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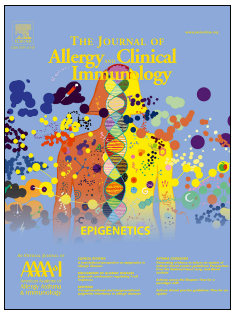

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Antibody-coated microbiota in nasopharynx of healthy individuals and hypogammaglobulinemia patients.

Short title: Ig coated bacteria in the human nasopharynx

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Capsule summary: We show that nasopharyngeal microbiota are coated by different immunoglobulin isotypes in healthy individuals and with IgG in X-linked agammaglobulinemia (XLA) patients treated with immunoglobulin replacement therapy.

Key Words: human nasopharynx microbiota; antibody-coated microbiota; sIgA; sIgM; IgD; IgG; healthy; X-linked (Bruton's) agammaglobulinemia patients; Immunoglobulin (IgG) replacement therapy; microbiota dysbiosis.

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The authors declare that they have no relevant conflicts of interest.

To the Editor:

Respiratory diseases are important causes of death, which collectively account for more than 1 in 10 of all deaths worldwide (WHO). Pathobionts, potential pathogenic bacteria which under normal circumstances are present as harmless microorganisms, can be found embedded in the nasopharyngeal commensal microbiota. Interestingly, the upper airway microbiome composition seems very similar to that of the lung, and pathobionts present in the nasopharynx can be a primary source of lower respiratory tract infections [1]. Several elements participate in respiratory mucosal barrier against invasive pathobionts including the commensal microbiota and glands that secrete and create an antimicrobial mucus barrier containing antimicrobial peptides, immunoglobulins and other soluble factors. Secretory immunoglobulin A (sIgA) and sIgM are transported through nasopharynx epithelial cells by polymeric Ig receptor (pIgR), and IgG by neonatal FcR (FcRn). Transepithelial transport of IgD has not been documented, but IgD is found in nasopharynx secretions [2] perhaps deriving from paracellular diffusion through cell junctions.

sIgA is the predominant antibody isotype in human nasopharyngeal secretions and it is assumed that the majority of sIgA targets the resident commensal microbiota, inhibiting their penetration via agglutination within the mucus layer, thereby reinforcing commensalism and increasing microbiota diversity[3]. High-affinity sIgA appears to neutralize microbial toxins and invasive pathogens in the absence of complement fixation[3]. Interestingly, Fadallah *et al*[4] reported recently that Selective IgA Deficiency (SIgADef) patients display mild dysbiosis with expansion of some pathobiont species in their feces as compared to healthy controls, although these patients are not abnormally susceptible to intestinal infections[4]. On the other

hand, recurrent pulmonary infections constitute the most prevalent comorbidity in SIgADef patients[4], suggesting that sIgA may have tissue-specific roles in immune defense, especially in the respiratory tract. A better understanding of immunoglobulins coating nasopharynx microbiota in healthy individuals could provide an important basis for the prevention of respiratory illnesses in some cases of humoral immunodeficiency.

To better understand the role for IgA (and other Ig isotypes) in regulating nasopharyngeal microbial communities, we analyze nasopharyngeal samples to detect Ig-bound microbes followed by 16S ribosomal RNA (rRNA) sequencing (Ig-seq) to determine the type of bacteria targeted by each Ig isotype. We stained nasopharyngeal samples with anti-human IgA, IgM, IgD and IgG antibodies and measured the immunoglobulin-bound populations using a flow cytometer-based assay[5]. The gating strategy for immunoglobulin staining of human nasopharynx microbiota is shown in Fig E1 (all methods are described in the Methods section in this article's Online Repository at www.jacionline.org). On average, a majority of the nasopharynx microbiota appears targeted by sIgA in healthy subjects (Fig E1A), although the frequency of $sIgA^+$ microbes is highly variable between individuals (Fig E1B). Next we performed flow sorting of IgA-bound nasopharyngeal microbiota followed by 16S rRNA sequencing (IgA-seq) on a fraction of samples (n=4) (Fig E2A). Beta diversity shows the extent of difference in microbiota community composition from different environments or conditions. The beta diversity, based on Bray-Curtis distance matrix and subjected to Principle Coordinate Analysis, suggest that bacteria derived from IgA^+ and IgA^- fractions cluster distinctly, however, permutational multivariate analysis of variance (PERMANOVA) of the two fractions was not significantly different (Fig E2A, B). The alpha diversity and the Shannon Diversity Index (a combined measure of evenness and number of bacteria) was lightly decreased in 16S rRNA sequences derived from IgA^- fraction (Fig E2C). However, both IgA^+ and IgA^- fractions exhibited equal distributions of rare and abundant genera and thus IgA appears to target nasopharyngeal commensals irrespective of their frequency (Fig E2D). These results suggest that under homeostatic conditions, sIgA bind to a broad repertoire of bacteria present in nasopharyngeal microbiota including both commensals and pathobionts [3].

