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# Immunoglobulin secretion influences the composition of chicken caecal microbiota

Jiri Volf[1], Bernd Kaspers[2], Benjamin Schusser[3,4], Magdalena Crhanova[1], Daniela Karasova[1], Hana Stepanova[1], Vladimir Babak[1] & Ivan Rychlik[1]✉

**The chicken caecum is colonised by hundreds of different bacterial species. Which of these are targeted by immunoglobulins and how immunoglobulin expression shapes chicken caecal microbiota has been addressed in this study. Using cell sorting followed by sequencing of V3/V4 variable region of 16S rRNA, bacterial species with increased or decreased immunoglobulin coating were determined. Next, we determined also caecal microbiota composition in immunoglobulin knockout chickens. We found that immunoglobulin coating was common and major taxa were coated with immunoglobulins. Similarly, more taxa required immunoglobulin production for caecum colonisation compared to those which became abundant in immunoglobulin-deficient chickens. Taxa with low immunoglobulin coating such as *Lactobacillus*, *Blautia*, [*Eubacterium*] *hallii*, *Megamonas*, *Fusobacterium* and *Desulfovibrio* all encode S-layer proteins which may reduce interactions with immunoglobulins. Although there were taxa which overgrew in Ig-deficient chickens (*e.g. Akkermansia*) indicating immunoglobulin production acted to exclude them from the chicken caecum, in most of the cases, immunoglobulin production more likely contributed to fixing the desired microbiota in the chicken caecum.**

Eukaryotic hosts are colonised by thousands of species belonging to different prokaryotes, protozoa, fungi or viruses. This inevitably leads to numerous interactions between host and its microbiota. Microbiota can be found in different host compartments, but the intestinal tract is the most densely populated, mostly by prokaryotes, what requires specific adaptations of both host and microbiota (due to used methodology, term microbiota will be used for designation of only prokaryotic component of the total microbiota in this study) in order to co-exist in an optimal balance. Eukaryotic hosts shape the composition of gut microbiota by the expression of bile salts, digestive enzymes or proteins with protective functions like mucins, antimicrobial peptides or immunoglobulins[1]and some of these molecules affect gene expression of gut microbiota[2–4]. Microbiota also affects its host by the expression of enzymes directly interacting with host structures such as mucin sulfatases, hyaluronidase or heparinase[5,6], or by release of metabolic byproducts such as short chain fatty acids[7,8]. Microbiota may also produce γ-aminobutyric acid, histamine or indole, all molecules with extensive biological functions[9–11]or may degrade feed polysaccharides of plant origin, which results in the additional release of biologically active molecules[12]. Altogether these events result in a delicate balance resulting in suppression of excessive growth of bacteria, which may have an adverse effect on host fitness. Collectively these events shift the balance into evolutionarily beneficial positions, not only for the host, but also for the associated microbial community.

A specific part of host interactions with microbiota is mediated by immunoglobulins. Immunoglobulins of IgA class belong among the major proteins present in mucosal surfaces of the intestinal tract and these molecules can also be found associated with human, mouse or chicken gut microbiota[13–16]. Expression of immunoglobulins in the intestinal tract is dependent on gut colonisation by live bacteria[17–19] since inoculation of germ free chickens with heat-killed microbiota did not result in immunoglobulin expression[20]. In agreement, the presence of B-lymphocytes and expression of immunoglobulins in the caecum of newly hatched chickens is delayed and the expression of immunoglobulins can be detected in the caecal tissue only from the second week of life[21–23]. The coating of gut microbiota by immunoglobulins may result in elimination as well as stabilisation of gut microbiota[14,24,25]. In an apparent contradiction, IgA deficiencies in humans are not associated with extensive differences in gut microbiota composition, likely due to expression of compensatory IgM[17,26]. Despite this,

[1]Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic. [2]Veterinary Faculty, Department for Veterinary Sciences, Ludwig Maximilians University Munich, Planegg, Germany. [3]Reproductive Biotechnology, TUM School of Life Sciences, Technical University of Munich, Munich, Germany. [4]Center for Infection Prevention (ZIP), Technical University of Munich, Freising, Germany. ✉email: rychlik@vri.cz

defects in immunoglobulin production are associated with an increased inflammatory response due to higher stimulation of gut epithelium by microbial molecular patterns like LPS or flagellin[2,3].

