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Master's thesis

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Anissa Zouzaf

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

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Determinants of IgA binding to gut bacteria - more than species identity*

Anissa Zouzaf[1,2], Johanna Kabbert[2], Patrick Vandormael[3] and Oliver Pabst[2]

[1]Hasselt University, Campus Diepenbeek, Agoralaan Building D – 3590 Diepenbeek, Belgium
[2]Institute of Molecular Medicine, Uniklinik RWTH Aachen, Pauwelsstraße 30 – 52074 Aachen, Germany
[3]Immunology and Infection research group, Biomedical Research Institute, Hasselt University, Campus Diepenbeek, Agoralaan Building C – 3590 Diepenbeek, Belgium

*Running title: *IgA specificity for gut microbiota*

To whom correspondence should be addressed: O. Pabst, Tel: +49 241 80-85496; Email: opabst@ukaachen.de

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ABSTRACT

The bacteria in our gut are in close interaction with our immune system. IgA is a key driver of microbiota-immune interactions, but the exact mechanisms of IgA-gut bacteria binding are unknown. It has previously been shown that IgA does not bind every single bacterium even though they are genetically identical. Therefore, we hypothesized that IgA binding capacity is affected by bacterial characteristics beyond the genetic identity. IgA plasma cell-derived monoclonal antibodies (mAbs) were generated from healthy and Crohn's disease (CD) donors. The binding capacity of mAbs and endogenous-IgA was characterized for 37 human fecal samples. Out of these, the most distinct binding patterns in terms of high binding capacity were selected for cell sorting of the mAb-positive -and negative fractions and 16S rRNA-based characterization. *De novo* clustering of the β-diversity in our sample library did not align with mAb binding profiles. In addition, the binding specificity of these mAbs was not significantly related to molecular species. Thus, mAb binding shows major heterogeneity between human donors that cannot easily be explained by differences in microbiota composition. A possible explanation is that IgA-binding to gut bacteria is heavily dependent on factors other than species composition. Further experiments will aim at an in-depth characterization of mAb-binding to gut bacteria.

INTRODUCTION

The structure of immunoglobulins

One of the most fascinating aspects of our gut microbiome is its peaceful coexistence and interaction with the host immune system. The human gut contains the largest plasma cell (PC) population, which predominantly secretes immunoglobulin A (IgA) in healthy individuals (1). Nevertheless, this immune response does not result in inflammation but establishes a homeostatic interrelationship with the microbiota (1-5).

In humans, there are two IgA isotypes; IgA1 and IgA2. In the gut, IgA1 is mainly found in the small intestine, whereas IgA2 is abundant in the large intestine (3, 4, 6). Under steady-state conditions, IgG and IgM are also present in the gut lumen.

Monomeric IgA consists of a fragment antigen-binding (Fab) fragment and a fragment crystallizable (Fc) region (7, 8). The Fab part is crucial for cognate antigen recognition, and it contains one constant and one variable part of the polypeptide heavy (H) and light (L) chain. The variable part is unique for each IgA and comprises the complementarity-determining (CDR) regions, with variability, diversity, and joining (V(D)J) fragments, interspaced with framework regions. In order to form the antigen-binding site, three CDRs of the H chain link together with three CDRs of the L chain. In contrast to the Fab part, the constant Fc part mediates effector-related functions such as activation of the complement system and binding to Fc-receptors, thereby enabling recognition of opsonized, i.e., antibody-coated, antigens.

T-cell dependent and independent activation of naive B cells

B cell activation and differentiation into antibody-secreting PCs is initiated by the encounter of a cognate antigen recognized by the B cell receptor (BCR). In the gut, this mainly happens in Peyer's patches (PP), which are the main inductive sites of the gut-associated lymphoid tissue (GALT). Subsequent to initial antigen binding, further steps in B cell activation may ensue in either a T-cell dependent (TD) or T-cell independent (TI) pathway (6, 8).

In the case of TI responses, signals such as microbial products will stimulate the proliferation of activated B cells (8). During differentiation of the activated B cells, different processes can take place. The first one is affinity maturation: to improve the affinity of the antibody for its antigen, somatic hypermutations might occur. The second one is isotype class switching which causes a switch to the IgA isotype and thereby the generation of IgA-secreting PCs.

TD antibody responses are induced in response to protein antigens, whereas TI responses frequently target fatty acids and polysaccharides (8). In TD responses, BCR internalization and major histocompatibility complex II (MHCII) presentation of the antigen will happen together with migration of the B cell to the interface of B cell follicles in GALT. Here, B cells can interact with T cells that have encountered parts of the same antigen via antigen presentation on, e.g., dendritic cells. For this interaction, B cells will display the antigen on MHCII and co-stimulatory signals such as CD40-CD40L interactions will mediate the TD response. This causes the generation of activated B cells and germinal center (GC) formation. In GCs, B cells undergo TD affinity maturation resulting in somatic hypermutations and class switch recombination to IgA (9).

Eventually, IgA plasmablasts can travel to the intestinal lamina propria, differentiate into resident PC and secrete IgA. In the gut, IgA primarily exists in a polymeric form by joining its H chain constant region with another monomeric secreted IgA (3, 4, 6). This happens with the help of mucosal PC-synthesized J-chains and disulfide bonding. Upon entering the external secretions of the body, secreted IgA needs to undergo structural changes. In the lamina propria of the gut, PC-secreted IgA binds to the polymeric Ig receptor (pIgR) expressed on the basolateral side of intestinal epithelial cells (IEC). Subsequently, the complex of pIgR and IgA is internalized, packaged into intracellular vesicles, and transported to the apical membrane. Concurrently, pIgR's polypeptide secretory component (SC) is covalently bound to secretory IgA (SIgA), and the rest of the pIgR is proteolytically cleaved. Finally, the complex gets released into the gut lumen. Therefore, SIgA is a dimeric hybrid antibody consisting of secreted IgA and the secretory part of pIgR. The SC plays a pivotal role in protecting the complex from proteolytic breakdown.

Intestinal IgA and gut microbiota: a match made in heaven

Dietary antigens, microbiota, and endogenous pathogens can induce IgA secretion (10, 11). In return, IgA can bind to components of luminal bacteria, such as flagellin, LPS and glycans (8). The percentages of IgA-coated bacteria in the gut vary depending on the location; ~10% of the bacteria are coated in the feces, whereas up to ~60% of the bacteria are coated with IgA in the proximal small intestine (1, 12).

Consequently, the roles of IgA in the gut are diverse; not only does it mediate immune reactions against unknown antigens, but it is also in charge of gatekeeping the intestinal bacterial balance by facilitating colonization of host-beneficial bacteria (2, 13).

Canonical and non-canonical binding

IgA has various binding modalities that can be grouped into canonical and non-canonical binding. The Fab region of an antibody mediates canonical binding to cognate antigens (6). In the bone marrow, antigen-independent recombinations take place: V(D)J fragments are randomly rearranged after activation of the recombination activation gene (*RAG*) (14). Because of this, B cells leave the bone marrow with a unique BCR. This process enables an enormous diversity of membrane-bound BCRs allowing for the recognition of millions of potential antigens in the host. So, any interaction between the antibody and its cognate antigen that involves the Fab-part of IgA is canonical.