Most SIgADef patients have a mild clinical phenotype[4], which may result from the ability of other Ig isotypes to functionally replace IgA. Increased IgD^+IgM^- B cell populations and increased soluble IgD levels have been described in healthy human upper respiratory mucosa [2]. Therefore, even if IgM and IgD have the same V region and the same antigen specificity, nasopharynx/respiratory B cells apparently switch from IgM to secrete IgD,

suggesting some functional advantage of the latter. The IgD hinge region linking is longer and more flexible than that of IgM thereby facilitating IgD binding to antigens at lower concentrations and in immune complexes [2]. Still, sIgM binds some residual bacteria in the nasopharynx (Fig E1), suggesting that IgM may participate in microbiota agglutination in the steady-state. On the other hand, we found that a vast majority (86%) of the nasopharynx microbiota is targeted by secreted IgD in healthy individuals (Fig E1). We observed that up to 60% of nasopharynx microbiota are double $SIgA^{+}IgD^{+}$ coated (Fig 1A, B). We performed flow sorting to isolate IgD-bound microbiota and 16S rRNA sequencing to identify the microbes specifically targeted by IgD (Fig 1C). The IgD^{+} $SIgA^{-}$ fraction showed a higher microbial diversity as compared to $SIgA^{+}IgD^{-}$ (Fig 1D). The beta diversity based on Bray-Curtis distances was also different (Fig 1E), suggesting that IgD bind specifically to bacteria not targeted by SIgA. $IgD^{+}SIgA^{-}$ fraction exhibited an enrichment in *Proteobacteria* phyla (including *Haemophilus and Moraxella* genus) and the pathobionts *Pseudomonas* and *Escherichia-Shigella* (Fig 1F). Previous studies showed that IgD somatic hypermutation (SHM) frequencies are correlated with higher rates of upper respiratory infection[6], suggesting that pathobiont exposures drive SHM of antigen experienced IgD^{+} B cells. Our results suggest that IgD can recognize a diverse array of both commensal and pathobiont bacteria inhabiting the human upper respiratory mucosa in a manner similar to sIgA. SIgADef patients have increase in IgD-producing B cells in the upper respiratory mucosa and increased IgD secretion[2], our results are consistent with the notion that IgD may provide a layer of mucosal protection during IgA deficiency.

Several studies demonstrated that mice and human IgG can react to conserved commensal antigens and coat both commensal and pathogenic bacteria[7]. However, Fadallah *et al*.,[5] recently showed that almost no bacteria in healthy human feces were coated with IgG. Despite the "absence" of IgG coated bacteria in feces, IgG present in serum of these individuals recognizes a wide range of commensals under homeostatic conditions[5]. In addition, SIgADef patients have a increased anti-commensal IgG response[5]. The reduction in IgG coated bacteria in feces does not exclude the possibility that IgG coated bacteria can be present within gut mucus, because IgG have the ability to attach to mucus proteins (e.g. mucin-bound IgG Fc binding protein)[8]. Significant quantities of IgG are secreted and detected within the mucus layer of human respiratory tract and we found that approximately 70% of the microbiota present in nasopharynx secretions are targeted by IgG in healthy individuals (Fig E1). These results demonstrate that human IgG may opsonize a wide range of nasopharynx microbiota under homeostatic conditions. 16S rRNA sequencing of IgG-bound

bacteria allowed identification of their taxonomy (Fig E3A). We observed that the IgG^+ fraction have reduced alpha diversity (Shannon index) comparatively to the IgG^- fraction (Fig E3B), suggesting that IgG bind selectivity to their target and are less polyreactive than IgA. The beta diversity based on Bray-Curtis distance was also significantly different in terms of both abundance and presence/absence of different OTUs (Fig E3C). IgG^+ fraction exhibited a significant decrease in binding in different genera, especially from *Proteobacteria* phylum (*Betaproteobacteria*, *Campylobacterales*, *Enterobacteriales*, *Pseudomonadales*) (Fig E3D). Members of *Proteobacteria* phylum are reported to be weak inducers of systemic IgG responses [5] and we found that IgG bind weakly to genera from *Proteobacteria* in nasopharynx of healthy individuals. Using the double binding with IgA and IgG antibodies we observed that about 54% of nasopharynx bacteria are double $SIgA^+IgG^+$ coated, and the level of IgG-only coated is low (16%) (Fig E3E, F). This data suggest that 77% of microbiota normally coated by SIgA are also coated with IgG, demonstrating a convergence of IgA and IgG responses toward the same microbial targets under homeostatic conditions. Sequencing of flow sorted IgG^+SIgA^- and $SIgA^+IgG^-$ bacteria (Fig E3G) showed that alpha diversity (Shannon index) (Fig E3H) and beta diversity (based on Bray-Curtis distance) (not showed) were not significantly different between IgG^+SIgA^- and $SIgA^+IgG^-$ fractions. We observed only a small enrichment on *Bacteroidetes* on IgG-only coated fraction (Fig E3I). Previous studies showed that human and mice IgG recognized *Bacteroides* that belongs to this phylum[7]. However, we conclude that IgG recognizes a diverse array of both nasopharynx pathobionts and commensal bacteria[7]. SIgADef patients have an increased anti-commensal IgG response associated with reduced systemic inflammation[5], our results are consistent with the notion that IgG may provide a layer of mucosal protection during IgA deficiency.

Immunoglobulin (IgG) replacement therapy (IVIg) has been used extensively in the treatment of primary and secondary immunodeficiency diseases. With IVIg therapy, X-linked agammaglobulinaemia (XLA-BTK) patients (characterized by a near-complete lack of Igs) have a significant reduction in the occurrence of respiratory tract infections and these patients may live a relatively healthy life[9]. We next studied the immunoglobulins coating of nasopharynx microbiota in IVIg treated XLA-BTK patients. XLA-BTK patients lack IgA, IgM and IgD nasopharynx microbiota binding (Fig 2A). On the other hand, IVIg therapy restore the secretion of nasopharynx IgG (not shown) and enhances IgG nasopharynx microbiota binding (Fig 2A). However, despite this increase in IgG coating, XLA-BTK patients exhibit nasopharyngeal microbiota dysbiosis. The beta diversity based on Bray-Curtis distance was significantly different in terms of both abundance and presence/absence of