The chicken intestinal tract differs from that of humans, pigs or mice. The short colon does not contain excess digesta and strictly anaerobic microbiota, like those present in distal parts of the intestinal tract of mice or humans, can be found in the chicken caecum. A further specific aspect of chickens is that anaerobic fermentation in the caecum is a discontinuous process as caeca are filled and emptied usually twice a day. The chicken caecum is filled with digesta from the small intestine, closed and anaerobic fermentation proceeds for approximately 12 h before the digesta is released to the colon and the whole process is repeated[27,28]. This may be a reason why different *Bacteroides* species colonise humans and chickens[29]. Due to these differences and since antibody production in the intestinal tract is considered as an essential part of interactions between host and its microbiota, in this study we tested which chicken microbiota members are or are not coated with immunoglobulins using flow cytometry cell sorting followed by 16S rRNA sequencing. Having available Ig-deficient knock-out chickens, we also tested whether colonisation of particular species was dependent on immunoglobulin production, and whether there was a correlation between coating by immunoglobulins and dependence of colonisation on immunoglobulin secretion.

## Results

### Immunoglobulin coating of chicken caecal microbiota

To define the extent of interactions between secreted immunoglobulins and gut microbiota in chickens, first we determined antibody coating of individual gut microbiota members. On average, $49.34 \pm 11.31\%$ of all bacterial cells were without immunoglobulin coating while $34.66 \pm 8.38\%$ of all bacteria were positive for immunoglobulins (Fig. 1A). Altogether, 3851 amplicon sequence variants (ASV) were detected in this experiment though to avoid issues with ASVs represented at low abundance, ASVs present in less than 5 original caecal digesta samples (out of 16 available) were excluded. Such subset was further reduced to ASVs which were present in 10 or more sorted samples out of 32 available. This resulted in 401 ASVs used in downstream analyses.

One hundred and seven ASVs, *i.e.* 26.68% ASVs of all, exhibited significantly increased or decreased immunoglobulin coating. Fifty-nine ASVs exhibited high immunoglobulin binding while 48 ASVs exhibited low immunoglobulin coating. Low immunoglobulin coated ASVs belonged to families Lactobacillaceae, Peptostreptococcaceae and Selenomonadaceae. High immunoglobulin coating was recorded in ASVs belonging to Ruminococcaceae, Oscillospiraceae, Rikenellaceae, Atopobiaceae and Prevotellaceae (Fig. 1A, B). ASVs belonging to Lachnospiraceae were the most common in our dataset. Within 89 ASVs belonging to Lachnospiraceae, 19 Lachnospiraceae ASVs exhibited high immunoglobulin binding and an additional 19 ASVs exhibited low immunoglobulin binding. Unlike other families, a high proportion of ASVs belonging to Lachnospiraceae exhibited differential immunoglobulin binding with the same number of high or low immunoglobulin binding ones.

Phylogenetic classification of all ASV with differential immunoglobulin coating together with an additional 100 of the most abundant ASVs with neutral immunoglobulin coating showed that within Firmicutes, extensive interactions with immunoglobulins were recorded in all major families including Lachnospiraceae, Ruminococcaceae, Oscillospiraceae, Erysipelotrichaceae and Selenomonadaceae, except for genus *Faecalibacterium* in which there was not a single ASV with high or low immunoglobulin binding (Fig. 1C). *Clostridium* sensu stricto 1 (*Clostridium disporicum* following manual BLAST comparison with GenBank database), *Lactobacillus*, *Enterococcus*, *Romboutsia* and *Turicibacter*, all colonising the small intestine[30], were characterised by low immunoglobulin coating. *Megamonas* and *Fusobacterium* also belonged among bacterial genera with low immunoglobulin coating. Similar bacterial counts of *Campylobacter* or *Helicobacter* were or were not coated with immunoglobulins, *i.e.* these genera did not belong to the taxa with high or low immunoglobulin binding. Out of Proteobacteria, *E. coli* exhibited neutral, *Desulfovibrio* exhibited no or low and *Sutterella* exhibited high immunoglobulin coating (Fig. 1C). Immunoglobulins interacted also with representatives of *Actinobacteria*. While *Olsenella* was characterised by high immunoglobulin coating, *Bifidobacterium* belonged among taxa with low immunoglobulin binding (Fig. 1D). In Gram negative Bacteroidetes, representatives of family Rikenellaceae and genus *Alistipes* belonged among taxa with high immunoglobulin binding. On the other hand, two representatives of Bacteroidaceae exhibited high affinity for immunoglobulins and the other two had low affinity (Fig. 1C).

