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Next-generation IgA-SEQ allows for high-throughput, anaerobic, and metagenomic assessment of IgA-coated bacteria

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Abstract

Background The intestinal microbiota plays a significant role in maintaining systemic and intestinal homeostasis, but can also influence diseases such as inflammatory bowel disease (IBD) and cancer. Certain bacterial species within the intestinal tract can chronically activate the immune system, leading to low-grade intestinal inflammation. As a result, plasma cells produce high levels of secretory antigen-specific immunoglobulin A (IgA), which coats the immunostimulatory bacteria. This IgA immune response against intestinal bacteria may be associated with the maintenance of homeostasis and health, as well as disease. Unraveling this dichotomy and identifying the immunostimulatory bacteria is crucial for understanding the relationship between the intestinal microbiota and the immune system, and their role in health and disease.

IgA-SEQ technology has successfully identified immunostimulatory, IgA-coated bacteria from fecal material. However, the original technology is time-consuming and has limited downstream applications. In this study, we aimed to develop a next-generation, high-throughput, magnet-based sorting approach (ng-IgA-SEQ) to overcome the limitations of the original IgA-SEQ protocol.

Results We show, in various settings of complexity ranging from simple bacterial mixtures to human fecal samples, that our magnetic 96-well plate-based ng-IgA-SEQ protocol is highly efficient at sorting and identifying IgA-coated bacteria in a high-throughput and time efficient manner. Furthermore, we performed a comparative analysis between different IgA-SEQ protocols, highlighting that the original FACS-based IgA-SEQ approach overlooks certain nuances of IgA-coated bacteria, due to the low yield of sorted bacteria. Additionally, magnetic-based ng-IgA-SEQ allows for novel downstream applications. Firstly, as a proof-of-concept, we performed metagenomic shotgun sequencing on 10 human fecal samples to identify IgA-coated bacterial strains and associated pathways and CAZymes. Secondly, we successfully isolated and cultured IgA-coated bacteria by performing the isolation protocol under anaerobic conditions.

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Conclusions Our magnetic 96-well plate-based high-throughput next-generation IgA-SEQ technology efficiently identifies a great number of IgA-coated bacteria from fecal samples. This paves the way for analyzing large cohorts as well as novel downstream applications, including shotgun metagenomic sequencing, culturomics, and various functional assays. These downstream applications are essential to unravel the role of immunostimulatory bacteria in health and disease.

Keywords Gut microbiota, IgA-SEQ, Intestinal inflammation, IgA-coated bacteria, Next-generation IgA-SEQ, High-throughput, Metagenomic shotgun sequencing, Culturomics, Inflammatory bowel disease, Cancer

Background

In recent years, it has become increasingly evident that the intestinal microbiota is essential for maintaining human health. The symbiotic relationship between the intestinal microbiota and the host contributes to the fermentation of food, protection against pathogens, and the development of the immune system, amongst others [1–3]. It is difficult to define a "healthy" gut microbiota since the composition varies highly between individuals, location within the gastrointestinal tract, and undergoes alterations while aging [2, 4]. It is known, however, that dysbiosis of the gut microbiota, caused by for example infections, changes in diet, or the use of medication, can deregulate bodily functions and contribute to various diseases [1]. For example, multiple studies have shown differences between the gut microbiomes of healthy individuals and inflammatory bowel disease (IBD) patients [5]. Even though questions remain concerning causality, studies have shown an association between IBD, microbiota dysbiosis, the immune system, and the integrity of the intestinal barrier [1, 6, 7]. Additionally, ever since the 1990s, when an association was found between *Helicobacter pylori* infections and the development of gastric cancer, microbiota dysbiosis has been linked to tumorigenesis [8]. Ever since several bacterial species have been associated with the development and progression of cancer [9, 10]. Moreover, studies in immunotherapy-treated cancer patients highlight the relevance of the microbiome composition for potent anti-tumor responses [11, 12].

Certain members of the intestinal microbiota chronically activate the immune system, resulting in plasma cell-derived, antigen-specific, IgA [13]. It is estimated that ~80% of the IgA-producing plasma cells in the body reside within the intestinal mucosa [14], where they produce 40–60 mg/kg/day of dimeric IgA in steady-state conditions [15, 16]. The produced high-affinity IgA is secreted into the lumen of the gut, where it binds, or "coats", the bacteria that initiated the immune response. A broader range of commensals can also be coated with IgA in a rather non-canonical manner, which is believed to be lower in affinity and specificity [3, 17, 18]. It is estimated that in healthy individuals 10–50% of the intestinal bacteria are coated with IgA, which is essential for gut homeostasis and health and may also promote gut microbial diversity [19–22]. In fact, population-based studies have identified IgA deficiency as a risk factor for autoimmune disorders, including IBD [23]. Additionally, IgA responses to the gut microbiota have been associated with other diseases, including multiple sclerosis, asthma, and allergies [24, 25].

We do not fully understand why certain bacterial species are coated with IgA. However, different downstream effects of IgA-coating have been reported, including the prevention or promotion of colonization of bacteria and the alteration of bacterial gene expression [26–28]. Interestingly, the percentage of IgA-coated bacteria is increased during inflammation, up to 90% [19]. To fully understand the biology behind IgA-coated bacteria and their role in human health and diseases, we need new tools to study these bacteria.

The current, most commonly used method to study IgA-coated bacteria was originally developed by Palm and De Zoete et al. and is known as IgA-SEQ [20]. This method combines fluorescent activated cell sorting (FACS) of IgA-coated bacteria with 16S rRNA gene sequencing to identify immunostimulatory bacteria. This method has proven successful in enhancing our understanding of IgA-coated bacteria and their role in disease [20, 21, 29]. For example, using this technology it was shown that highly IgA-coated bacteria drive intestinal disease and can exacerbate DSS-induced colitis in germ-free mice [20]. Additionally, IgA-SEQ was performed on stool samples from a large cohort of IBD patients to identify predictors for IBD progression [29]. By identifying immunostimulatory bacteria in IBD patients with peripheral spondyloarthritis, IgA-SEQ uncovered key players linking mucosal and T_H17-dependent inflammation, giving insights into the mechanistic understanding of this common extraintestinal manifestation in IBD patients [21]. IgA-SEQ has also been used to study other human microbiotas, including those of the nose, oral cavity, colostrum, and mature human milk [30–34]. Building on the original IgA-SEQ method, Jackson et al. developed the IgAScores R package, which aids the interpretation of IgA-SEQ datasets by adjusting for the compositional

nature of relative abundance data to accurately quantify IgA binding per taxon [35].

Unfortunately, the current IgA-SEQ method has certain disadvantages. FACS-sorting is a lengthy and low-throughput process. Moreover, the number of bacteria that can be sorted within a reasonable timeframe is limited, restricting the downstream applications to 16S rRNA gene sequencing. Moreover, the FACS-sorting process is a rather rigorous process, taking place in aerobic conditions, which results in diminished survival rates of sorted anaerobic bacteria, precluding the possibility of culturing these bacteria in vitro.

In this study, we therefore established and validated two new magnet-based IgA-SEQ approaches, which can be used in anaerobic, high-throughput settings. We systematically compared the original FACS-based IgA-SEQ protocol to the two magnet-based IgA-SEQ approaches and showed that the magnetic 96-well plate-based next-generation IgA-SEQ (ng-IgA-SEQ) approach is consistently reliable across different experimental settings, including the sorting of IgA-coated bacteria from human fecal samples. Additionally, ng-IgA-SEQ allowed for novel, previously unattainable, downstream applications namely, shotgun metagenomic sequencing of IgA-coated bacteria (due to the ability to obtain greater numbers of IgA-coated bacteria) and culturing of IgA-coated bacteria directly from fecal samples (by isolating IgA-coated bacteria under anaerobic conditions). Our improved ng-IgA-SEQ technology and its novel downstream applications open new avenues to elucidate the role of IgA-coated bacteria in human health and disease.