different OTUs (Fig 2B) and have also a low alpha microbial diversity (Shannon index) comparatively to healthy individuals (Fig 2C). XLA-BTK patients exhibited a significant enrichment in *Proteobacteria* phyla (Fig 2D) and different pathobiont genera, especially *Streptococcus* (and *Streptococcus pneumoniae* specie, not shown), *Moraxella*, *Alloiococcus*, *Bacteroides,* mucus-degrading *Mucispirillum* among others commensals (Fig 2F). For example, IgG bind weakly to genera from *Proteobacteria* phylum in healthy (Fig E3D), suggesting that the lack of IgA/IgD binding (Fig 1F)[4] contributes to the *Proteobacteria* phyla expansion in XLA BTK patients. These results suggest that IVIg (IgG) therapy can not fully control pathobiont (e.g. *S. pneumoniae*) carriage load in nasopharyngeal cavity of XLA-BTK patients. Our results suggest an incomplete efficacy of IVIg (IgG) therapy in these patients (that may be related to the absence of IgA and IgM/IgD in these preparations) which raises the subsequent risk of recurrent sinus and respiratory tract infections[9]. However, the present study is consistent with a protective role of IgG antibodies in the nasopharyngeal mucosa in the context of IgA, IgM and IgA deficiency. IVIg therapy can help in control respiratory infections in primary immunodeficiency diseases patients by promoting pathogens opsonization, inducing complement activation and activation of Fc receptors for IgG if the opsonized bacteria reach the epithelium or the circulation to disseminate to systemic organs. However, these patients might also benefit from prophylactic IgA administration.

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FIGURE LEGENDS

FIG. 1. Nasopharynx IgD recognize a broad spectrum of pathobionts/commensals. A, Double binding of IgD and SIgA antibodies to nasopharynx microbiota. **B,** The antibody binding to microbiota is highly variable between healthy subjects (n = 12). **C,** Strategy for the BugFACS and 16S rRNA sequencing (n = 4). **D,** The alpha diversity, calculated using the Shannon index, in different sorted fractions. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test. **E,** Principal coordinate analysis (PCoA) of 16S RNA sequencing Operational Taxonomic Units (OTU) of $IgD^{+}SIgA^{-}$ and $SIgA^{+}IgD^{-}$ sorted fraction along the first two principal coordinate (PC) axes based on Bray-Curtis distance. **F,** Differential abundance analysis showing taxa at phylum/class/order/family/genus level which it is found to be overrepresented in the $IgD^{+}SIgA^{-}$ comparatibly to $SIgA^{+}IgD^{-}$ fraction.

FIG. 2. Immunoglobulin (IgG) replacement therapy (IVIg) "restore" the IgG binding to pathobionts/commensals but did not rescue the nasopharynx microbiota dysbiosis of X-linked agammaglobulinemia (XLA BTK) patients. A, The IgG binding to nasopharynx microbiota in healthy controls (n = 12) and X-linked agammaglobulinemia (XLA BTK) patients under immunoglobulin (IgG) replacement therapy (n = 5). Median values are indicated. **B,** Principal coordinate analysis (PCoA) of 16S RNA sequencing Operational Taxonomic Units (OTU) of the healthy controls and XLA BTK patients along the first two principal coordinate (PC) axes based on Bray-Curtis distances and the respective PERMANOVA test. **C,** The alpha diversity, calculated using the Shannon index, in healthy and XLA BTK patients. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test. **D, E,** Bar plot showing % of microbiota phylum (**D**) and genus (**E**) level in healthy and XLA BTK patients. **F,** Volcano plot displaying differential nasopharynx microbiota genus between XLA BTK patients and healthy. The vertical axis (y-axis) corresponds to the mean expression value of log 10 (q-value), and the horizontal axis (x-axis) displays the log 2 fold change value. The red dots represent the up-regulated genus in XLA BTK patients; the blue dots represent the genus downregulated. Positive x-values represent up-regulation and negative x-values represent down-regulation.