#### *Microbiota analysis in immunoglobulin-deficient chickens—animal identification*

Next, we were interested whether immunoglobulin production influences gut microbiota composition. To test this hypothesis, knockout chickens deficient in immunoglobulin expression were used[31]. Classification of wild-type, heterozygotes and Ig-deficient homozygote chickens was performed as described previously[31]. The phenotype of predicted genotypes was then confirmed by ELISA quantification of IgY and IgA in the blood serum (Fig. 2A) and by real-time RT PCR targeted to constant regions of IgY, IgA, IgM and Ig light chain transcripts in the caecal tissue (Fig. 2B). Both ELISA and RT PCR showed that the level of immunoglobulin expression was the same in the wild-type and heterozygous chickens while no immunoglobulin expression was detected in knockouts (Fig. 2). Wild-type and heterozygous chickens were therefore grouped in a single group hereafter called wild-type chickens and compared to the Ig-deficient mutants.

#### *Chicken response to the absence of immunoglobulin production*

In additional experiment we checked whether chickens compensated for the absence of immunoglobulin expression by quantifying the expression of 58 genes by real-time RT PCR in the caecal tissue. These genes were originally identified as responsive to *Salmonella* Enteritidis infection thus being inflammatory markers[23] or responded to inoculation of chickens with caecal contents of adult hens[16]. Of these, 32 genes exhibited