On the other hand, non-canonical binding is not Fab-restricted and refers to the binding of antibodies involving any structure apart from the Fab (6). As for non-canonical IgA binding, these

are the glycans associated with the Fc region, SC, hinge region, and J chain of IgA. Bacteria have many glycostructures that can cause non-canonical IgA binding (15). Nakajima *et al*. observed glycan-mediated interactions between antibodies and gut bacteria (16).

The importance of being specific

The IgA binding specificity for gut microbiota has been a controversial and much-disputed subject within the field of gut immunology. It has long been thought that highly specific mAbs were only targeted at pathogens (17). However, more recently, it has been shown that the immune system can recognize many different antigens, including self-antigens and intestinal microbial antigens (18-20).

To test the IgA response to the intestinal microbiota, Benckert *et al.* determined the reactivity profile of human IgA-secreting PCs (2). First, they determined the IgA-encoding sequences of IgA PCs. Next, they cloned and expressed the respective mAbs to test their antigen-directed reactivity. Their results showed that all IgA PC-derived mAbs, from the repertoire of three healthy human donors (HD), carried a high number of somatic mutations and underwent strong antigen-mediated selection. Furthermore, 25% of the antibodies were polyreactive and bound to a diverse array of self and non-self antigens. The term polyreactive implies that an antibody can react to a variety of structurally unrelated antigens, e.g., insulin and lipopolysaccharides (LPS) (6, 19). Polyreactive binding of antibodies to structurally related antigens can involve both the Fab-part and other structures of the antibody (6). Nevertheless, this study failed to take into account the diversity of the human microbiome. The rate of polyreactive mAbs was measured using an enzyme-linked immunosorbent assay (ELISA) with pre-defined antigens, such as LPS derived from Escherichia Coli (E. Coli). The gut microbiome is much more diverse than this experimental setting, and the chance that the mAbs find their destined candidate in this setting is low.

Next, Bunker *et al.* reported that murine IgA is not catered towards specific bacterial taxa but is broadly reactive to a diverse and defined subset of bacteria (19, 20). Besides, this IgA polyreactivity was not related to microbial and dietary antigens, and somatic mutations did not significantly alter IgA reactivity. Overall, the authors concluded that IgAs are poorly selective, polyreactive antibodies in mice. Nonetheless, a few questions arise from this conclusion. Somatic mutations are needed for the active selection of specific IgAs by the immune system. The mice used in these experiments were young (six weeks) and therefore carried fewer mutations in their IgA compared to older mice and adult humans. In fact, the frequency of mutations in IgA observed in aged mice is similar to the mutation frequency in young children (6). Therefore, these experiments in mice insufficiently reflect the situation in adult humans. Furthermore, this polyreactive behavior is found in germline-like antibodies that did not undergo affinity maturation yet (19).

In contrast, Kabbert *et al.* found that in a set of healthy human and CD donor-derived mAbs polyreactivity was not correlated with bacteria binding (1). A lot these mAbs were highly mutated and cross-species reactive. Cross-species reactivity implies that one unique mAb can bind to one unique antigen on many different, structurally unrelated species. A lot of these somatic mutations were likely acquired through TD affinity maturation in the GALT, before entering the intestinal lamina propria. Here, activated B cells have been probed to produce specific antibodies. In contradiction to this, somatic mutations lead to a broader specificity instead of a more restrictive specificity. They took out the mutations of the antibodies, which reverted them back to the germline-like configuration, and they did not bind to microbiota anymore and also did not exert polyreactive capacities. This shows that somatic mutations are needed for binding of mAbs to gut bacteria. Furthermore, they also investigated the Fab-dependent canonical binding reactivity of mAbs to gut microbiota and showed that canonical binding can cause cross-species reactivity. For this, they used recombination fusion protein IgA PC-derived mAbs, which have a Fc part with the isotype IgG1 and a unique Fab part with the isotype IgA1 or IgA2. Consequently, the differences in bacteria binding between these mAbs display the differences in canonical Fab-dependent binding. The question now is, what are the epitopes to which these recombinant mAbs bind in humans?

A recurring phenomenon has been observed in mice and humans, where certain bacterial species are sometimes coated and sometimes not coated with intestinal IgA (1, 12). This coating, or the lack

thereof, is telling for the functional and pathological properties of these bacteria (1, 12, 21, 22). The molecular mechanisms of this selective IgA binding specificity, however, are unknown. Our recently published and unpublished experiments imply a cross-species reactivity for gut IgA-microbiota interactions (1). In other words, IgA PC-derived mAbs are specific and unique for one PC in the lamina propria, and these mAbs can bind to different bacterial species that are not genetically related. The binding epitopes of these mAbs, and the mechanisms behind cross-species reactive IgA-microbiota interactions, are still largely unknown.

This research aims at elucidating how IgA cross-species reactivity is established in humans. We hypothesize that the specificity of human IgA is caused by a combination of the genetic identity of bacterial strains, isolated from human fecal samples, and a bacterial state unrelated to the species identity. As for now, a molecular explanation for the term 'bacterial state' is lacking. We use this term to describe factors influencing the availability of IgA binding epitopes on the bacterial surface. These factors may include intrinsic differences such as metabolic adaptations of bacterial gene expression and growth. Moreover, these factors may also include extrinsic mechanisms such as the modification of the bacterial surface by host factor binding or enzymatic activities in the gut.

EXPERIMENTAL PROCEDURES

Sample acquisition – An existing collection of 37 human fecal samples from the centralized bio-material bank of the Medical Faculty of the RWTH Aachen University (RWTH cBMB) was used. Sampling was done according to the regulation of the RWTH cBMB and the Ethics Vote 206/09 of the Ethics Committee of the Medical Faculty of RWTH Aachen University.

Monoclonal antibody (mAb) isolation from human gut plasma cells – Recombination fusion protein IgA PC-derived mAbs were generated from three HD and three CD donors, as described before (1, 2).

Labeling of the mAbs with Alexa Fluor™ 647 (AF647) – Human donor-derived mAbs were directly labeled with AF647 using the Alexa Fluor™ 647 Antibody Labeling Kit (Invitrogen, A20186) according to the manufacturer's instructions.

Bacteria isolation – Human fecal samples from the RWTH cBMB are stored at -80°C and were thawed and homogenized in 1 ml filtered Hank's balanced salt solution (HBSS, Gibco, ThermoFisher Scientific) supplemented with 2% goat serum (Sigma-Aldrich). Homogenized samples were then centrifuged at 500 rpm for 1 min and washed three-four times with filtered HBSS over a nylon gaze (Sephar NITEX) until there was no debris left in the supernatant. Next, the clear supernatant was centrifuged at 8,000 rpm for 8 min to obtain a bacterial pellet. This pellet was resuspended in 200 µl filtered HBSS/goat 2% and passed through a gaze. Lastly, a 1:100 dilution was made in HBSS/goat 2% to adjust the optical density of the suspension at a wavelength of 600 nm (OD600) to 0.25 ± 0.05 (5×10^7/ml bacteria) (1).