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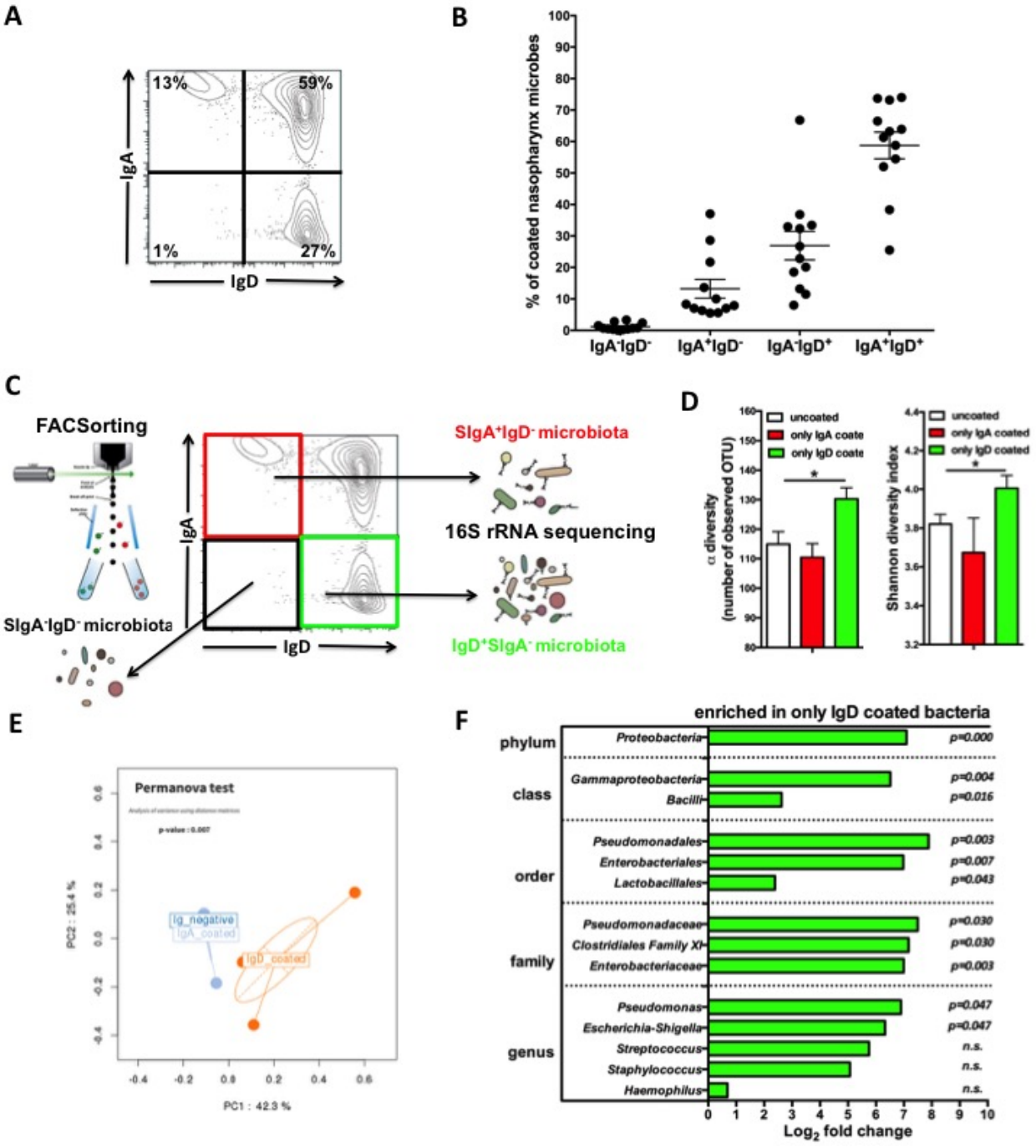