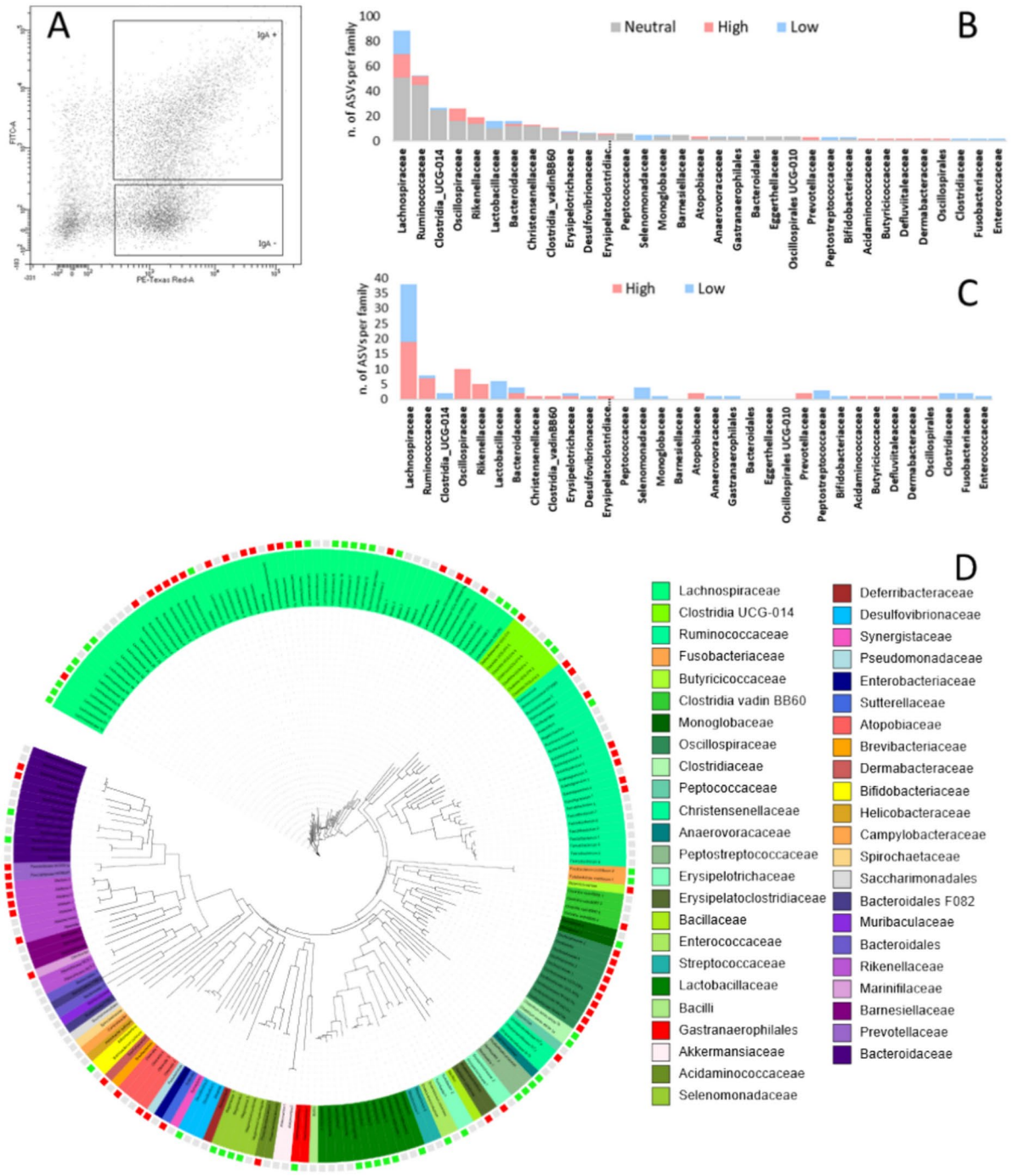


**Fig. 1**. Immunoglobulin coating of individual ASVs. Panel **A**, gating and sorting Ig positive and Ig negative bacterial cells from the chicken caecum. Panel **B**, immunoglobulin coating of individual ASVs belonging to particular families. Blue columns, ASVs with low immunoglobulin coating, red columns, ASVs with high immunoglobulin coating. Mind that grey color does not indicate ASVs with no immunoglobulin binding but equal proportion of bacterial cells with and without bound immunoglobulins. If an ASV cannot be classified to a family, higher taxonomic designation is shown. Panel **C**, the same as in panel **B**, except for the fact that only differentially abundant ASVs are shown. Significant difference was defined as $p < 0.05$ using non-parametric Wilcoxon paired test with Pratt's modification. Panel **D**, all ASVs with differential immunoglobulin coating together with additional 100 the most abundant ASVs without any preference for immunoglobulin binding were Clustal aligned using their partial 16S rRNA sequences. Red squares, ASVs with high immunoglobulin binding. Green squares, ASVs with low immunoglobulin binding. Grey squares, ASVs with no preference for immunoglobulin binding.