Methods

Bacterial strains

In this study the following bacterial strains were grown overnight at 37 °C in LB broth, containing the hereafter indicated antibiotics: pUMS-PelB-mCherry *E. coli*, hereafter named mCherry$^+$*E. coli*, which constitutively expresses mCherry fused to a PelB signal peptide (kanamycin resistant (30 µg/ml)) and GFP$^+$/ΔSpA/ΔSbi *S. aureus* Newman, hereafter named GFP$^+$*S. aureus*, which constitutively expresses GFP (green fluorescent protein) and lacks staphylococcal protein A (SpA) and second immunoglobulin-binding protein (Sbi) (chloramphenicol resistant (10 µg/ml) [36]. Both bacterial strains were a generous gift from Prof. Dr. Suzan Rooijakkers.

E. coli/*S. aureus* experimental setup

The optical density at 600 nm (OD_{600}) was measured of overnight bacterial cultures and washed bacteria were diluted to the desired OD of 0.5. *S. aureus* was labeled with a monoclonal recombinant IgA antibody against wall teichoic acid (anti-WTA-4497 [37], 1:40 dilution, stock concentration: 591 µg/ml, produced in EXPI293F cells as described in De Vor et al. [37]) in PBS supplemented with 0.15% Albumin Bovine Fraction V (BSA) (SERVA, #11,930.03) for 30 min on ice. Next, bacteria were stained with PE anti-human IgA (Miltenyi Biotec, clone IS11-8E10, #130–113-476) (1:10) for 30 min on ice and subsequently labeled with MojoSort™ Mouse anti-PE nanobeads (Biolegend, #480,080) (1:10) for 30 min on ice. Different ratios of *E. coli* and *S. aureus* were mixed together before magnetic sorting (see below). Tested average percentages of *S. aureus* were ~2.5%, 12.5%, and 35%.

Spiking experimental setup

For the spiking experiments [35] an OD_{600} of 0.025 *S. aureus* per condition was labeled as described above and added to 5×10^7 Syto9$^+$ human fecal-derived bacteria (see "Human fecal samples" section below) before magnetic sorting of IgA-coated *S. aureus*.

Human fecal samples

Human fecal samples were collected by the Amsterdam University Medical Center (Medical Ethical Review Committee number 2016.135) [38]. The donors were adults who underwent an endoscopy, showing no abnormalities. Inflammation was excluded by microscopic evaluation of mucosal biopsies. Three human fecal samples were collected by the University Medical Center Utrecht in GutAlive containers (MicroViable Therapeutics) [39] to preserve an anaerobic environment after sample collection (Medical Ethical Review Committee numbers TC-bio 18–123 and TC-bio 23–200).

Fecal sample preparation

Fecal samples were prepared as previously described [20]. Briefly, 50–100 mg of fecal sample was homogenized in 1 ml PBS and centrifuged (25×*g*, 15 min, 4 °C) to remove large debris. Fecal bacteria in the supernatant were removed (100 µl) and washed using PBS containing 1% BSA (staining buffer). 1/100th of the sample was stained with Syto9 Green Fluorescence dye (Invitrogen, #S34855) (3.3 µM final concentration, 5 min, room temperature) for the quantification of bacteria using flow cytometry (MACSQuant) VYB (Miltenyi Biotec). 5×10^7 bacteria were kept separate for 16S rRNA gene sequencing analysis of the pre-sort sample. For the IgA-isolation protocols, 5×10^7 Syto9$^+$ bacteria were incubated in blocking buffer (staining buffer containing 20% Normal Mouse Serum (Jackson ImmunoResearch, #015–000-120) (20 min, on ice) and subsequently stained with PE anti-human IgA (1:10 or 1:75, 30 min, on ice) in 100 µl staining buffer. For the magnetic isolation protocols, samples were next labeled with MojoSort™ Mouse anti-PE

nanobeads (Biolegend, #480,080) (10 µl/sample, 30 min, on ice) in staining buffer.

For shotgun metagenomic sequencing the protocol was scaled up as follows: 200 µl of fecal bacteria-containing supernatant was split into two samples for the remainder of the protocol. Samples were incubated in blocking buffer as described above and stained with PE anti-human IgA (1:20) in 200 µl staining buffer. Next, samples were labeled with 40 µl MojoSort™ Mouse anti-PE nanobeads. After the isolation duplicates were added together again for metagenomics shotgun sequencing (see below).

Magnetic IgA$^+$ bacterial isolation

For sorting using the 96-well plate format, stained samples were resuspended in 800 µl separation buffer (PBS containing 0.5% BSA and 2 mM EDTA (Honeywell, #3610)). Twenty-five microliters were kept separate for flow cytometry analysis of the pre-sort sample. The remainder of the sample was placed in a deep well 96-well plate (VWR International BV, #732–4900) and the plate was placed on a tabletop Magnetic Stand-96 (Invitrogen, #AM10027). After 10 min the supernatant was carefully removed and collected for FACS analysis (IgA$^-$ fraction). The magnetic bead pellet was washed three times with 800 µl of separation buffer, before resuspending in PBS (IgA$^+$ fraction). The pre-sort, IgA$^-$, and IgA$^+$ fractions were analyzed by flow cytometry.

To allow for sufficient numbers of isolated IgA$^+$ bacteria for metagenomic shotgun sample preparation: the supernatant after the first incubation was placed in a clean well and underwent the isolation protocol again.

For the column-based sorting, stained samples were resuspended in 3 ml separation buffer. One hundred fifty microliters were kept separate for flow cytometry analysis of the pre-sort sample. The MACS MultiStand and QuadroMACS™ separator (Miltenyi Biotec) were used. LS columns (Miltenyi Biotec, #130–042-401) were pre-washed with separation buffer according to company protocol before loading the samples. The flowthrough was collected for flow cytometry analysis (IgA$^-$ fraction). The columns were washed three times before eluting the magnetically labeled fraction (IgA$^+$). The pre-sort, IgA$^-$, and IgA$^+$ fractions were analyzed by flow cytometry. 16S rRNA gene- and shotgun metagenomic (96-well plate protocol only) sequencing was performed on the pre-sort and isolated IgA$^+$ samples.

Flow cytometry and fluorescence-activated cell sorting

All flow cytometry analyses were performed using the MACSQuant VYB (Miltenyi Biotec). Fluorescence-activated cell sorting (FACS)-based sorting of IgA$^+$ bacteria from human fecal samples was performed using the Sony MA900 (Core Flow cytometry Facility, UMC Utrecht). 2×10^6 PE$^+$ events were sorted using a 100-µm sorting chip and "purity" mode. The purity of the sorted samples was checked at the MACSQuant VYB.

DNA extraction and quantification

Generally, DNA extraction for 16S rRNA gene sequencing was performed using the QIAamp fast DNA stool mini kit (Qiagen) as previously described [40], with minor modifications. In short, bacteria were lysed using 0.1 mm zirconium beads (Lab Services) in 450 µl InhibitEx buffer (Qiagen). After two rounds of bead beating, with an intermediate cooling step on ice, and an incubation at 95 °C for 7 min, the DNA stool mini kit protocol was resumed at the proteinase K treatment step. To increase yields, the DNA extraction for metagenomic shotgun sequencing of the magnetically sorted samples and 16S rRNA gene sequencing of the FACS-sorted samples followed the same bead beating and heat incubation step as described above in PowerBead Solution (Qiagen), followed by the DNeasy UltraClean Microbial Kit (Qiagen). DNA elution was performed using Buffer EB (Qiagen, #1,014,609).

Total DNA was quantified by Bacteria Quantification (BQ-) real-time PCR based on Liu et al. [41] or Quant-iT™ PicoGreen dsDNA Assay kit (Invitrogen). For the BQ-PCR, 2 µl of DNA template was added to 5 µ l TaqPath mastermix,CG (Applied Biosystems, #A15298), 5 µ M probe (6-FAM/ZEN) 5′-CAGCAG CCGCGGTA-3′ (Iowa Black®FQ)) and 10 µM of both the forward (5′-CCTACGGGDGGCWGCA-3') and reverse (5′-GGACTACHVGGGTMTCTAATC-3′) primers (IDT DNA technologies) [41]. Samples were run on a StepOnePlus RT-PCR system (Thermo Fisher) using the following program: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. DNA concentrations were calculated based on a standard curve using the Zymobiomics DNA standard (Zymo Research, #D6306).