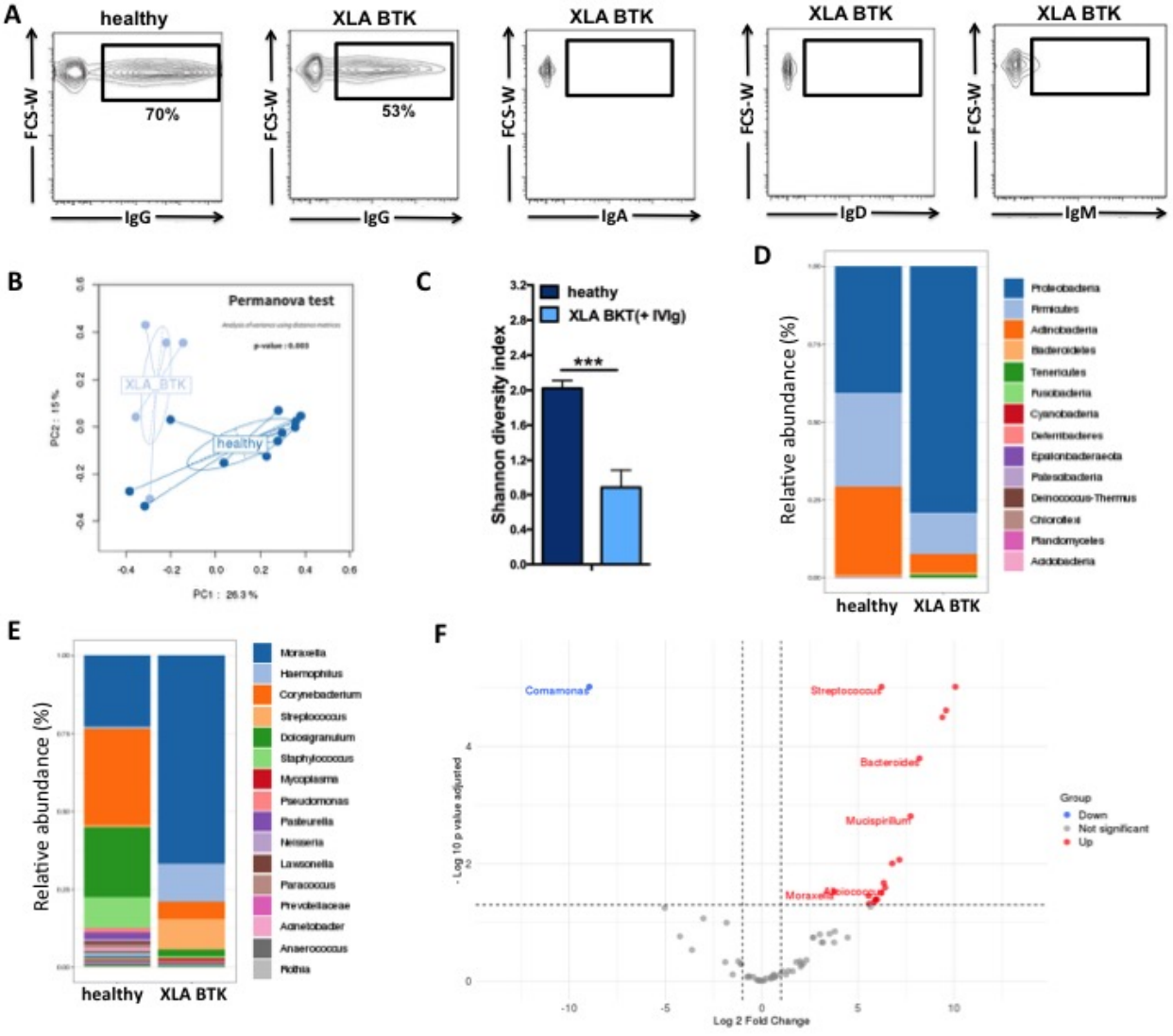
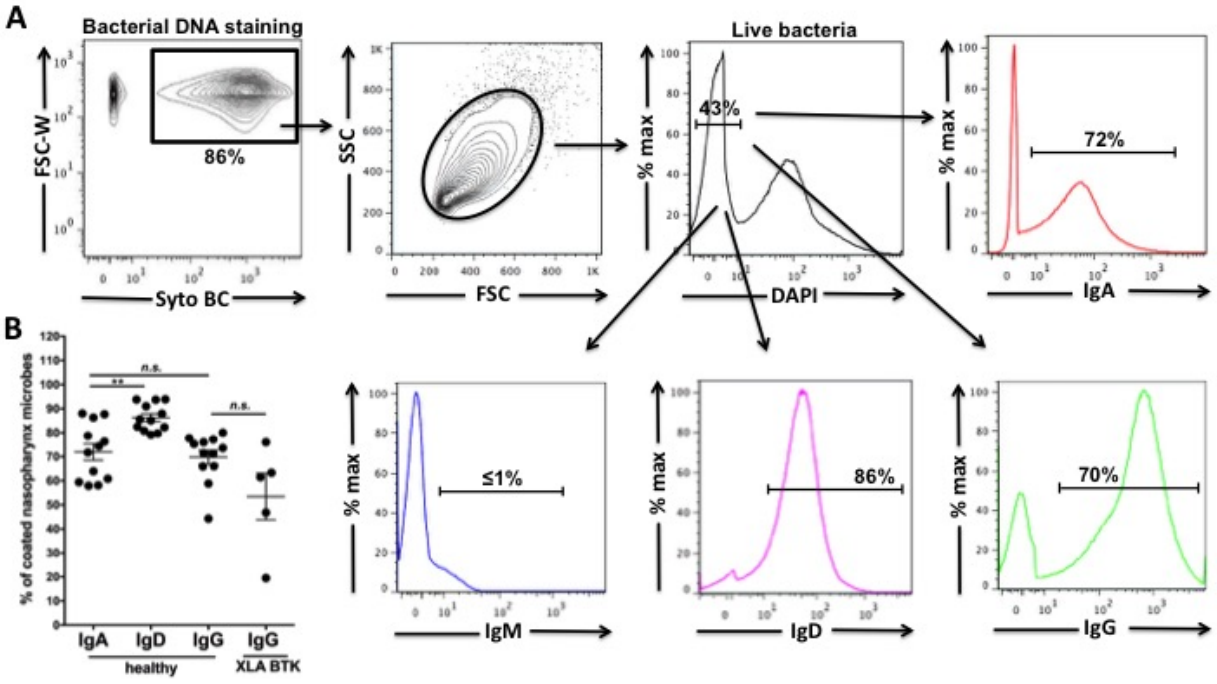
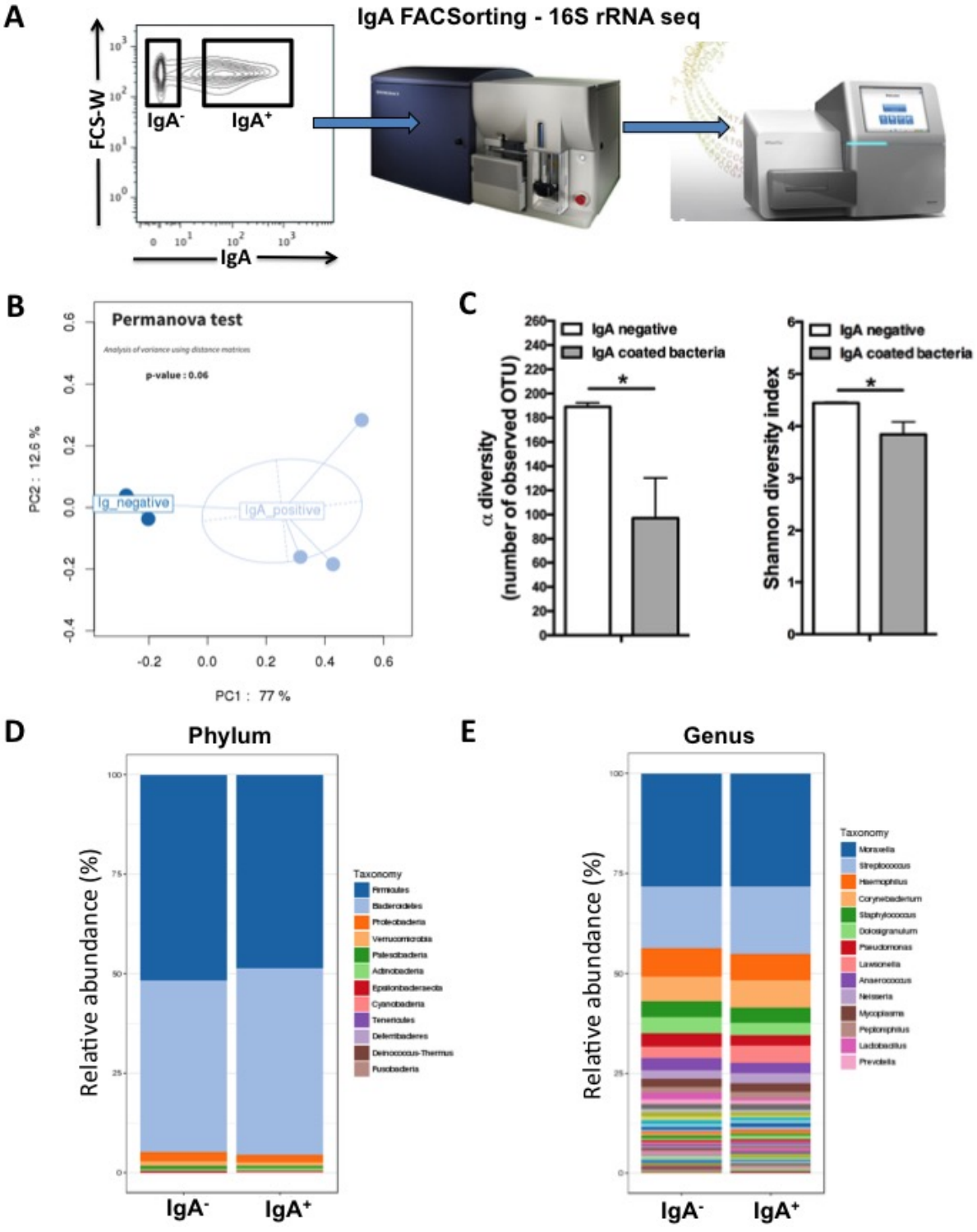