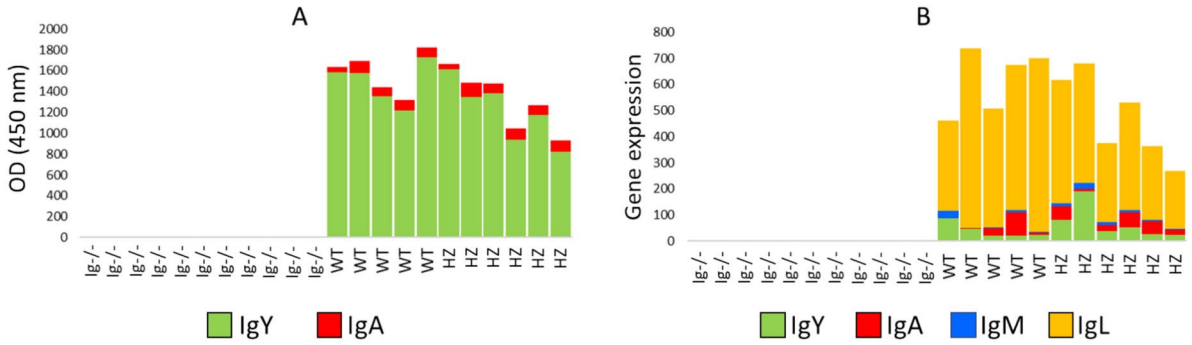


**Fig. 2**. Immunoglobulin expression determined by ELISA and RT PCR. Genotype classification of wild-type (WT), heterozygotes (HZ) and Ig-/- homozygote chickens was performed as described previously[31]. Immunoglobulin expression for each chicken included in this study was then verified in blood serum by ELISA (Panel **A**) and in caecal tissue by real time RT-PCR (Panel **B**). Both protocols confirmed that Ig-deficient homozygote chickens clearly differed from the wild-type and heterozygous chickens by a total lack of expression of IgY, IgA, IgM and Ig light chain (IgL).

significantly different expression between wild-type and Ig-deficient chickens, though fold change exceeded a factor of 2 only in 9 of them. ISG12(2) (also called IFI27) with 5.8×higher expression in the Ig-deficient compared to wild-type chickens was the gene with the highest differential expression. IL17, IL22, IL8, TLR4 and Gal4 were approximately two-fold induced in the Ig-deficient chickens while IL1β, IL6, MMP7, AVD, ES1 or C4 did not change in expression indicating minor activation of the mucosal gut immune system but without corresponding inflammation. Polymeric immunoglobulin receptor protein (pIgR) belonged among the proteins with the highest expression and remained highly expressed also in the absence of immunoglobulin production (Fig. 3).

*Caecal microbiota in wild-type and Ig-deficient chickens*

Although we aimed to introduce complex adult microbiota by spreading faecal material from adult hens over the floor 2 days before chick placement, sequencing showed that genera *Bacteroides*, *Parabacteroides*, *Barnesiella*, *Megamonas*, *Phascolarctobacterium* or *Parasutterella* characteristic for adult-type microbiota, though present in faecal material of donor birds, were essentially absent from microbiota of chickens (Fig. 4A and Supplementary file S1). We can exclude inability of chickens to become colonised by adult type of microbiota due to their age[32–34] and chicken colonisation was therefore dependent on environmental sources only.

Microbiota composition of the wild-type chickens did not differ from microbiota of heterozygous chickens while these samples differed from the samples originating from the Ig-deficient chickens (Fig. 4A). Microbiota of wild type chickens consisted of higher number of bacterial species and exhibited higher species diversity than microbiota of Ig-deficient chickens (Fig. 4B). Representatives of 10 phyla were detected in at least one chicken. Of these, 6 phyla were differently abundant in wild-type and Ig-deficient chickens. Firmicutes and Actinobacterota were more abundant in microbiota of wild-type chickens. Patescibacteria represented at overall low abundance was also more abundant in the wild-type chickens. On the other hand, Cyanobacteria, Desulfobacterota and Verrucomicrobiota were more abundant in microbiota of Ig defective chickens (Fig. 4C). Since Cyanobacteria sequences commonly originate from plant chloroplast DNA as a consequence of undigested feed of plant origin, these sequences were manually BLAST compared with 16S rRNA GenBank database and *Vampirovibrio chlorellavorus* was identified as the closest database entry, though with 85% similarity only. Search against the whole GenBank database then resulted in the same result, *i.e.* did not show similarity to any plant chloroplast sequences.

There were 29 families with an average representation higher than 0.1% of total microbiota either in wild-type or Ig defective chickens of which 18 were differently abundant (Fig. 4D). Coriobacteriaceae, Clostridiaceae, Streptococcaceae, Enterobacteriaceae, Atopobiaceae, Peptostreptococcaceae, Bifidobacteriaceae, Peptococcaceae, [Eubacterium] coprostanoligenes group, Bacilli RF39, Lachnospiraceae and Ruminococcaceae (arranged according to decreasing fold difference) were significantly more abundant in microbiota of wild-type chickens while Akkermansiaceae, Izemoplasmatales, Desulfovibrionaceae, Gastranaerophilales, Bacillaceae and Clostridia vadin BB60 group were more abundant in microbiota of Ig-deficient chickens (Fig. 4E). While the families more abundant wild-type chickens comprised those common to chicken caecal microbiota, families enriched in Ig-deficient chickens (Izemoplasmatales, Bacillaceae, Gastranaerophilales or Akkermansiaceae) are less frequently associated with chicken gut microbiota.