16S rRNA gene sequencing

For 16S rRNA gene sequencing, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified and sequenced using the Illumina MiSeq instrument and Reagent Kit v3 (600 cycles) according to Fadrosh et al. [42]. Non-template controls, negative controls, and mock communities (whole bacteria and DNA standards) (Zymo Research, #D6300; #D6310) were used from the beginning of the DNA isolation up to the data analysis and matched with the expected mock compositions.

Shotgun metagenomic sequencing

Shotgun metagenomic sequencing was performed on isolated DNA samples (between 50 and 540 ng total) without additional DNA amplification steps, using the NovaSeq X platform (Illumina) upon library preparation using TruSeq DNA Nano (350 bp insert) with a run configuration of 2 × 100 bp with an output of 5 Gb per sample by Macrogen.

Culturomics

The anaerobic magnetic 96-well plate-based isolation was performed in an anaerobic chamber (Coy Laboratories) with an environment of 5% H_2, 10% CO_2, and 85% N_2. 10^5 fecal-derived bacteria (quantified by performing FACS analysis on an aliquot stained with Syto9) before and after IgA^+ isolation were plated on Gut Microbiota Medium (GMM) agar plates [43] and incubated at 37 °C under anaerobic conditions. After 48 h, 1 ml of GMM broth was added to the plates to collect the bacteria for 16S rRNA gene sequencing.

Data analysis and statistical methods

For the analysis of the microbial community based on 16S rRNA gene sequences, we adjusted a recently published Python-based workflow for Illumina sequence data, designated Tourmaline [44]. Tourmaline is built using the Snakemake workflow management system and includes QIIME2 and DADA2 for sequence variant detection. We adapted the workflow for the use of the 16S rRNA dual-index primers and the use of not yet demultiplexed fastq files as input (https://gitlab.com/malbert.rogers/tourmaline/-/releases/v2.1). In the configuration file, we adjusted the truncation settings to–p-trunc-len-f 215 –p-trunc-len-r 235 and selected the SILVA 138.1 database as a reference [45]. We set a minimal threshold of 1000 reads per sample, the average reads per sample for FACS-sorted samples: ± 20,000; for plate-based sorting: ± 25,000; for column-based sorting: ± 30,000.

The shotgun metagenomics data were analyzed using the HUMAnN 3.9 pipeline, including MetaPHlAn 4.1 for taxonomic profiling and MetaCyc for functional pathway analysis [46]

We used R version 4.4.0 (2024–04-24) to perform differential abundance (DA) analysis on pathways, including four different statistical tools: analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2), ALDEx2, MaAslin2, and LinDA [47–50]. For calculating the fold change undetected pathways were given a pseudo-count of 1 and copies per million were calculated and used to calculate the fold change (IgA^+/pre-sort).

IgA^+ Probability scores were calculated for bacteria that were present in at least two independent samples as previously described by Jackson et al. [35]. The gut microbial CAZyme repertoires were analyzed by using Cayman [51].

All graphs were made as written in the figure legends using GraphPad Prism Version 10.2.3.

Results

Establishing two magnet-based IgA-sorting methods

To improve the current IgA-SEQ approach, we first focused on standardizing and optimizing magnet-based sorting protocols for human fecal samples. Here, we use two distinct magnet-based sorting approaches: a Magnetic Stand-96, suited for a 96-wells plate configuration (hereafter referred to as the plate-based format), and the QuadroMACS™ Separator, designed for column-based sorting (hereafter referred to as the column-based format), both in combination with nanobeads. Each of these sorting methods offers unique advantages; the plate-based format enables simultaneous sorting of 96 samples, while the column-based format provides the potential for automation by using one of the automated cell separation systems of Miltenyi Biotec. Both approaches are therefore suitable for high-throughput sorting.

We optimized both isolation methods using a controlled setup involving a mixture of $mCherry^+$ *E. coli* and GFP^+ *S. aureus*. To create a mixed population of IgA^- and IgA^+ bacteria, we coated *S. aureus* with an *S. aureus*-specific IgA antibody (anti-WTA-4497) [37]. Subsequently, we separated the IgA^- *E. coli* and IgA^+ *S. aureus* using the plate- and column-based magnetic sorting methods (Fig. 1A). Throughout this study we will refer to the samples before sorting as the "pre-sort" samples, the sorted uncoated bacteria as the "IgA^-" fraction (supernatant or flowthrough for the plate- or column-based format, respectively), and the sorted IgA-coated bacteria as the "IgA^+" fraction.

IgA-coating of bacteria in healthy human fecal samples typically ranges from 10 to 50% [19–21]. In our experimental setup, we tested various ratios, ranging from approximately 2.5 to 35% IgA^+ bacteria. Additionally, we included a negative (100% *E. coli*) and positive (100% *S. aureus*) control to detect any potential non-specific effects (Supplementary Figure S1). Flow cytometry data showed that both plate- and column-based protocols effectively separated IgA^+ bacteria from a mixture of two bacterial strains without significant non-specific binding (Fig. 1B, C, Supplementary Figure S1). The purity of the IgA^+ fraction was lowest when starting with a low percentage of IgA^+ bacteria (pre-sort ~2.5% IgA^+), reaching ~89% IgA^+ after sorting for both protocols. However, as the initial proportions of IgA^+ bacteria increased, the purity of the sorted IgA^+ fraction improved to ~98%. The column-based sorting protocol consistently yielded the highest purity of the IgA^- fraction (> 99%), independently

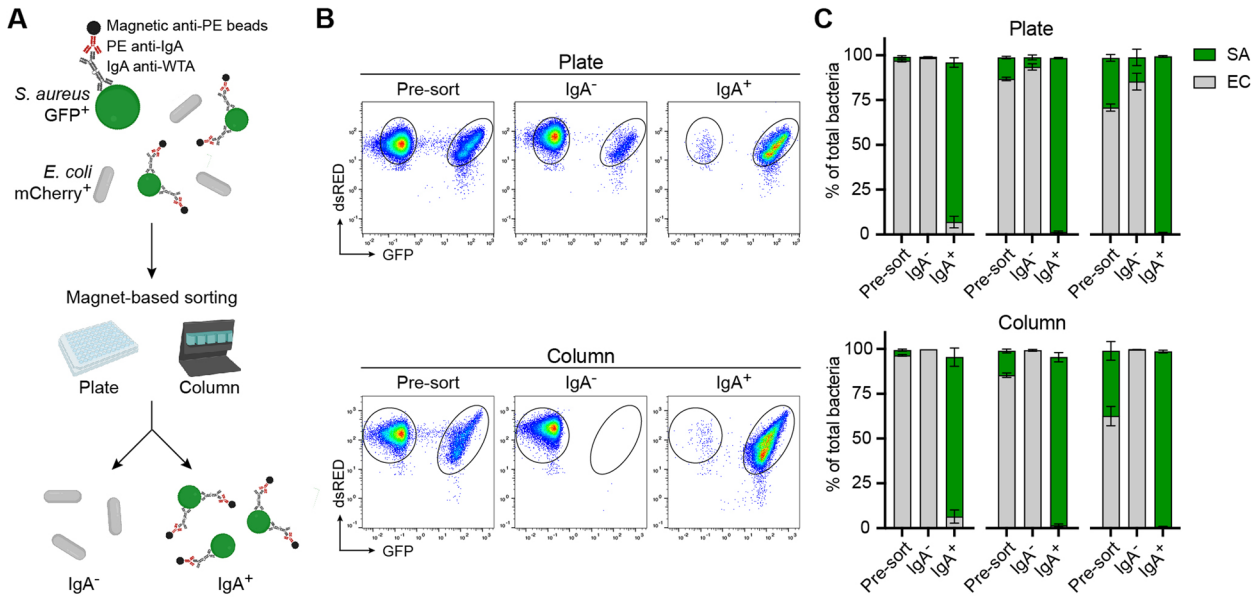

Fig. 1 Establishing two magnet-based IgA-sorting methods. **A** Experimental setup. *E. coli* (mCherry$^+$) and labeled *S. aureus* (GFP$^+$) are combined in varying ratios before magnetically sorting IgA$^+$ bacteria using the 96-well plate- and column-based formats. The pre-sort, IgA$^-$ and IgA$^+$ fractions are collected and analyzed by flow cytometry. **B**, **C** Representative dot plots (**B**) and purity (**C**) of the pre-sort and magnetically sorted IgA$^-$ and IgA$^+$ fractions. dsRED (mCherry (*E. coli*)/PE (IgA)) is shown on the *Y*-axis and GFP (*S. aureus*) on the *X*-axis (**B**). Average pre-sort percentages of *S. aureus*: 2.6%; 12.7%; 32% (left; middle; right graphs). SA: *S. aureus*, EC: *E. coli*. Experiment was performed in triplicates

of the starting material, whereas the plate-based sorting yielded a purity between 86 and 99% for the IgA$^-$ fraction. The negative control (*E. coli* only) showed some non-specific capturing of uncoated bacteria using the column-based approach, whereas a minimal number of uncoated bacteria were captured using the plate-based approach (Supplementary Figure S1).