A
13%
59%
1%
27%
IgA
IgD
B
% of coated nasopharynx microbes
IgA-IgD-
IgA+IgD-
IgA-IgD+
IgA+IgD+
C
FACSorting
SIgA-IgD- microbiota
IgA
IgD
SIgA+IgD- microbiota
16S rRNA sequencing
IgD+SIgA- microbiota
D
uncoated
only IgA coate
only IgD coate
α diversity (number of observed OTU)
Shannon diversity index
E
Permanova test
p-value : 0.007
Ig_negative
IgA_coated
IgD_coated
PC2 : 25.4 %
PC1 : 42.3 %
F
enriched in only IgD coated bacteria
phylum
Proteobacteria
p=0.000
class
Gammaproteobacteria
p=0.004
Bacilli
p=0.016
order
Pseudomonadales
p=0.003
Enterobacteriales
p=0.007
Lactobacillales
p=0.043
family
Pseudomonadaceae
p=0.030
Clostridiales Family XI
p=0.030
Enterobacteriaceae
p=0.003
genus
Pseudomonas
p=0.047
Escherichia-Shigella
p=0.047
Streptococcus
n.s.
Staphylococcus
n.s.
Haemophilus
n.s.
Log2 fold change


A
healthy
XLA BTK
XLA BTK
XLA BTK
XLA BTK
FCS-W
70%
53%
IgG
IgG
IgA
IgD
IgM
B
Permanova test
XLA_BTK
healthy
PC2 : 15 %
PC1 : 26.3 %
C
healthy
XLA BKT(+ IVIg)
Shannon diversity index
***
D
Relative abundance (%)
Proteobacteria
Firmicutes
Actinobacteria
Bacteroidetes
Tenericutes
Fusobacteria
Cyanobacteria
Deferribacteres
Epsilonbacteraeota
Patescibacteria
Deinococcus-Thermus
Chloroflexi
Planctomycetes
Acidobacteria
healthy
XLA BTK
E
Relative abundance (%)
Moraxella
Haemophilus
Corynebacterium
Streptococcus
Dolosigranulum
Staphylococcus
Mycoplasma
Pseudomonas
Pasteurella
Neisseria
Lawsonella
Paracoccus
Prevotellaceae
Acinetobacter
Anaerococcus
Rothia
healthy
XLA BTK
F
Comamonas
Streptococcus
Bacteroides
Mucispirillum
Moraxella
- Log 10 p value adjusted
Log 2 Fold Change
Group
Down
Not significant
Up




A
IgA FACSorting - 16S rRNA seq
FCS-W
IgA-
IgA+
IgA
B
Permanova test
Analysis of variance using distance matrices
p-value : 0.06
PC2 : 12.6 %
PC1 : 77 %
Ig_negative
IgA_positive
C
IgA negative
IgA coated bacteria
α diversity (number of observed OTU)
Shannon diversity index
D
Phylum
Relative abundance (%)
Taxonomy
Firmicutes
Bacteroidetes
Proteobacteria
Verrucomicrobia
Patescibacteria
Actinobacteria
Epsilonbacteraeota
Cyanobacteria
Tenericutes
Deferribacteres
Deinococcus-Thermus
Fusobacteria
IgA-
IgA+
E
Genus
Relative abundance (%)
Taxonomy
Moraxella
Streptococcus
Haemophilus
Corynebacterium
Staphylococcus
Dolosigranulum
Pseudomonas
Lawsonella
Anaerococcus
Neisseria
Mycoplasma
Peptoniphilus
Lactobacillus
Prevotella
IgA-
IgA+