There were 1024 ASVs present in at least one of the wild-type or Ig-deficient chickens. To decrease background noise caused by ASVs represented at low abundance, we did not consider ASVs which were present in less than 8 samples out of 22 available. Following such reduction, downstream analysis has been performed with 386 major ASVs. Of these, 172 were differently abundant ($p < 0.05$), 120 more abundant in caecal microbiota of the wild-type chickens and 52 more abundant in the immunoglobulin-deficient chickens. Colonisation of nearly a half (44.5%) of the major microbiota members was therefore affected by immunoglobulin production and a positive dependence of colonisation on immunoglobulin production was 2.3×more frequent than a negative one.

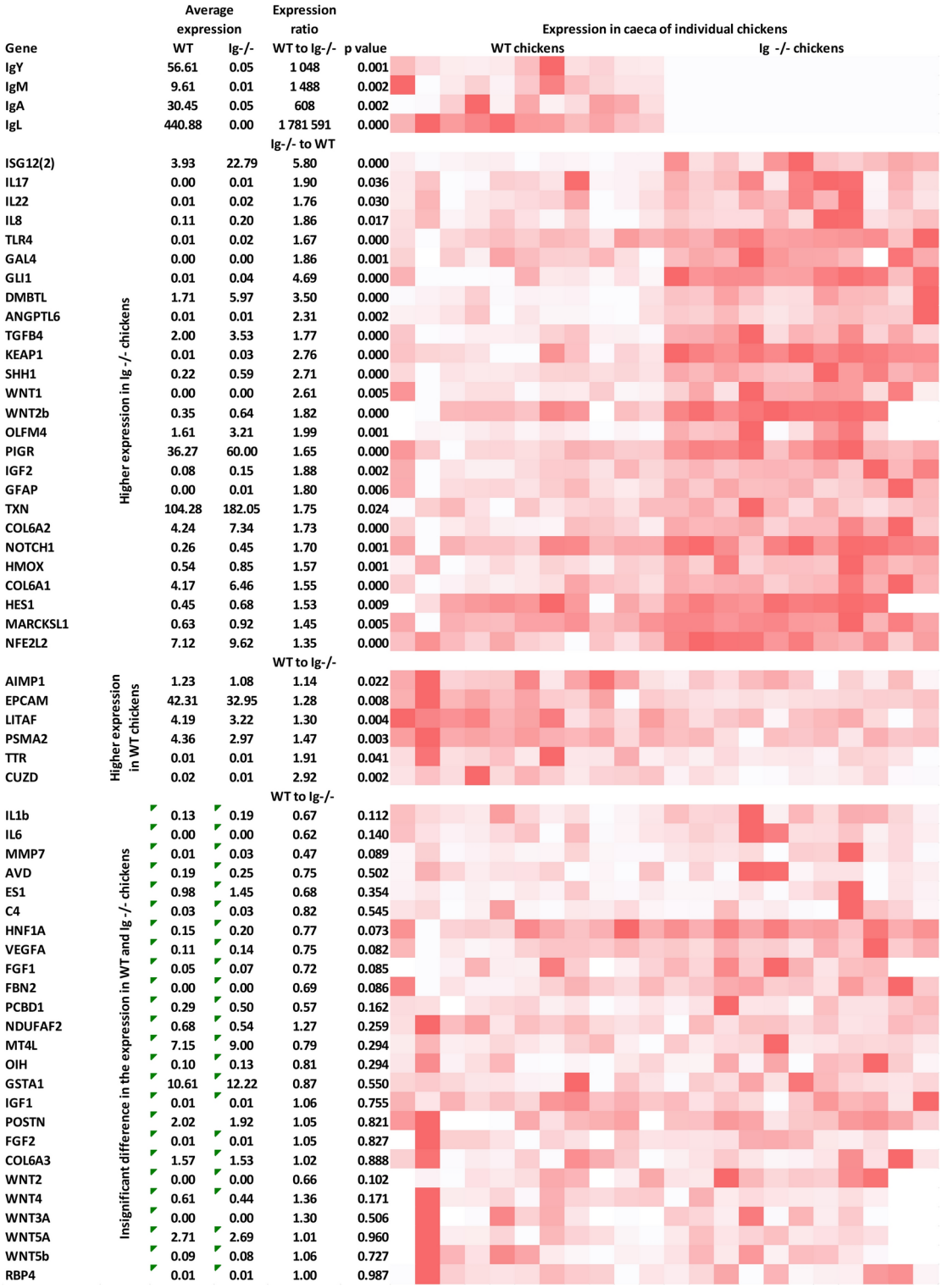


**Fig. 3**. Gene expression in the chicken caecum in the wild-type and Ig-deficient chickens. The differences in gene expression in the chicken caecum of wild-type and immunoglobulin-deficient chickens rarely exceeded a factor of 2 and never exceeded a factor of 6 (except for immunoglobulins themselves).

Differently abundant ASVs belonged to families Lachnospiraceae, Ruminococcaceae, Oscillospiraceae, Clostridia UCG-014 and Clostridia vadin BB60 group. ASVs belonging to Lachnospiraceae, Ruminococcaceae and Oscillospiraceae usually required expression of immunoglobulins for their colonisation, *e.g.* 38 different ASVs belonging to Lachnospiraceae were more abundant in microbiota of wild-type chickens while only 7 ASVs from Lachnospiraceae were more abundant in Ig-deficient chickens (Fig. 5A and B). ASVs which required the