In conclusion, our optimized magnet-based high-throughput protocols show great potential in sorting IgA-coated bacteria from a bacterial mixture.

To validate our magnet-based IgA-sorting protocols in a complex fecal microbial community, we performed a control experiment as proposed by Jackson et al. [35]. Here we aimed to specifically isolate a spiked IgA-coated bacterial strain from fecal-derived microbiota. We pre-labeled GFP$^+$ *S. aureus* with IgA and anti-IgA antibodies before spiking these bacteria into three different unstained human fecal-derived bacterial suspensions (Fig. 2A). By doing so, even though a portion of the human fecal-derived bacteria are IgA-coated, they will not be sorted since they lack the labeling with the anti-IgA antibody that enables binding to the magnetic beads, ensuring selective sorting of IgA-coated *S. aureus*. *S. aureus* constituted ~28% of the total bacterial community in the spiked fecal sample (Fig. 2B–D), which is within the biologically relevant range. Flow cytometry analysis of the spiked fecal samples showed a high purity of the IgA$^+$ fraction after sorting (~97.5% and ~91.5% for the plate- and column-based methods, respectively). The purity of the IgA$^-$ fraction was notably higher when using the column-based protocol (~99%) while it was lower using the plate-based protocol (~89%). The number of beads used in our plate-based method is the limiting step to isolating more IgA$^+$ bacteria from one sample; therefore, increasing the purity of the IgA$^-$ fraction is possible by increasing the number of used beads per sample.

16S rRNA gene sequencing was performed on both the pre-sort and IgA$^+$ fractions of spiked fecal samples (Fig. 2D, Supplementary Figure S2A). These data confirmed our findings, demonstrating the efficiency of both magnet-based protocols, and the superiority of the plate-based method in sorting IgA-coated bacteria from complex microbial communities.

Comparative analysis of different IgA-SEQ protocols

Next, we aimed to compare our magnet-based IgA-SEQ protocols with the original FACS-based IgA-SEQ protocol using a set of 10 human fecal samples. We isolated IgA-coated bacteria from the same samples using the plate-, column-, and FACS-based sorting protocol. Additionally, we used two different concentrations of anti-IgA antibody for the plate-based protocol, to investigate whether we can specifically select for highly coated bacteria by titrating the amount of anti-IgA antibody. Flow cytometry analysis showed high purity of the IgA$^+$ fraction using both the FACS- and plate-based protocols

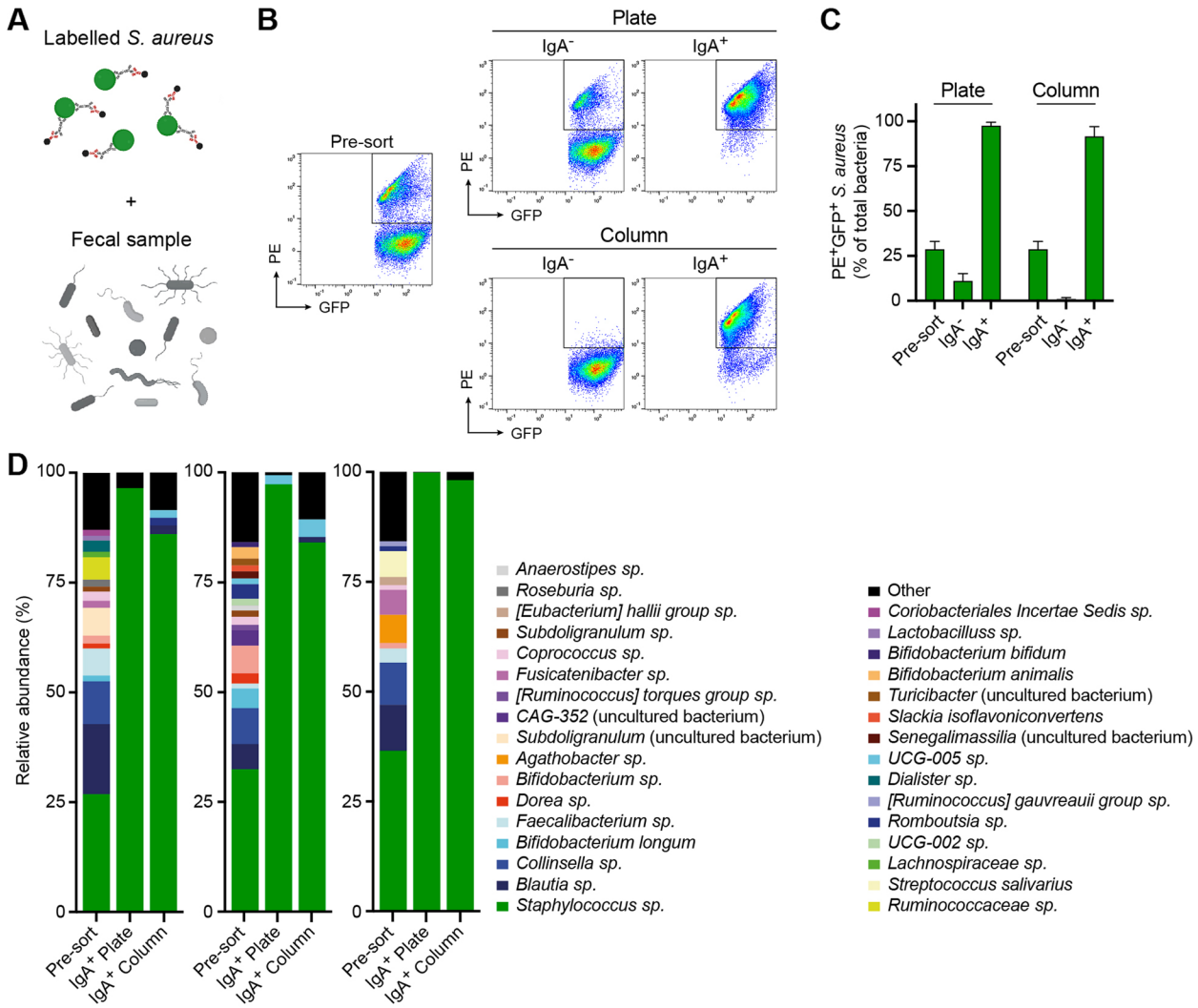

Fig. 2 Validation of the magnet-based IgA-sorting methods. **A** Experimental setup. Labeled *S. aureus* (GFP$^+$) is combined with homogenized fecal-derived bacteria before magnetic sorting of IgA$^+$ bacteria. **B**, **C** Representative dot plots (**B**) and purity (**C**) of the pre-sort, IgA$^-$ and IgA$^+$ fractions using the plate- and column-based formats. PE (IgA) is shown on the *Y*-axis and GFP (*S. aureus* and Syto9 (living fecal bacteria)) is shown on the *X*-axis (**B**). **D** Average relative abundance of the bacterial species (> 1%) in the pre-sort and IgA$^+$ fractions after plate- and column-based sorting based on 16S rRNA gene sequencing results of three spiked human fecal samples. Bacterial species < 1% are grouped under "Other". $N = 3$, experiments are performed in triplicates

(Fig. 3A, B, Supplementary Figure S3A). However, flow cytometry analysis of the column-based isolation protocol showed no discernible difference between the IgA-coated fraction before and after isolation, indicating that this method is not functional when working with "real-world" fecal samples (Fig. 3A, B). This might be due to the physical features of the columns, which might prevent proper isolation of the IgA$^+$ bacteria from complex fecal samples. Titrating the anti-IgA antibody had minimal effects on the purity of the IgA$^+$ fraction; however, it lowered the overall percentage of PE$^+$ bacteria (Fig. 3B), suggesting that lowering the anti-IgA antibody indeed selects for bacteria with higher levels of IgA coating. These data were confirmed by 16S rRNA gene sequencing of both the pre-sort and IgA$^+$ fractions (Supplementary Figure S3B).