Bacterial flow cytometry staining– The concentration of the directly labeled mAbs was measured with NanoDrop. Each mAb was diluted in sterile HBSS to a concentration of 5 µg/ml. In separate tubes, mouse anti-human IgA Pe (1:50, Miltenyi Biotec, clone IS11-8E10, 130-093-128), goat anti-human IgG Fc_γ AF647 (1:150, Jackson ImmunoResearch, 109-605-098), and mouse anti-human IgM AF647 (1:150, Southern Biotech, clone SA-DA4, 9020-31) were diluted in sterile HBSS. As respective controls, the isotypes mouse IgG2a,κ Pe (1:100, Biolegend, clone MOPC-173, 400212), goat IgG AF647 (1:150, Southern Biotech, 0109-31), and mouse IgG1 AF647 (1:150, Southern Biotech, 0102-31) were also diluted in sterile HBSS. Next, 3 µl or 9 µl of bacterial suspension was added to each condition for flow cytometric or fluorescence-activated cell sorting (FACS) analysis, respectively. This was followed by a dark incubation of 30 min on ice. The samples were then stained with 50 µl of Syto-9 nucleic acid stain (1:500, ThermoFisher), incubated for 10 min on ice, and washed in 1 ml HBSS at 13,000 rpm for 8 min at 4°C. The supernatant was discarded, pellets were resuspended in 60 µl HBSS and transferred to a flow cytometry tube (Greiner Bio-One, 102201) for flow cytometric analysis.

Flow cytometric analysis bacteria – A minimum of 300,000 events were obtained for each sample on the LSRFortessa (BD Biosciences, BD FACSDiva™ Software). Each analysis was performed on two or more technical independent replicates. Unstained bacteria were analyzed to determine the gating strategy.

Flow cytometric bacterial sort purification – FACS samples were prepared as described above and sorted on the FACSAria II (BD Biosciences, BD FACSDiva™ Software). IgA hi^+/ IgA^- and mAb hi^+/mAb^- events were gated and sorted into collection tubes. Depending on the individual samples, between 200,000 and 500,000 events were sorted first, for each gate, using the purity sort setting and an ND filter. A second round of purity sorting was done for the hi^+ events only in order to increase the purity of the sorted sample. After each purity sort, the purity of the sample was checked. A minimum of 100,000 hi^+ events was acquired. Next, 50,000 Syto-9 positive events were sorted and, together with unsorted bacterial input material, used for the DNA library. In addition, sheath fluid was collected to determine the presence of bacteria in the sort stream and fluidics of the FACSAria II. The sorted fractions were centrifuged at 13,000 rpm for 8 min at 4°C, and pellets were stored at -80°C for further analysis.

Bacterial DNA extraction – Metagenomic DNA was isolated by adding 600 µl of DNA stabilizer (Invitek) and 500 mg of autoclaved zirconia beads (⌀100 µm) to the frozen human fecal aliquots. Next, 500 mL of 5% N-lauroylsarcosine solution and 200 mL 4M guanidine-thiocyanate were added to the samples to facilitate cell lysis and inhibit DNA degradation, respectively. The samples were incubated at 70°C for 1h with constant, moderate shaking (700 rpm). For mechanical cell lysis by bead-beating, the FastPrep-24 with cooling adapter was used; 6.5 m/s, 40s, three cycles. Subsequently, 15 mg of polyvinylpyrrolidone (Merck) was added to the lysed samples to catch impurities that can inhibit downstream enzymatic reactions. This solution was then centrifuged at 15,000 g at 4°C for 4 min. The supernatant, containing the DNA, was transferred to a new tube and centrifuged again at 15,000 g, 4°C for 4 min. Lastly, 5 µl of RNase was added to the sample followed by incubation at 37°C for 20 min with constant, moderate shaking to obtain an RNase-free suspension. This DNA-containing suspension was purified using the NucleoSpin gDNA kit (Machery-Nagel) according to the manufacturer's instructions. The DNA concentration was measured using the NanoDrop.

Polymerase chain reaction (PCR) – The hypervariable regions V3 and V4 of the 16S ribosomal RNA (rRNA) were amplified in two subsequent amplification reactions (15+10 cycles), using the 341F-785R primers (23). PCR quality was checked via gel electrophoresis and the PCR products were purified using magnetic beads (AMPureXP, Beckman Coulter). In order to multiplex in equimolar amounts, DNA concentration was measured using the Quant-iT PicoGreen dsDNA kit (Invitrogen) on a Qubit-3 fluorometer (ThermoFisher). Since DNA concentration is proportional to the amount of DNA, the samples were normalized and pooled in an equimolar amount of 2 nM

16S rRNA gene amplicon sequencing – The multiplexed samples were sequenced on an Illumina MiSeq in paired-end mode (2×220 bp) using the Rapid v2 chemistry. 16S rRNA sequencing preparations and analysis were performed by the Institute of Molecular Microbiology, Uniklinik RWTH Aachen, as part of the Q02 project of CRC1382.

Statistical analysis – Statistical analysis for flow cytometric data was performed in R using the pheatmap package agglomerative clustering algorithm. 16S rRNA sequencing raw sequencing data was processed with the IMNGS (imngs.org) tool, and analysis was done using the Rhea script in R (24). Additional statistical analyses were done in GraphPad Prism. The normal distribution of the data was assessed using the Shapiro-Wilk test.

RESULTS

Experimental setup and establishment of a bacterial flow cytometry gating strategy – The mechanisms underlying IgA binding to intestinal bacteria under steady-state conditions are poorly understood. In this study, the binding of a collection of IgA PC-derived mAbs was screened on a set of human stool samples to define binding patterns of intestinal IgA for human gut bacteria (**Fig. 1**). Human fecal (HF) samples were obtained as part of the Cultimic study (Cul), and replicate aliquots of HF material were obtained for 37 donors. HF samples were pooled and aliquoted because this reduces the intra-individual differences and thus the variability in mAb-bacteria binding capacity. However, working with HF samples also implies some limitations, namely the interindividual differences in bacterial composition, which is less prominent in murine material.

Alongside this, HD and CD donor-derived mAbs were isolated from intestinal IgA PCs. This generated a library of over a hundred mAbs, of which four HD and four CD mAbs were chosen for this study after pre-screening their microbiota binding capacity using murine and human fecal material ((1), **Supplementary Table 1**). The mAbs used herein are fusions of the original IgA-derived Fab fragment linked to the human Fc IgG1 part and conjugated to the fluorochrome AF647 (1).

Bacteria were isolated from all 37 HF samples and stained with the eight selected mAbs to map the binding reactivity of IgA PC-derived mAbs to different members of the microbiota. In addition, coating of HF bacteria with endogenous IgA, IgG, and IgM was also analyzed.

