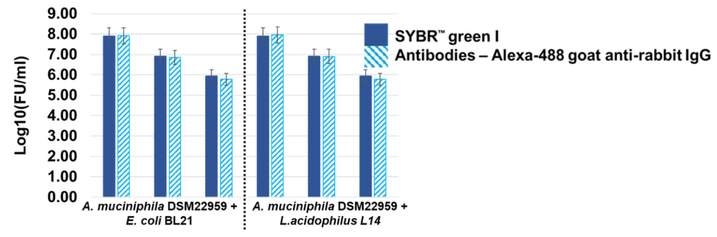


Figure 3. Quantification of different cells concentrations of *A. muciniphila* DSM 22959 using SYBR ™ green I staining, before being mixed with *E. coli BL21* or *L. acidophilus L14 cells*. Specific quantification of *A. muciniphila* cells, in the mixed cell suspensions, was performed by antibodies labeling and compared with SYBR ™ green I data. Analytical imprecision is not higher than 5%. FU, fluorescent units.

C) The developed method can detect *A. muciniphila* in enrichment cultures [2] **(Fig. 4)**

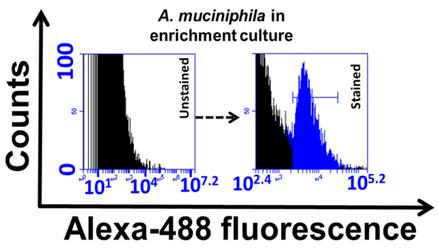
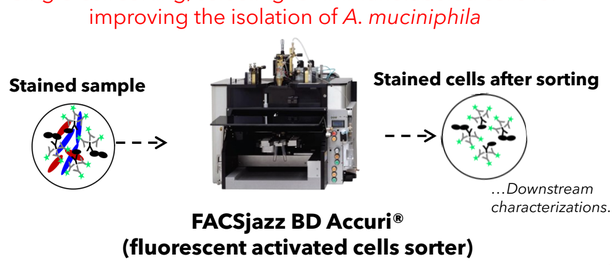


Figure 4. Flow cytometry histograms show the detection of *A. muciniphila* in enrichment cultures after antibody staining. Antibody-stained cells were identified in the blue gate.

Future prospective:
Single-cell sorting, selecting for immunolabeled cells for improving the isolation of *A. muciniphila*



4. References

[1] Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S (2007) Intestinal integrity and *A. muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol. 73 (23): 7767-70.

[2] Derrien M, Vaughan EE, Plugge CM, de Vos WM (2004) *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int. J. Syst. Evol. 54: 1469–1476

[3] Yang A, Yen C (2012) PCR Optimization of BOX-A1R PCR for Microbial Source Tracking of *Escherichia coli* in Waterways. Journal of Experimental Microbiology and Immunology 16: 85-89