To further compare the different isolation methods, we used the 16S sequencing data to determine the levels of bacterial IgA-coating by calculating the IgA$^+$ Probability score [35], which defines the likelihood of a specific bacteria being coated by IgA. A ranked distribution of the IgA$^+$ Probability scores of all bacterial species from all samples shows that the plate-based isolation method is more sensitive in identifying IgA-coated bacteria,

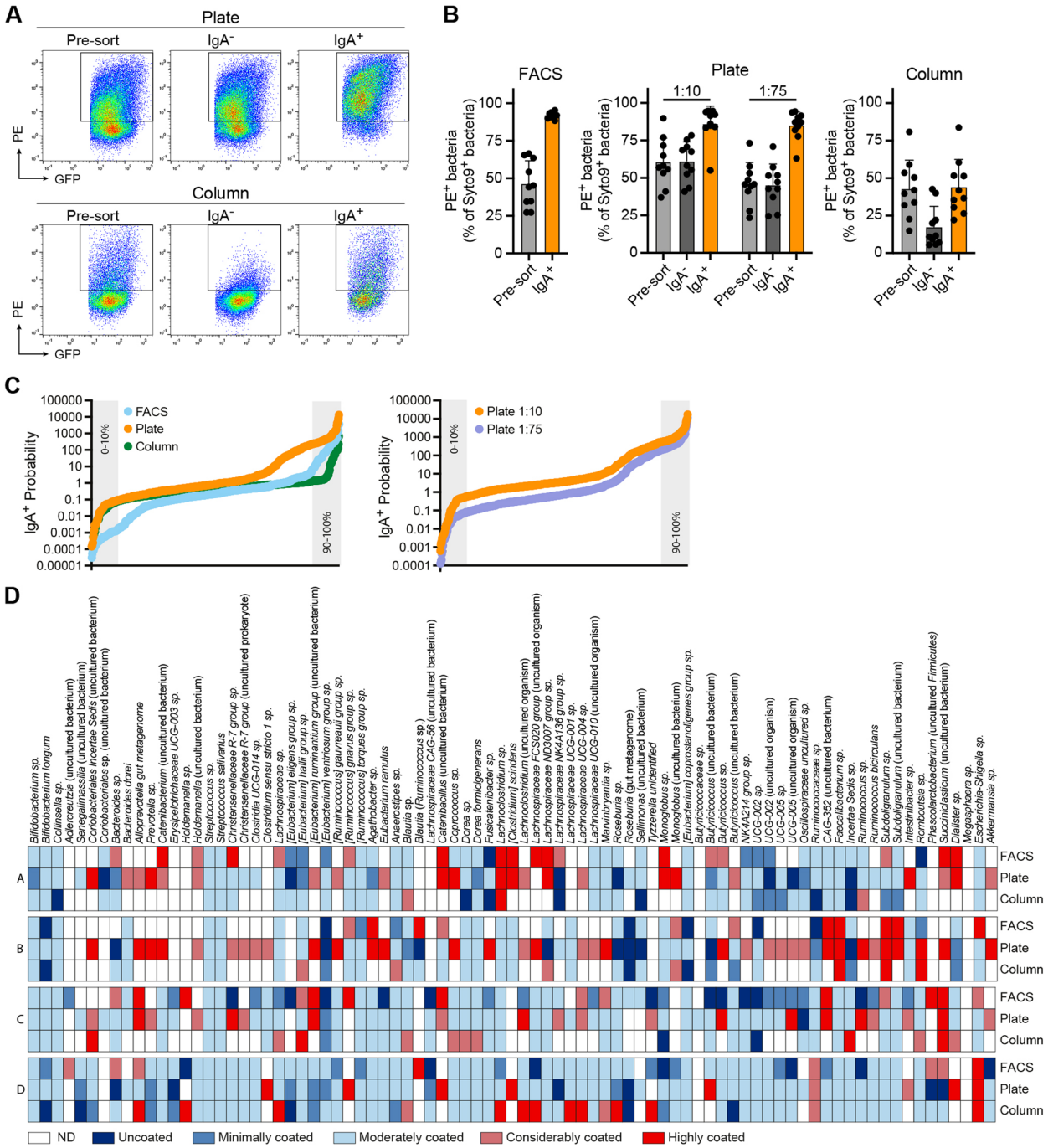

Fig. 3 Comparative analysis of different IgA-SEQ methods. **A** Representative dotplots of the pre-sort, IgA$^-$ and IgA$^+$ fractions using the plate- (PE anti-IgA antibody 1:10 diluted) and column-based formats. PE (IgA) is shown on the *Y*-axis and GFP (Syto9 (living fecal bacteria)) is shown on the *X*-axis. **B** Purity of the pre-sort, IgA$^-$ and IgA$^+$ fractions after the FACS- plate-, column-based isolation methods. For the plate-based method, two different dilutions (1:10 (left) and 1:75 (right)) of the anti-IgA antibody were used. **C** The IgA$^+$ Probability scores of all bacterial species found in all samples of the indicated isolation methods are shown. The X-axis depicts all identified bacteria sorted based on the IgA Probability score. The bottom and top 10% are classified as uncoated and highly coated bacteria, respectively. 10–20% is classified as minimally coated and 80–90% as considerably coated. **D** Heatmap of the IgA$^+$ Probability scores of four human fecal samples (A–D) showing the levels of IgA coating of bacterial strains found in at least two independent samples using the three different isolation protocols (FACS; plate 1:75; column). Bacteria not detected (ND) in the 16S rRNA gene sequencing results are colored white. See Supplementary Figure S4 for the heat maps of the other six samples. $N = 10$, experiments are performed in triplicates

independent of the level of IgA-coating, compared to the FACS-based method, as shown by the left-shift of the data line (Fig. 3C). As suggested above, titrating the anti-IgA antibody in the plate-based method indeed allows us to alter the sensitivity of the method and select for bacteria with higher levels of IgA-coating (Fig. 3C). As suggested by our earlier data, the column-based method performed worse at identifying IgA-coated bacteria, but provided highly pure IgA^- bacteria.

Using the distribution of the combined IgA^+ Probability scores, we classified the bottom 20% as uncoated (0–10%) and minimally (10–20%) coated and the top 20% as considerably (80–90%) and highly coated (90–100%) for each individual method (Fig. 3C, D, Supplementary Figure S4). The FACS- and plate-based methods aligned in roughly 60% of cases regarding the level of IgA-coating on the identified bacterial species. Interestingly, the plate-based method identified on average more IgA-coated bacterial species compared to the FACS-based method. This is most likely due to the bias of the FACS-sorting gate which might exclude low IgA-coated bacteria in combination with the higher number of bacteria sorted using the plate-based method, thereby allowing the identification of lower abundant IgA-coated bacteria. These data show that the plate-based next-generation (ng)IgA-SEQ is an agile and suitable alternative to the FACS-based IgA-SEQ.

Shotgun metagenomic sequencing using next-generation IgA-SEQ

Elucidating the role that immunostimulatory bacteria play within the intestinal tract can significantly advance our understanding of the interplay between the intestinal microbiota, the immune system, and diseases like IBD. While previously developed tools such as IgA-SEQ began to uncover the importance of the interactions between the microbiota and the immune system, many questions remain unanswered due to the limited downstream applications of such tools and limited bacterial classification depth inherent to 16S rRNA gene sequencing. With our improved ng-IgA-SEQ protocol, we now have the opportunity to unravel the intricate mechanisms of immunostimulatory bacteria through previously unattainable downstream applications.