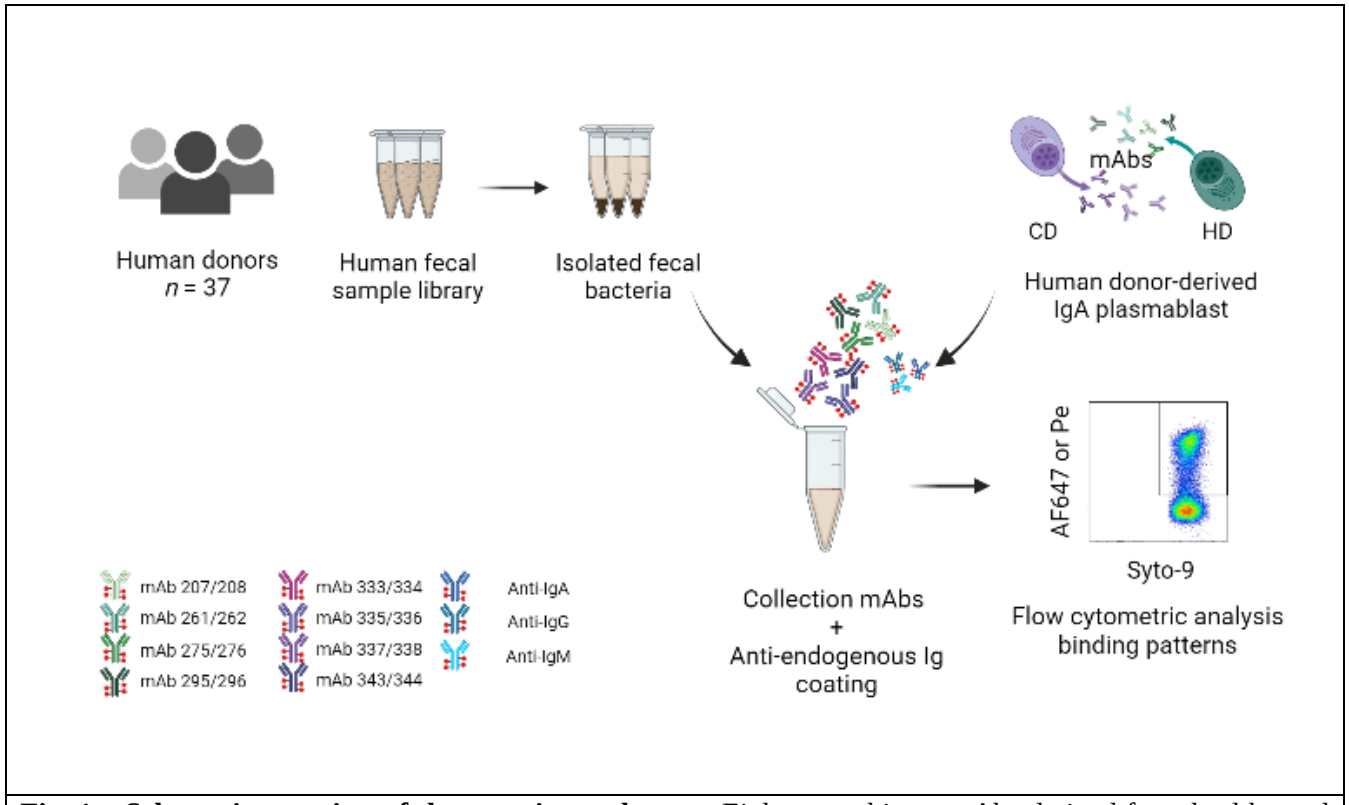

Fig. 1 – **Schematic overview of the experimental setup**. Eight recombinant mAbs derived from healthy and Crohn's disease IgA PCs, previously screened by Kabbert *et al.*, were screened for their reactivity to samples from a library of 37 human fecal samples (Cultimic study) with flow cytometry. Image created with Biorender. *CD; Crohn's disease, HD; healthy donor, mAbs; monoclonal antibodies, Ig; immunoglobulin*.

To establish a validated gating strategy, unstained human fecal samples were analyzed (**Fig. 2A,B**). Unstained bacteria did not show fluorescence in the green fluorescent Syto-9 channel, whereas samples stained with Syto-9 only showed a clear Syto-9 positive population. This positive population was then gated further to investigate the mAb^+, anti-IgG^+, anti-IgM^+ (**Fig. 2A**) and anti-IgA^+ (**Fig. 2B**) populations. The respective isotypes controls were taken into account for antibodies against endogenous IgA, IgG, and IgM. Based on previous studies, flow cytometric IgA-bacteria interactions were categorized as low (<1%), intermediate (1-5%), and high binding capacity (>5%) interactions (1).

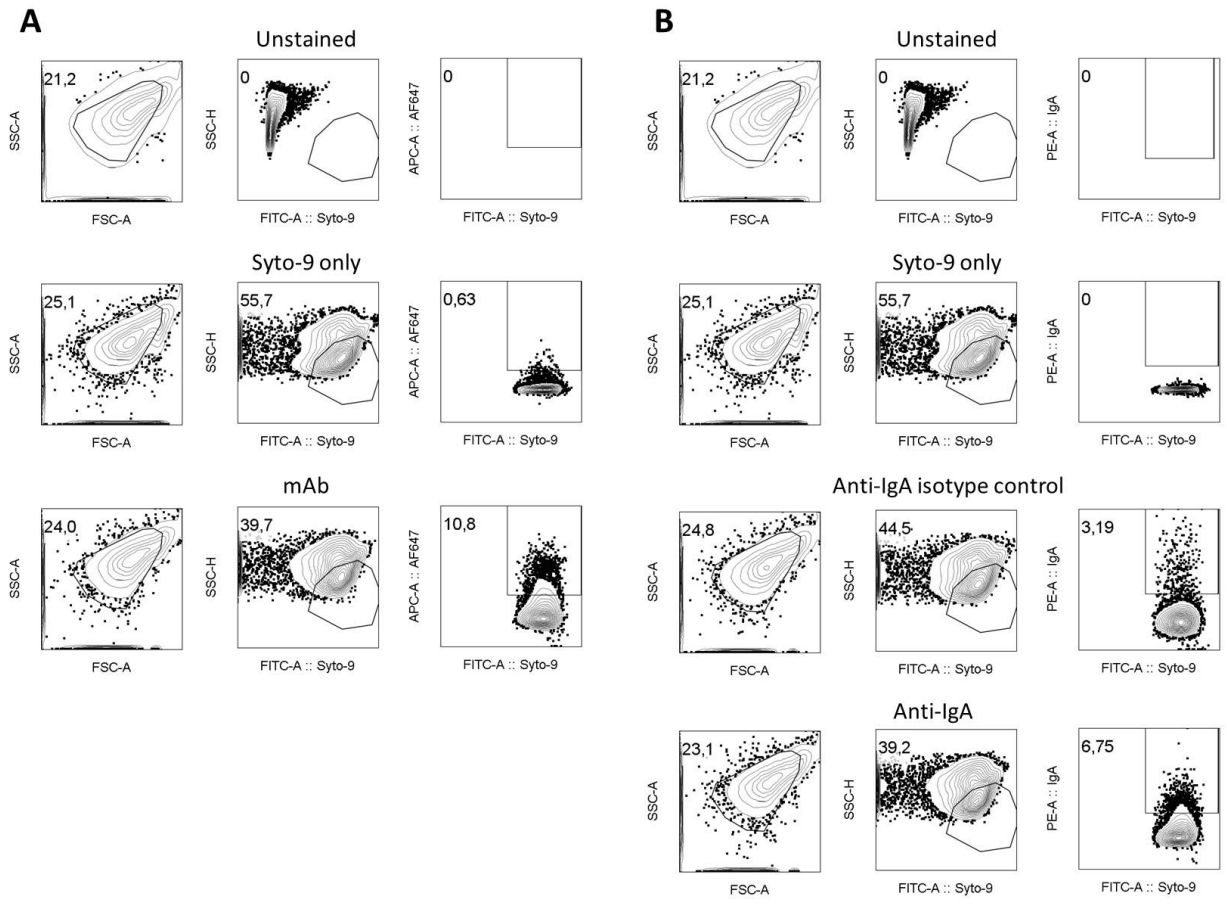

Fig. 2 – **Establishment of a flow cytometric gating strategy for fecal bacteria. A)** Bacteria were isolated from the feces of human donors. Syto-9 nucleic acid dye was used to stain live and dead bacteria. Samples stained with Syto-9 only were used as a background control to set the gates for each individual sample (<1%). **B)** For the endogenous IgA coating, anti-IgA-Pe isotype control was used to set the gates. A similar strategy was used for anti-endogenous IgG and IgM. *mAb; monoclonal antibody, IgA; immunoglobulin A*