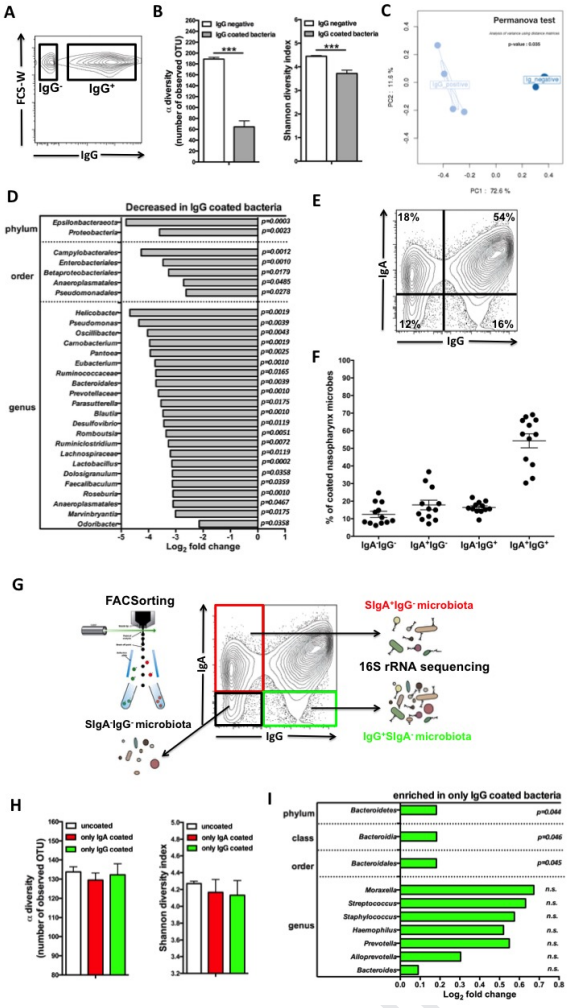


FIG. E1. A, Gating strategy for the microbiota flow cytometric assay. We used the DNA dye SYBR Green to preferentially stain microbes allowing us to gate on ($SYBR^{hi}$). We then used specific secondary antibodies to characterize the anti-commensal antibody (IgA, IgM, IgD and IgG) response. **B,** The antibody binding to microbiota is highly variable between healthy subjects (n = 12) and X-linked agammaglobulinemia (XLA-BTK) patients under immunoglobulin (IgG) replacement therapy (n = 5). Median values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test.

FIG. E2. Nasopharynx SIgA binds a broad spectrum of pathobionts/commensals. **A,** Strategy for the IgA-BugFACS and 16S rRNA sequencing. **B,** Principal coordinate analysis (PCoA) of 16S RNA sequencing Operational Taxonomic Units (OTU) of IgA^+ and IgA^- sorted fraction along the first two principal coordinate (PC) axes based on Bray-Curtis distance and the respective PERMANOVA test. **C,** The alpha diversity, calculated using the Shannon index, in IgA^+ and IgA^- sorted fraction. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test. **D,** Bar plot showing % of microbiota phylum in IgA^+ and IgA^- sorted fraction. **E,** Bar plot showing % of microbiota genus in IgA^+ and IgA^- sorted fraction. Data are representative of 4 healthy individuals.

FIG. E3. Nasopharynx IgG recognize a broad spectrum of pathobionts/commensals. A, Strategy for the IgG-BugFACS sorting (n = 4). **B,** The alpha diversity, calculated using the Shannon index, in IgG^+ and IgG^- sorted fraction. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test. **C,** Principal coordinate analysis (PCoA) of 16S RNA sequencing Operational Taxonomic Units (OTU) of IgG^+ and IgG^- sorted fraction along the first two principal coordinate (PC) axes based on Bray-Curtis distance and the respective PERMANOVA test. **D,** Differential abundance analysis showing taxa at phylum/order/genus level which it is found to be downrepresented in the IgG^+ comparatibly to IgG^- fraction. **E,** Double binding of SIgA and IgG antibodies to nasopharynx microbiota. **F,** The antibody binding to microbiota is highly variable between healthy subjects (n = 12). **G,** Strategy for the BugFACS and 16S rRNA sequencing (n = 4). **H,** The alpha diversity, calculated using the Shannon index, in different sorted fractions. Mean and standard error of mean (SEM) values are indicated, and subgroups are compared with a nonparametric Mann-Whitney test. **I,** Differential abundance analysis

showing taxa at phylum/class/order level which it is found to be overrepresented in the $IgG^{+}SIgA^{-}$ comparatibly to $SIgA^{+}IgG^{-}$ fraction.

METHODS

Patient recruitment, sample collection and processing

Nasopharynx specimens were obtained with sterile dry swabs (COPAN LQ Stuart Transport Swab; COPAN Italia S.p.A, Brescia, Italy), which are rotated five times around the inside of each nostril while applying constant pressure. Nasopharynx swabs were collected in the office under strict aseptic conditions with sterile gloves and instrumentation. Laboratory protocols were standardized and staff members were trained in sample preparation protocols [1]. All swabs will be frozen (-80°C) upon collection from healthy individuals at local site. The 12 healthy donors were recruited originally as part of the *Milieu Intérieur* cohort [1] (BioTrial, Rennes, France), but were technically excluded from the main cohort due to blood sampling criteria. The clinical study was approved by the Comité de Protection des Personnes - Ouest 6 (Committee for the protection of persons) on 13 June 2012 and by the French Agence Nationale de Sécurité du Médicament (ANSM) on 22 June 2012. The study is sponsored by the Institut Pasteur (Pasteur ID-RCB Number: 2012-A00238-35) and was conducted as a single center study without any investigational product. The protocol is registered under ClinicalTrials.gov (study# NCT01699893). X-linked agammaglobulinemia patients were recruited at Hôpital Necker-Enfants Malades (French National Reference Center for Primary Immunodeficiencies). Pathogenic mutations in the Bruton tyrosine kinase (BTK) were identified in all 5 cases. At diagnosis, they had IgG plasma levels below 100 mg/ml with no detectable IgA and IgM, while all 5 had no detectable blood B cells ($CD19^+$). At time of study, patients were aged 14 to 28 years (median 15). They were receinving regular IgG substitution with a through level above 800 mg/dl (800 to 1385). None had antibiotic therapy. Two are perfectly healthy while 3 have minimal bronchiectasis. Most recent immunological investigations were unremarkable a part for the absence of detectable B cells. The age and gender is not significantly different between X-linked agammaglobulinemia patients and healthy. Written informed consent was obtained from all patients and/or parents. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Nasopharynx swabs processing

Swabs were thawed and vortexed for 1 minute at 2500 rpm to insure complete sample recovery. Samples was transferred in a 96 well deep-well plate and centrifuged at 16.000g for 10 minutes at 4°C to pellet cells and accompanying microbes. Supernatants were recovered and stored at -80°C until analysis using a TECAN Evo 75 robot.