By scaling up our plate-based ng-IgA-SEQ protocol, we successfully isolated between 50 and 300 ng of DNA from IgA-coated bacteria. This allowed us to perform proof-of-concept shotgun metagenomic sequencing on the pre-sort and IgA^+ fractions derived from ten human fecal samples, increasing the classification depth compared to 16S rRNA gene sequencing (Fig. 4, Supplementary Figure S5). IgA^+ Probability scores were calculated and showed that IgA-coating patterns are diverse among these human fecal samples (Fig. 4A, Supplementary Figure S5B). Generally, some samples show higher levels of overall IgA-coating than others. High levels of the coating were observed for several bacterial species, including *Bifidobacterium pseudocatenalatum* (in five out of six samples it was detected), *Firmicutes* SGB4303 (7/10), *Veillonellaceae* SGB5809 (3/4), and *Akkermansia muciniphila* (3/6). Low levels of IgA-coating on the other hand, were found in bacterial species including *Lachnospiraceae* SGB4964 (7/7), *Clostridiaceae* SGB4269 (5/9), and *Ruminococcus bicirculans* (5/7). The IgA^+ Probability scores of the 16S- and shotgun metagenomic sequencing data show similar medium to low coating for bacteria including *Bifidobacterium longum*, *Collinsella* sp., *Streptococcus* sp., *Anaerostipes* sp., and *Dorea* sp. Patterns of high IgA coating can be observed for specific individuals in both sequencing results including for *Bifidobacterium* sp. (sample D), *Collinsella* sp. (F), *Holdemanella* sp. (E), and *Akkermansia* sp. (E & I). The shotgun metagenomic sequencing data provides a previously inaccessible in-depth insight into the nuances of IgA-coating at the bacterial species and strain level which 16S rRNA gene sequencing might overlook.

Pathway analysis of shotgun metagenomic sequencing data might be essential to elucidate the mechanisms that allow for IgA-coating and bacterial immunostimulatory behavior. As a proof-of-concept, we performed pathway analysis on the shotgun metagenomic sequencing data of the pre-sort and IgA^+ fractions. This analysis showed markable changes in several pathways in the IgA-coated fraction compared to the pre-sort fecal sample (Fig. 4B, Supplementary Figure S5C). Some pathways, including pathways related to vitamin K biosynthesis, were virtually undetected in the pre-sort samples but were significantly enriched in the IgA^+ fraction. Other pathways, for example, mannan degradation and peptidoglycan biosynthesis, showed the opposite effect and were decreased in the IgA^+ fraction (Supplementary Figure S5C).

Besides general pathway analysis, we also analyzed the presence of Carbohydrate-Active enzymes (CAZymes) and their associated modules in the pre-sort and IgA-coated fractions using the Carbohydrate Active Enzymes database (http://www.cazy.org/) [52] (Fig. 4C). CAZymes encompass different classes of enzymes that breakdown, synthesize and/or modify glycoconjugates, oligosaccharides and polysaccharides. Some bacterial species express CAZymes, such as glycoside hydrolases (GHs), which can degrade mucins into a carbon source that can be utilized by these bacteria. Alterations in mucin degradation by the gut microbiota have been associated with several diseases, including IBD [53]. Therefore, we hypothesize that CAZyme expression might also be correlated with immunostimulatory bacteria. We focused on known

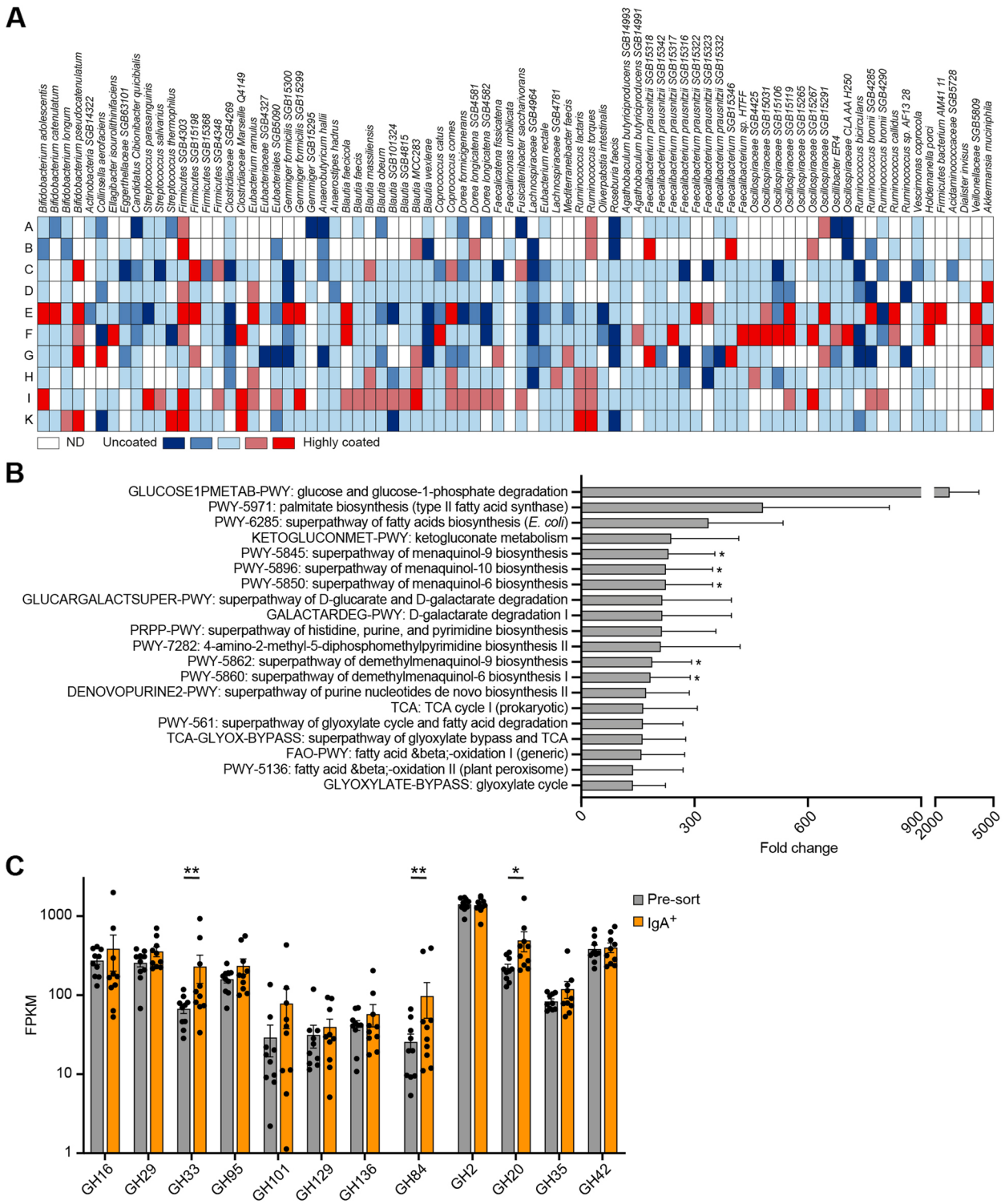

Fig. 4 Shotgun metagenomic sequencing using next-generation IgA-SEQ. **A** Heatmap of the IgA$^+$ Probability scores of 10 human fecal samples (A-I, K) showing the levels of IgA coating of bacterial strains found in at least two independent samples using the magnet-based ng-IgA-SEQ protocol. Bacteria not detected (ND) in the shotgun metagenomic sequencing results are colored white. **B** Pathway analysis shows the most enriched pathways in the IgA$^+$-fraction compared to the pre-sort fecal sample. * indicate significantly upregulated pathways as determined by LinDA. **C** Mucin *O*-glycan CAZyme analysis in the pre-sort and IgA$^+$ fraction depicted in fragments per kilobase of transcript per million mapped reads (FPKM). GH16-GH136 (left) are active on terminal mucin capping structures, GH84 (middle) is active on gastric mucins and GH2-GH42 (right) are active on mucin *O*-glycan backbones [44]. Wilcoxon test was used for statistical analysis. *$p < 0.05$; **$p < 0.01$. $N = 10$

mucin-degrading CAzymes, such as the GH33, which includes sialidases crucial for the first steps of mucin degradation, expressed by for example *Akkermansia muciniphila* and several *Bacteroides* species [54]. Interestingly, certain families of GHs, including GH33, GH84, and GH20, indeed showed an increased abundance in the IgA-coated fraction compared to the pre-sort samples (Fig. 4C). Even though we only used a small set of samples for these proof-of-concept experiments, this data does highlight the possible association between CAZymes, immunostimulatory bacteria and, potentially, diseases.