IgA-derived mAbs show distinct binding patterns to human feces-derived bacteria – Once the gating strategy was defined, the binding of the IgA PC-derived mAbs was screened on a set of HFs. In total, 37 samples were analyzed with eight different mAbs, of which 17 samples were repeated. Flow cytometric analysis showed that both HD and CD-derived mAbs display high, intermediate, and low binding to different donor samples (**Fig. 3A**). As for the mean binding percentages, there was no significant difference between the HD and CD mAb subset (p>0.05) (**Fig. 3A**). Furthermore, there was variation in the binding profiles of the mAbs for each individual donor (**Fig. 3B**).

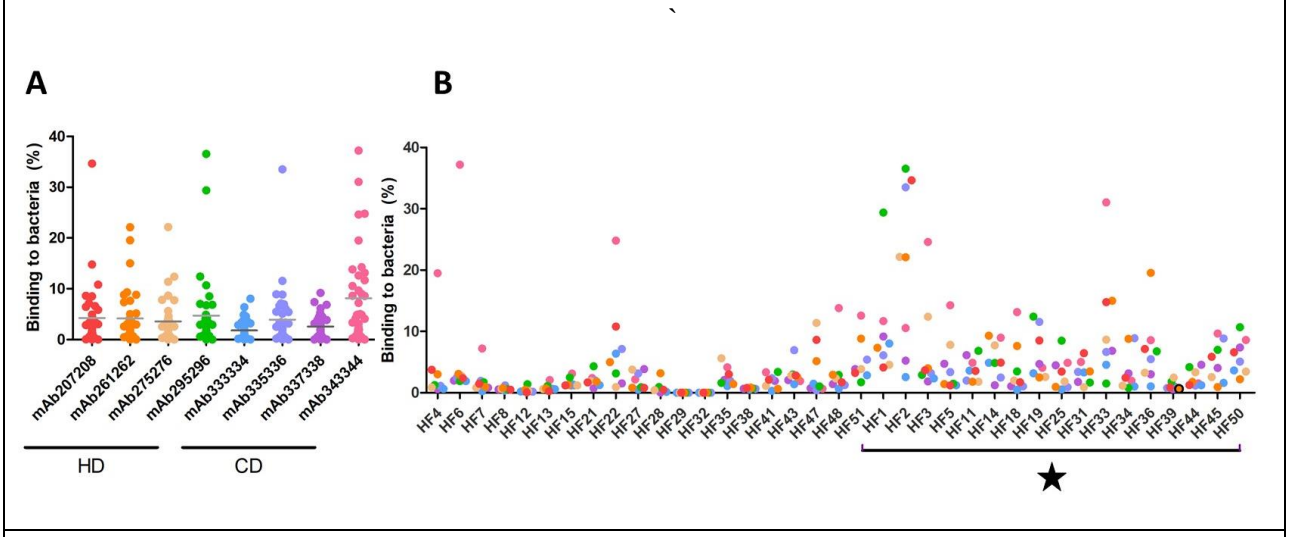

Fig. 3 – **Human donor-derived fecal bacteria bound by mAbs reveals inter-donor variation in mAbs binding profiles. A)** Bacteria were isolated from human fecal samples from 37 donors and stained with directly labeled mAbs. There were no significant differences between the binding capacity of HD and CD mAbs for intestinal bacteria (p>0.05) as determined by a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Grey horizontal lines indicate the mean binding percentages. **B)** Dot plot depicting the different binding percentages of all mAbs for each individual donor (n = 37) shows variation between samples. Data are expressed as percentages, and the mean was taken in the case of two independent replicates (samples marked with a star ★). *HF; human feces, mAb; monoclonal antibody, HD; healthy donor, CD; Crohn's disease*

Individual microbial compositions did not correlate with mAb binding in humans – To investigate the variation of mAb binding between samples, the microbial configuration differences between samples on species level were investigated. The cumulative relative abundance of operational taxonomic units (OTUs) for each sample was investigated from phylum to family level, showing a diverse microbial composition for each sample in the Cultimic study (**Fig. 4**). OTUs are used as a proxy to refer to distinct bacterial species. 16S rRNA does not allow differentiation between closely related bacterial species. Thus, an OTU is generated by clustering of sequencing reads at 97% similarity, which is then taxonomically assigned. Consequently, each OTU is at least 3% different from another OTU. There is some variation between the donors on the phylum level, although most donors have a fecal microbiota centered around Firmicutes and Bacteroidetes (**Fig. 4**). Differences between donors show even more prominently on the family level (**Fig. 4**).