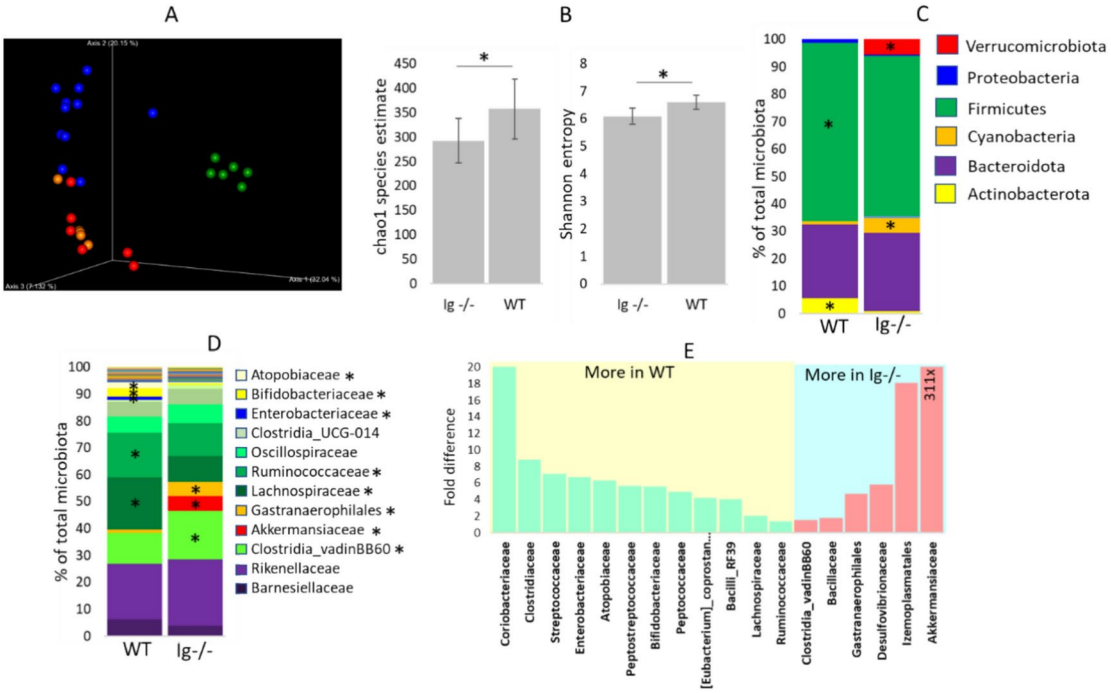


**Fig. 4**. Microbiota composition in the wild-type, heterozygous and Ig-deficient chickens. Panel **A**, PCoA clustering showed a separation of the microbiota in the faecal material of adult hens (green dots) spread over the floor of animal house used for raising wild-type (red dots), heterozygous (orange dots) and Ig-deficient chickens (blue dots). Panel **B**, chao1 species estimate and Shannon diversity of microbiota from wild type and Ig -/- chickens. Panel **C**, caecal microbiota composition of wild-type and Ig-deficient chickens at the phylum level. Panel **D**, caecal microbiota composition in the wild-type and Ig-deficient chickens at the family level (only major families are identified by color coding). Panel E, fold difference in families differently abundant in caecal microbiota of wild-type and Ig-deficient chickens. Column height for Akkermansiaceae is not to scale as the abundance of this family in Ig-deficient chickens was 311 times higher than in the wild-type controls. *—significant difference defined as $p < 0.05$ in t-test (for Panel **B**) or Mann–Whitney test (for panels **C** and **D**).

expression of immunoglobulins for their colonisation belonged to genera *Anaerostipes*, *Anaerotignum*, *Blautia*, *Butyricicoccus*, *Drancourtella*, *Flintibacter*, *Gemmiger*, *Mediterraneibacter*, *Pseudoflavonifractor*, *Roseburia*, *Subdoligranulum*, *Faecalibacterium*, *Bifidobacterium*, *Olsenella*, *Enorma*, *Collinsella* or *Escherichia*. On the other hand, ASVs belonging to families Clostridia UCG-014, Clostridia vadin BB60 group and Gastranaerophilales, and genera *Akkermansia*, *Anaeromassilibacillus* and *Desulfovibrio* were more abundant in microbiota of Ig-deficient chickens (Fig. 5C).