Collectively, these data show that using our next-generation IgA-SEQ method we can perform shotgun metagenomic sequencing on IgA-coated bacteria, which will be essential in increasing our understanding of these bacteria at previously unobtainable depth.

Anaerobic culturomics of IgA-coated bacteria

Numerous members of the intestinal microbiota are (facultative) anaerobes. Therefore, the majority of bacteria derived from fecal samples do not survive the sample preparation using the original FACS-based IgA-SEQ protocol, due to the aerobic conditions and the additional rigorous nature of the FACS-sorting. Our ng-IgA-SEQ method is adaptable to various laboratory settings since it uses a tabletop 96-well plate magnet and can easily be performed within an anaerobic chamber. We tested ng-IgA-SEQ-based enrichment of IgA-coated bacteria under anaerobic conditions by performing the entire plate-based isolation method, including sample preparation and staining, and subsequent culturing of the IgA-coated bacteria under anaerobic conditions (Fig. 5A). For these proof-of-concept experiments we used fecal samples collected using GutAlive devices [39], designed to create an anaerobic environment upon sample collection, thereby enhancing the survival of anaerobic bacteria post-collection.

Subsequently, the pre-sort and IgA^+ bacteria were plated on GMM agar plates and cultured in anaerobic conditions. After 48 h, the cultured bacteria were collected and 16S rRNA gene sequencing was performed to identify the bacterial strains (Fig. 5A). While the diversity of the cultured bacteria varied substantially between fecal samples, a distinct shift was observed in the cultured bacteria with and without enrichment for IgA-coated bacteria (Fig. 5B). Several similarities can be observed between the 16S rRNA gene sequencing data (Supplementary Figure S3B) and the culturomics data (Fig. 5B). For example, both *Coprococcus* sp. and *Bifidobacterium longum* are enriched in the IgA^+ fraction in sample C, while *Blautia* sp. in sample B and *Collinsella* sp. and *Bacteroides* sp. in sample A are decreased in the IgA^+ fraction. These experiments provide evidence that our next-generation IgA-SEQ approach effectively isolates viable IgA-coated bacteria, thereby paving the way for novel, functional downstream applications.

Discussion

Bacteria are essential for homeostatic conditions and general health in humans; however, we do not fully understand this symbiotic relationship. The largest and most diverse microbial community is found within the human intestinal tract. The host immune system plays a pivotal role in the protection against pathogens and in maintaining gut homeostasis. Immunostimulatory bacteria within the intestinal tract, including pathogens and commensals, can activate the immune system, resulting in the production of secretory IgA. However, why certain bacterial species are coated with IgA and their exact role in health and disease remains unclear.

In the current study, we developed and validated two magnet-based isolation methods for IgA-coated bacteria using a tabletop 96-well plate-based format and the column-based Magnetic-Activation Cell Sorting system. Both isolation systems accurately sort IgA-coated bacteria from a pre-defined bacterial mixture. However, only the 96-well plate-based approach consistently achieved the same success using a set of human fecal samples. The validation experiments using the column-based approach indicated that uncoated bacteria persistently adhere to the column, despite repeated washing, posing a significant challenge. Nonetheless, the column-based method does offer an opportunity for isolating the IgA-uncoated fraction, as the flowthrough is highly effectively depleted of all IgA-coated bacteria. To increase the purity of the IgA^- fraction using the plate-based method, further optimization of the number of magnetic beads is required, as this is the limiting step in the plate-based sorting method. Increasing the number of magnetic beads will increase the purity of the IgA^- fraction, however, this may compromise the purity of the IgA^+ fraction due to potential increased non-specific binding.

Previous studies have tested or used magnet-based technologies to isolate Ig-coated bacteria from different types of samples as well [35, 55, 56]. In this study, we have optimized and validated the magnet-based isolation protocol specifically for human fecal samples on different levels, including the amount of starting material, antibody and magnetic bead concentrations, washes, and time on the magnet. Our results are consistent with the findings of Jackson et al., who also refrained from using column-based isolation technologies, due to the limited enrichment using human fecal samples [35]. Bourgonje et al. isolated IgG-coated bacteria using the column-based method; however, the enrichment was rather low

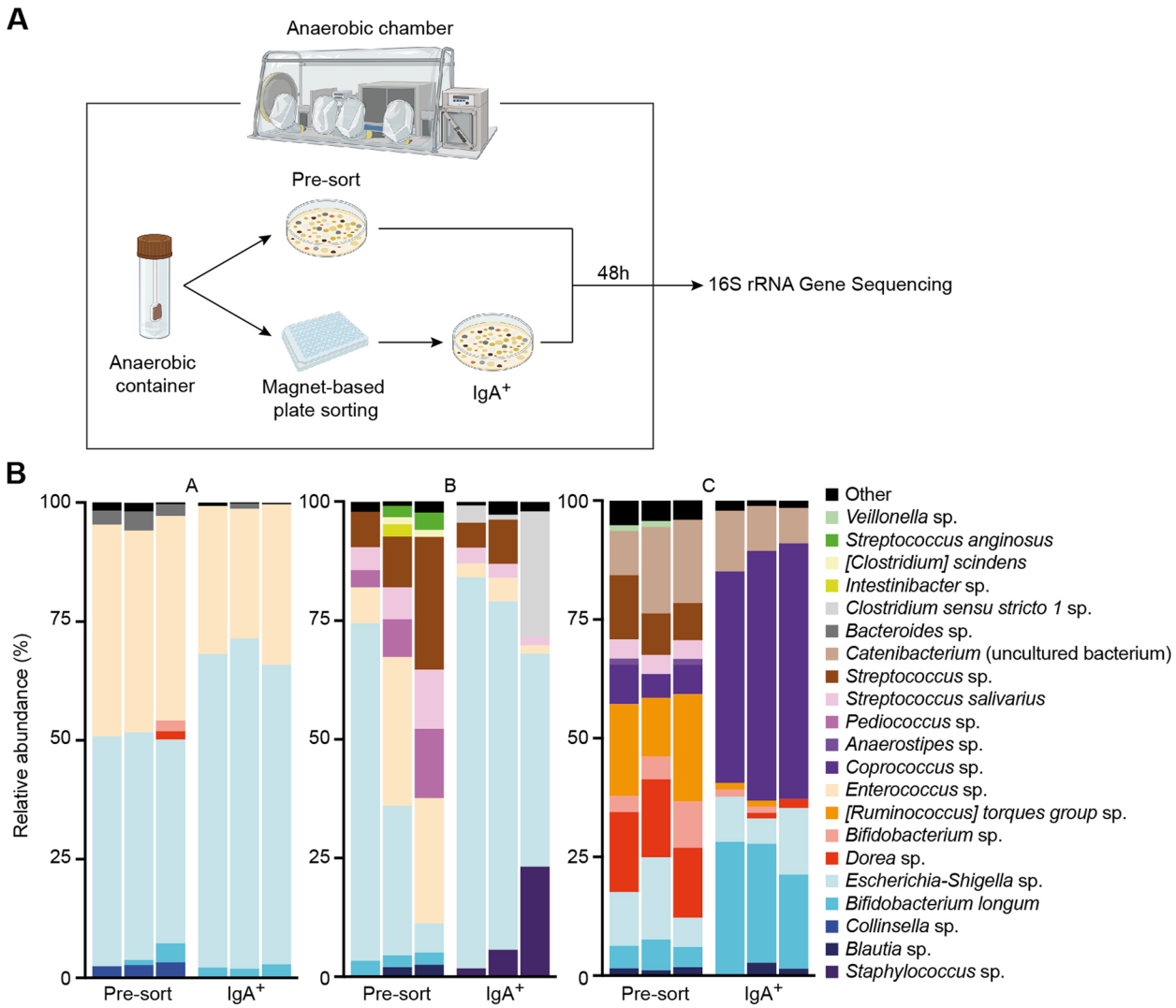

Fig. 5 Anaerobic culturomics of IgA-coated bacteria. **A** Experimental setup. An anaerobic collection container was used to collect human fecal samples. The entire plate-based IgA-isolation protocol and subsequent culturing were performed in anaerobic conditions. **B** Average relative abundance of the bacterial species (> 1%) in the pre-sort and IgA^+ fractions using the magnet-based plate sorting after culturing for 48 h based on 16S rRNA gene sequencing results of three human fecal samples (A–C). Bacterial species present < 1% are annotated under "Other". *N* = 3, plated and sequenced in triplicates

(38 > 45%), confirming a potential downside of using the column-based method for human fecal samples [55]. The successful use of the column-based technique using other types of material is not excluded by our work; Gupta et al. show an efficient enrichment of IgA-coated bacteria from mouse fecal samples (to roughly 90%) and this technique might also work for other, less complex, microbiota samples [56]. Further research and thorough validation are necessary to determine which technique is best for each specific material.