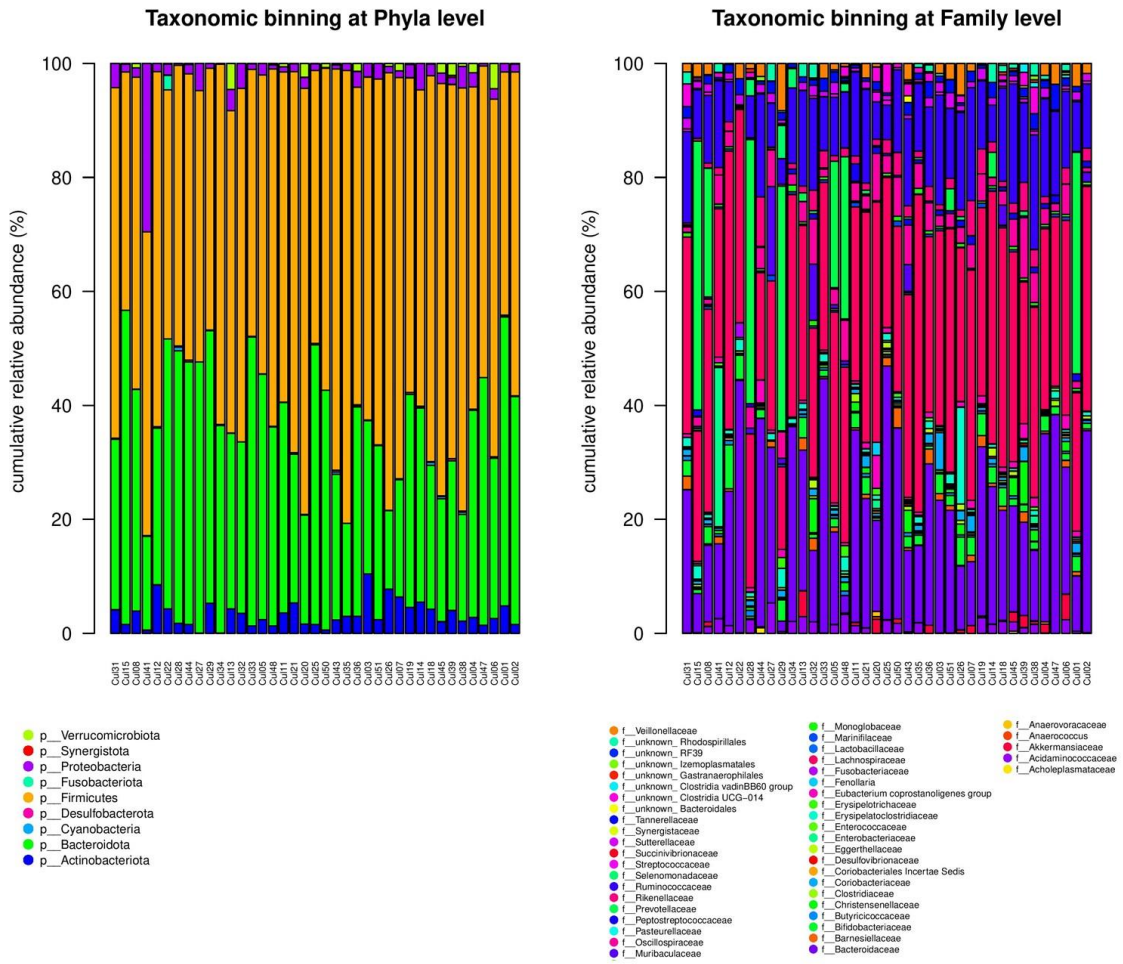

Fig. 4 – **Taxonomic binning of 16S rRNA gene amplicons reveals diverse microbial communities in Cultimic study samples.** The diversity within the Cultimic study samples is shown as the taxonomic binning of 16S amplicon sequences for each of the individual HFs at the phylum (left panel) and family (right panel). Each bar represents one individual. The colors correspond to the phyla and families, as shown in the legends. *Cul; Cultimic study number.*

To visualize the variation between the samples (β-diversity), Meta Nonmetric Multidimensional Scaling (metaNMDS) plots based on the generalized UniFrac distances were used (**Fig. 5A**) (25). In addition, *de novo* clustering of the β-diversity of the 37 samples did not visually correlate with mAb binding (**Fig. 5B**).

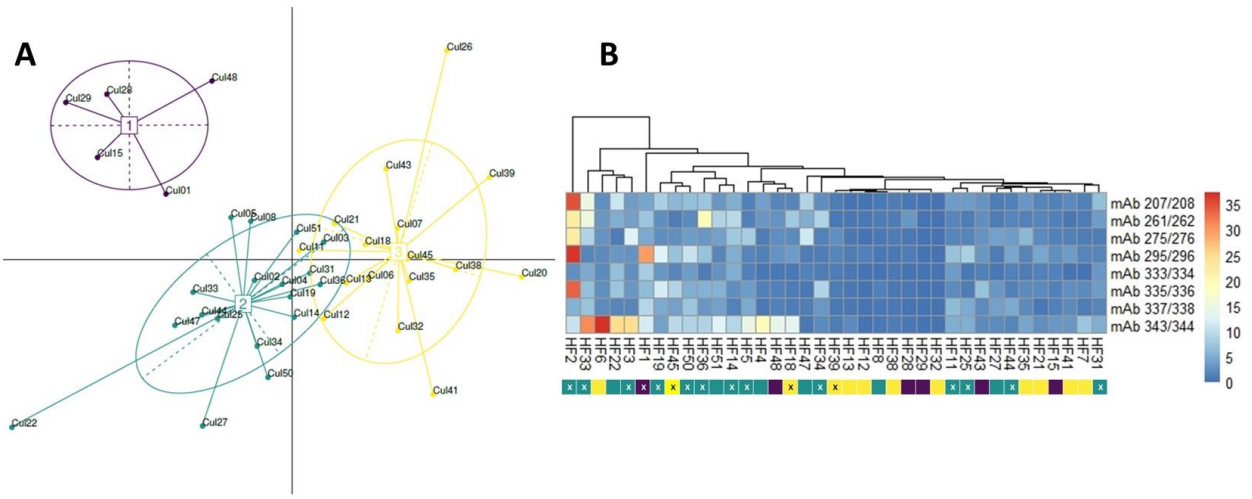

Fig. 5 – **MetaNMDS plot of the *de novo* clustering of the β-diversity in the Cultimic study does not correlate with positive mAb binding. A)** *De novo* clustering analysis of the human fecal samples shows the β-diversity of the bacterial communities within the Cultumic study. The distance between the samples in the microbial profiles is based on generalized UniFrac distances (p<0.001) (N. Treichel, 2021). **B)** Heatmap showing the percentage of mAb-bound bacteria for each sample. Bacteria were isolated from human fecal samples from 37 donors and stained with directly labeled mAbs. Agglomerative dendrogram clustering is based on the similarity of the positive fraction mAb-binding percentage between donors. Data are expressed as percentages, and the mean was taken in the case of two independent replicates (n = 17, indicated with 'x'). Percentages range from 0.0% to 37.2%. The column annotation corresponds to the NMDS cluster number of each individual sample by color. *Cul; Cultimic study number, mAb; monoclonal antibody, HF; human fecal sample.*

Given the relative abundances of OTUs, a serial comparison analysis between the samples was done. For each sample, the correlation between the binding percentage to each mAb individually in relation to the most abundant OTUs in the samples is shown in **Fig. 6**. The size of the bubble in combination with the color indicates whether specific mAb binding is positively (blue) or negatively (red) correlated with specific OTUs. According to the adjusted p-values, there is no significant correlation between OTUs and mAb binding (p>0.05) (**Supplementary Table 2**). However, some OTUs, e.g., OTU_1 and OTU_3, seem more likely to bind all the mAbs, whereas other OTUs, such as OTU_13 and OTU_15, seem less likely to be bound by mAbs, although not statistically significant (**Supplementary Table 2**)

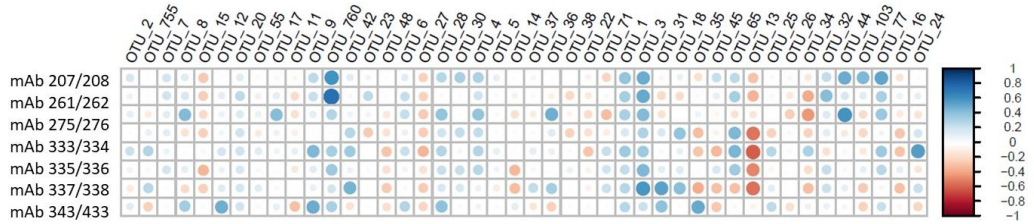

Fig. 6– **Bubble chart shows the statistical comparison of taxonomic composition and mAb binding between groups.** According to the adjusted p-values, there is no significant correlation between OTUs and mAb binding. The legend on the right and the corresponding colors indicate the Pearson's correlation coefficient r. Non-parametric Mann-Whitney pairwise comparison test corrected for multiple testing using the Benjamini-Hochberg method. *OTU; operational taxonomical unit, mAb; monoclonal antibody*