Bacterial Flow Cytometry

Bacterial species-specific antibody against microbiota were assessed by a flow cytometry assay was described previous[2]. Briefly, the bacterial pellet was washed and resuspended in flow cytometry buffer (1x-PBS, 2% BSA, 0.02% w/v sodium azide) with SYBR Green (1/10000 (v/v) dilution, Invitrogen) incubated 30 min on ice. After, bacterial suspension was washed twice and pelleted by centrifugation at 16.000g for 10 min. Supernatant was removed and pellets were resuspended in 100 µL flow cytometry buffer with mouse anti-human IgA-PE (clone IS11-8E10), anti-human IgD-PerCP-Cy5.5 (clone IAG-2), anti-human IgM-APC (clone SA-DA4) and anti-human IgG-BUV395 (clone G18-145) at a final IgG concentration of 10 µg/mL in a 96-well V-bottom plate and incubated for 20 min on ice. Suspensions were washed once with 100 uL of flow cytometry buffer and cells pelleted by centrifugation, then resuspended in flow cytometry buffer with DAPI (Life Technologies) prior to flow cytometry. Acquisition of cells events was performed using a FACS LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA) and analysis was performed with Flow-Jo software software (TreeStar, Ashland, OR). Staining of live bacteria was visualized by gating on SYTO $BC^{+}FSC^{+}SSC^{+}$ $DAPI^{-}$ cells.

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Different immunoglobulin-binding microbiota were sorted[3] (purity ≥ 99%) using a FACSAria II (BD Biosciences) into eppendorf tube in a laminar flow biocontainment hood (BioProtect IV Safety Cabinet, Baker Co.). The gating strategies used to collect different bacterial populations are shown in Fig. E1. Threshold settings were set to the minimal allowable voltage for sidescatter (SSC). Isolated bacteria were pelleted (15.000×g, 10 minutes, 4 °C) and frozen at -80°C before sequencing of bacterial 16S rRNA genes.

Bacterial DNA isolation and 16S rDNA sequencing

In our study, we are performing the extraction of total genomic DNA from swabs samples, using the TECAN Freedom EVOware workstation, and the NucleoSpin® 96 Genomic DNA kit (Macherey-Nagel ®). For DNA extraction, a "negative" control was included containing buffers. Briefly, the pellets that remain in the deep well plate were incubated with Ready-Lyse™ Lysozyme Solution (250 U/µl) (Epicentre, Hessisch Oldendorf, Germany) for 30 minutes at 37°C. Next, we incubated the suspension with Proteinase K/buffer T1 working solution at 55°C overnight until the samples are completely lysed. We add 20 µg of glycogen (DNA carrier) and we follow the DNA extraction kit protocol. Finally

we eluted the DNA in 25 µl and the DNA box is immediately frozen at -80°C at local center until its use. The concentration of extracted DNA is determined using TECAN (QuantiFluor® ONE dsDNA System, Promega), and DNA integrity and size were also confirmed with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The V3-V4 region of the bacterial 16S rRNA region were PCR amplified with V3-340F (CCTACGGRAGGCAGCAG) and V4-805R (GGACTACHVGGGTWTCTAAT) containing a primer linker, primer pad, unique 8-mer Golay barcode which was used to tag PCR products from respective samples and negative control. PCR reactions consisted of 18 µl of AccuPrime Pfx SuperMix (12344-040; Invitrogen), 0.5 µl of each primers and 1 µl of DNA (10 ng). PCR was carried out as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 15 s and 72 °C for 1 min, and a final extension step at 72 °C for 10 min on a Biorad thermocycler. PCR products were cleaned using NucleoMag magnetic purification beads (MACHEREY-NAGEL Kit) following the protocol, quantified with the QuantiFluor® ONE dsDNA kit (Promega), and pooled in equal amounts of each PCR product. Library pools were loaded at 12pM with a 15% PhiX spike for diversity and sequencing control, onto a v3 300-bp paired end reads cartridge for sequencing on the Illumina MiSeq NGS platform.

Sequence processing and statistical analysis

After removing reads containing incorrect primer or barcode sequences and sequences with more than one ambiguous base, we recovered from 53 samples a total of 3.090.252 reads (58.307 reads on average). The bioinformatics analysis was performed as previously described [4]. Briefly, amplicons were clustered into operational taxonomic units (OTU) with VSEARCH (v1.4) and aligned against the SILVA database. The input amplicons were then mapped against the OTU set to get an OTU-abundance table containing the number of reads associated with each OTU. The normalization, statistical analyses and multiple visualization were performed with SHAMAN (SHiny application for Metagenomic Analysis (shaman.c3bi.pasteur.fr) based on R software.

Statistical analysis

GraphPad Prism was used for statistical analysis. Thus, non-parametric tests (Mann Whitney U-test) were used where applicable.

METHOD REFERENCES

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Additional information can be found at: http://www.pasteur.fr/labex/milieu-interieur