For future research, we propose next-generation IgA-SEQ by using the magnetic, 96-well plate-based isolation approach to sort IgA-coated bacteria from fecal samples. This method has been demonstrated to be versatile and consistently reliable across various experimental setups. Importantly, its suitability for high-throughput applications renders it much faster and more user-friendly than the FACS-based approach, especially when analyzing large cohorts. FACS-sorting 2 million IgA^+ bacteria from human fecal samples took 30–60 min per sample, depending on the percentage of IgA-coated bacteria. Using the plate-based isolation method, 96 samples can be processed simultaneously within 2 h, showing the time efficiency of our improved technology. Additionally, the sorting-gate, necessary to FACS-sort IgA-coated bacteria, may inadvertently

exclude low IgA-coated bacteria from downstream analyses. The yield of IgA-coated bacteria is significantly higher when using the plate-based isolation method compared to the FACS-based approach, allowing for the identification of previously overlooked low abundant IgA-coated bacteria and the metagenomic analysis of these samples. The adaptable nature of the next-generation IgA-SEQ protocol also allows the isolation of different sets of IgA-coated bacteria, depending on the experimental setup. Additionally, further optimizing the number of beads per sample will allow the user to isolate specific numbers of IgA-coated bacteria. Furthermore, the small size of the magnet enables its use in various laboratory settings, for instance within an anaerobic chamber. These advantages pave the way to a largely unexplored field of research, aiming to decipher the intricate mechanisms of immunostimulatory bacteria within the intestinal tract.

Due to the nature of the FACS-based IgA-SEQ method, previous studies have relied on 16S rRNA gene sequencing data. While such data provides insights into the genera responsible for immunostimulatory effects, it performs rather poorly in the classification of bacterial species, resulting in gaps in our understanding. With the implementation of the ng-IgA-SEQ technology, we successfully isolated sufficient bacterial DNA to perform direct shotgun metagenomic sequencing. This enabled the identification of the immunostimulatory bacteria at species and strain levels and thereby allowed the identification of crucial differences between individual bacteria, which might have been overlooked in the past. Examples can be taken from comparing the IgA^+ Probability scores derived from our 16S- and shotgun metagenomic-sequencing data. For instance, bacterial species that cluster together in the 16S IgA-SEQ results, such as *Bifidobacterium*, *Blautia*, and *Ruminococcus* are separated into multiple species in the shotgun metagenomic sequencing results. Importantly, these data show that species within the same genus are not always coated with IgA in a similar fashion, as seen for different *Bifidobacterium* and *Ruminococcus* species. Further research is needed to understand the difference regarding IgA coating for specific bacterial species within the same genus.

Using next-generation IgA-SEQ we successfully isolated viable, anaerobic bacteria. Logically, not all bacterial species thrive under the same conditions, therefore diversifying the culture conditions will be beneficial to culture a broader range of IgA-coated bacteria. It is important to note that this culturomics approach is not a comprehensive substitute for identifying all IgA-coated bacteria. Rather, it serves as a valuable tool for isolating specific, viable bacterial strains for further downstream applications including functional studies.

Although outside of the scope of this study, due to the adaptable nature of the magnet-based ng-IgA-SEQ method, it is feasible to study other proteins expressed or coated on bacteria, by simply replacing the anti-IgA antibody for another antibody of interest, e.g. IgG. Additionally, due to the sensitivity of ng-IgA-SEQ, it is possible to isolate and subsequently study IgA-coated bacteria from low(er) bacterial abundant samples, including breast milk, and nasal, oral, or vaginal swabs.

In summary, our magnet-based, high-throughput next-generation IgA-SEQ method unveils numerous opportunities to further elucidate the role of immunostimulatory bacteria in health and disease. This may lead to a more thorough understanding of the bacterial properties that result in the induction of specific IgA, their impact on the intestinal tract, and the discovery of novel biomarkers or therapeutic strategies for conditions like IBD and cancer.

Conclusions

Our next-generation magnetic, 96-well plate-based high-throughput, IgA-SEQ technology poses many advantages compared to the original FACS-based IgA-SEQ approach. It is faster, allows for high-throughput applications, yields more bacterial DNA, and can be easily employed in various lab settings. This allows for previously unachievable downstream applications including shotgun metagenomic sequencing and culturomics, enabling the exploration of many unresolved questions surrounding immunostimulatory bacteria within the gut microbiota.

Abbreviations

BSA	Albumin bovine fraction V
CAZyme	Carbohydrate-active enzyme
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
GH	Glycoside hydrolase
GMM	Gut microbiota medium
ng-IgA-SEQ	Next-generation IgA-SEQ
IBD	Inflammatory bowel disease
ICI	Immune checkpoint inhibitor
IgA	Immunoglobulin A
MACS	Magnetic-activated cell sorting
S. aureus	*Staphylococcus aureus*
Sbi	Second immunoglobulin-binding protein
SpA	Staphylococcal protein A

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01923-9.

Additional file 1: Supplementary Figure S1. Establishing two magnet-based IgA-sorting methods. Supplementary Figure S2. Validating the magnet-based IgA-sorting methods. Supplementary Figure S3. Comparative analysis of different IgA-SEQ methods Part I. Supplementary Figure S4. Comparative analysis of different IgA-SEQ methods Part II. Supplementary Figure S5. Shotgun metagenomic sequencing using next-generation IgA-SEQ

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Authors' contributions
M.R.d.Z. conceived the study. M.R.d.Z. and B.O. supervised the study. M.v.G. and J.L. designed and performed experiments, analyzed data, and wrote the manuscript. A.C. performed experiments and analyzed data. S.G. and M.C.V. performed DNA isolations and 16S rRNA gene sequencing. M.R.d.Z. and J.T. analyzed sequencing data. S.B., N.K.H.d.B, R.J.V. and K.P.M.S. enrolled patients and collected samples. E.C.B. offered guidance and technological insights. All authors co-wrote the manuscript.

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Data availability
The 16S rRNA gene sequencing and metagenomic sequencing data have been deposited in the European Nucleotide Archive repository under the study accession PRJEB75807 (https://www.ebi.ac.uk/ena/browser/view/PRJEB75807).

Declarations

Ethics approval and consent to participate
Informed consent for the participation and fecal sample collection was provided by donors prior to enrolment. The sample collection was approved by the VU University Medical Center (Medical Ethical Review Committee number 2016.135) and the University Medical Center Utrecht (Medical Ethical Review Committee numbers TC-bio 18–123 and TC-bio 23–200).

Competing interests
N.K.H.d.B has served as a speaker for AbbVie and MSD and has served as consultant and principal investigator for TEVA Pharma BV and Takeda. N.K.H.d.B has received a (unrestricted) research grant from Dr. Falk, TEVA Pharma BV, MLDS and Takeda (not related to the submitted work). K.P.M.S. has a consulting/advisory relationship with Abbvie and Sairopa. K.P.M.S. has received research funding from TigaTx, Bristol Myers Squibb, Philips, Genmab and Pierre Fabre (all paid to institution and not related to the submitted work. B.O. has received funding from Takeda, Pfizer, Galapagos, Ferring, Celltrion, BMS, and AbbVie (not related to the submitted work), and served on the advisory boards of Takeda, Galapagos, Janssen, AbbVie, BMS, and Ferring. M.R.d.Z. is an inventor on patents for IgA-SEQ.

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