Sort purification strategy for further 16S rRNA analysis of the mAb positive and negative fractions based on the distinctness of binding patterns– In order to define the nature of mAb binding to gut bacteria in more depth, samples were selected for sort purification of mAb binding and non-mAb binding bacteria. Samples were chosen based on the distinctness of the mAb binding pattern (**Fig. 7A**). Distinctness was based on the shape of the mAb binding pattern and evaluated by two independent researchers. The observed variation in binding capacity is likely due to individual differences in microbiota composition and other factors that can influence the availability of mAb binding epitopes. Samples with a high binding capacity for one or more mAbs were duplicated. However, a high mAb positive fraction binding capacity does not imply a sortable bacteria population. For these 17 samples, the binding of antibodies against endogenous IgA, IgG, and IgM coating was also checked (**Fig. 7B**). Endogenous antibodies did not mark overt differences between different donor samples. However, gut bacteria were significantly more likely to be coated with IgA, as compared to IgG and IgM (p<0.05) (**Fig. 7B**).

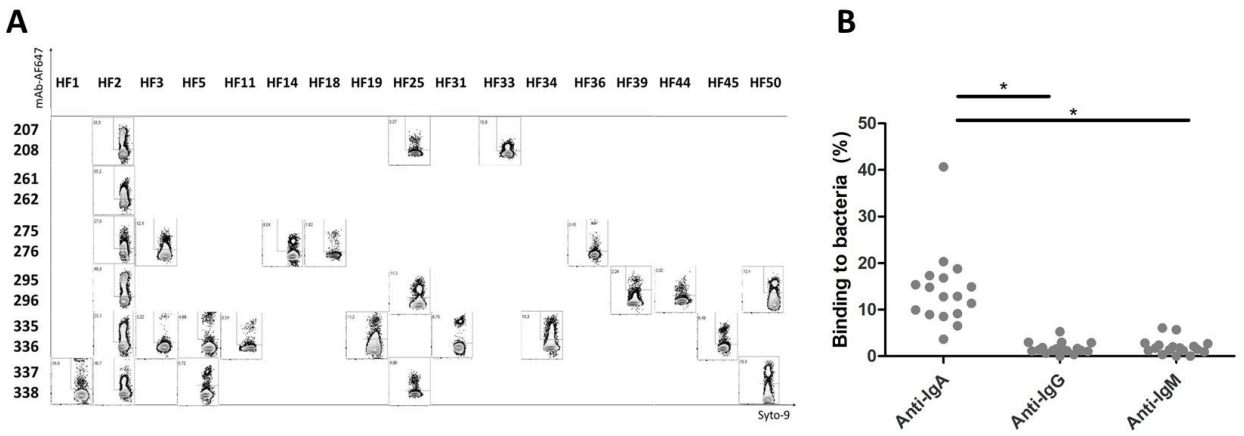

Fig. 7 – **Distinct binding patterns for mAb binding in the library of human fecal samples, but not for endogenous Ig-coating. A)** Representative FACS plots showing distinct mAb and endogenous binding patterns to human fecal bacteria of 17 samples. Bacteria were stained with Syto-9 nucleic acid dye and the microbiota reactivity of the AF647 directly labeled mAbs (rows) was analyzed using flow cytometry. **B)** Endogenous IgA, IgG, and IgM coating show highly variable binding patterns to human feces-derived bacteria. Bacteria were significantly more coated with IgA as compared to IgG and IgM (*p<0.05) as determined by a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. *HF; human feces, mAb; monoclonal antibody*

For the selected samples, mAb-positive and negative fractions were sorted for further 16S rRNA analysis (**Fig. 8**). Sorting was done in the purity setting, enabling enrichment of the samples by only sorting positive events with high confidence. After each sort, the purity of the mAb^+/IgA^+ and mAb^-/IgA^- bacteria were checked (>90%). Two subsequent purity sorts were needed for the positive fractions to obtain more than 90% purity. To serve as a control for follow-up 16S rRNA sequencing, mock sorts/samples have been obtained that did not contain any bacteria but had been generated at the sort day under identical conditions compared to the proper samples. Each sample was sorted in duplicate, and after each sort, samples were spun down and stored at -80°C for further analysis. These samples are currently being analyzed by 16S rRNA sequencing.

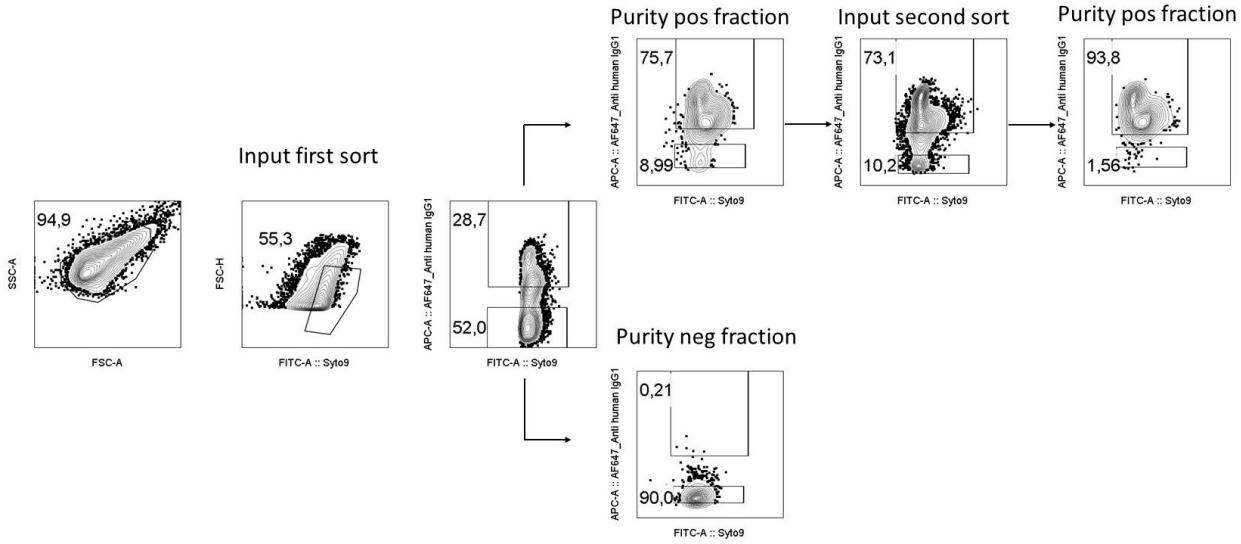

Fig. 8 – **Bacterial sort purification strategy.** The bacterial input was analyzed, and gates were set according to the Syto-9 control and isotype control, in the case of anti-endogenous IgA. For the mAb/IgA$^+$ fraction, two subsequent purity mode sorts were done. After each sort, the purity of the sample was checked (>90%). Final fractions were spun down and stored at -80°C for further analysis.

DISCUSSION

The main goal of the current study was to map IgA-gut bacteria interactions in relation to the 16S rRNA sequencing-based microbiota composition. Here we showed that eight mAbs derived from human intestinal PCs have distinct binding patterns to different members of the microbiota. Each of the eight antibodies detected gut bacteria; however, the binding intensity and the fraction of bacteria bound by a given antibody largely varied between the individual human donor samples. This observation might foremost reflect differences in microbiota composition; different individuals will have a distinct microbiota composition. If this was the case, one might expect that differences in antibody binding patterns might align with similarities in microbiota composition. I.e., donors that share a similar microbiota composition, as determined by 16S rRNA sequencing, might also share similar antibody binding profiles. Conversely, donors that show a somewhat different microbiota composition might also show dissimilar antibody binding. However, this was not the case. Instead, the overall 16S rRNA species composition of the individual human fecal samples was not predictive for these mAb binding patterns.

We speculate that besides microbiota composition and genetic identity of the bacterial species present in a given donor, additional factors determine IgA binding to gut bacteria. Antibodies can bind to distinct epitopes, and the expression and availability of these epitopes might vary depending on the bacterial and host state.

Gut bacteria dynamically regulate their gene expression profile, e.g., in response to environmental conditions such as oxygen and substrate availability (26, 27). Thus depending on the metabolic and growth state, antibody epitopes might be available on the bacterial surface. Moreover, host effects might shape bacterial gene expression and metabolic pathways (28). Therefore, we consider these factors as intrinsic because they affect the expression of antibody epitopes by regulatory process in bacteria.

On the other hand, extrinsic factors might affect the binding of antibodies to gut bacteria. For example, host factors such as proteins of the complement system or antimicrobial peptides can bind to the bacteria surface and might thereby

create new antibody epitopes. Moreover, the gut lumen is rich in proteolytic and glycan modifying activities. Thus host factors might act on the bacterial surface and thereby affect epitope availability. A particular likely target might be core glycan structures. Glucosidases removing terminal sugars from the bacterial surface might expose underlying core glycans that act as epitopes for IgA.

Effectively a combination of intrinsic and extrinsic factors might combine to shape the binding of IgA to the gut microbiota.

HD as well as CD mAbs both had distinct binding patterns for members of the gut microbiota, suggesting that changes in the inflammation state of the gut did not cause major disruptions in the immune cell repertoire, as previously observed (1, 29). This implies that cross-species antibodies are prevalent in both healthy and inflamed conditions, underlying their importance. It is unclear whether these mAbs exert specific pro- or anti-inflammatory properties, or both, by binding to gut bacteria. There are multiple functionalities of bacteria-coating IgA, including immune exclusion and intracellular neutralization, thereby modifying local immune responses in a non-inflammatory way (30, 31). IgA can also contribute to the initiation of inflammation by the formation of immune complexes (32). Consequently, this triggers IgA Fc receptor FcαRI (FcαRI) mediated inflammation. FcαRI-induced inflammation might play a role in various chronic inflammatory disorders, including IBD.

On the other hand, it has been shown that disease activity did play a role in shaping IgA/IgG-gut bacteria responses in patients with CD and Colitis Ulcerosa (33). Therefore, it would be interesting to include samples from donors with different disease states and antibiotic regimes into our HF library. Our study showed that bacteria were significantly less likely to be coated with IgM and IgG as compared to IgA. Van der Waaij *et al.* determined the percentage of coated bacteria in human feces, both for IBD patients and HD (34). Overall, both IBD patients' and HD's fecal bacteria had high IgG and IgM coating percentages, around 20-90%. However, no isotype controls nor multiple blocking steps were used to cancel out background events, as it was the case in our experimental setup.

Taxonomic binning of the donor samples showed that they are most abundant in Firmicutes and Bacteroidetes. As shown previously, the Firmicutes and Bacteroidetes phyla make up 90% of the total gut microbiota (35). Moreover, the inter-donor variation on the family level could be traced back to different OTUs. An OTU, or molecular species, clusters the 16S rRNA sequencing reads at 97% similarity. The representative sequence for each OTU is then taxonomically assigned. The classification of OTUs into taxonomic groups can be done in different ways, e.g., Lowest Common Ancestor (LCA) (24, 36). Notwithstanding, this method also poses its limitations, given that a large number of bacteria have not been characterized yet. Therefore, many OTUs encompass unknown species and represent incomplete taxonomic classification.

Furthermore, our findings may be somewhat limited by the experimental setup. There is no golden standard for gating bacteria; they defy the limits of flow cytometry due to their relatively small size. It is not excluded that fungi, yeast, or archaea are present in the primary gate. However, Syto-9 only stains bacteria and further 16S rRNA sequencing can also show this. Another possibility might be *in vitro* cultivation of known bacterial species and characterization of mAb binding in different stages of bacterial development and under different environmental circumstances. Different developmental stages might trigger various surface molecules, whereas factors such as temperature have been shown to influence the diffusion of Bacillus subtilis membrane proteins to microdomains in the membrane (37, 38).

Moreover, the human gut contains mostly strictly anaerobic bacteria (39). As a result, these populations get lost in our workflow. A culture-based method could potentially resolve this issue. Therefore, a collaborative project with BIOASTER (Paris, France) was established to anaerobically sort bacteria into culture plates and cultivate them, allowing for a more in-depth analysis of the species in the samples.

CONCLUSION

Altogether, we found evidence suggesting that IgA binding to gut bacteria is not only dependent on the genetic species identity but also on additional characteristics. This evidence will likely be solidified by analysis of the 16S rRNA data of the sort purified bacterial fractions.

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SUPPLEMENTAL MATERIAL

Supplementary Table 1 – Overview of the selected directly labeled recombination fusion protein IgA PC-derived mAbs. The number between brackets refers to the Fab heavy and light chain.

HD mAbs	CD mAbs
HD2a88 (207/208)	CD2a70 (333/334)
HD3a14 (261/262)	CD2a127 (335/336)
HD3a103 (275/276)	CD2a146 (337/338)
HD3a147 (295/296)	CD3a549 (343/344)

HD; healthy donor, CD; Crohn's disease, mAbs; monoclonal antibodies

Supplementary Table 2– Statistical metrics of the serial group comparisons between mAbs and OTUs. According to the adjusted *p*-values, there is no significant correlation between OTUs and mAb binding. Non-parametric Mann-Whitney pairwise comparison test corrected for multiple testing using the Benjamini-Hochberg method. A *p*-value<0.05 is considered statistically significant.

		OTU760	OTU3	OTU13	OTU44
	n	19	31	20	13
mAb 207/208	Pearson's r				0.5924
	p-value				0.0329
	Adj. *p*-value				0.638
mAb 261/262	Pearson's r	0.7423	0.5071		
	p-value	3e−04	0.0036		
	Adj. *p*-value	0.0963	0.2856		
mAb 275/276	Pearson's r	0.5972			0.5222
	p-value	0.0069			0.0182
	Adj. *p*-value	0.3051			0.4566
mAb 295/296	Pearson's r			−0.5304	
	p-value			0.0161	
	Adj. *p*-value			0.4369	
mAb 333/334	Pearson's r			−0.6032	
	p-value			0.0049	
	Adj. *p*-value			0.2856	
mAb 337/338	Pearson's r		0.5846	−0.5482	
	p-value		6e−04	0.0123	
	Adj. *p*-value		0.0973	0.3946	

mAbs; monoclonal antibodies