## Comparison of immunoglobulin coating in cell sorting experiment and ASV abundance in wild-type or Ig-deficient chickens

When the two experiments were compared, only 22 ASVs were differently abundant both in the cell sorting and Ig-deficient chicken experiment (Table 1). Ten ASVs with high immunoglobulin binding and increased abundance in wild-type chickens included *Mediterraneibacter catenae* (previously [*Ruminococcus*] *torque*), *Lachnoclostridium edouardi*, *Anaerobium acetethylicum* (all family Lachnospiraceae), *Subdoligranulum variabile*, *Ruminococcus albus* and *Gemmiger gallinarum* (all family Ruminococcaceae), *Oscillibacter massiliensis* (Oscillospiraceae), *Merdibacter massiliensis* (family Erysipelotrichaceae), *Butyricicoccus pullicaecorum* (family Butyricicoccaceae) and *Paludihabitans psychrotolerans* (order Oscillospirales).

Nine ASVs with low immunoglobulin binding but requiring immunoglobulin production (at low abundance in Ig-deficient chickens) included *Clostridium disporicum* (family Clostridiaceae), *Blautia hydrogenotrophica*, *Anaerobutyricum soehngenii*, *Hominisplanchenecus faecis*, *Drancourtella massiliensis* and two ASVs of *Mediterraneibacter catenae* (all family Lachnospiraceae), and *Romboutsia timonensis* and *Lentihominibacter faecis* (both order Peptostreptococcales).

Two ASVs with low immunoglobulin binding and decreased abundance in microbiota of wild-type chickens included isolates from Lachnospiraceae and Gastranaerophilales, which were loosely related to *Fusicatenibacter saccharivorans* (95.77% 16S rRNA similarity to this species) and *Vampirovibrio chlorellavorus* (84.83% 16S rRNA similarity), respectively. *Papillibacter cinnamivorans* (Oscillospiraceae, 92.36% 16S rRNA similarity) was the only ASV which exhibited high immunoglobulin binding but preferential colonisation of immunoglobulin-deficient mutants.

Due to the low number of identical ASVs detected in both experiments, finally we checked for taxonomic relatedness and immunoglobulin binding or abundance in wild-type or Ig-deficient chickens. ASVs

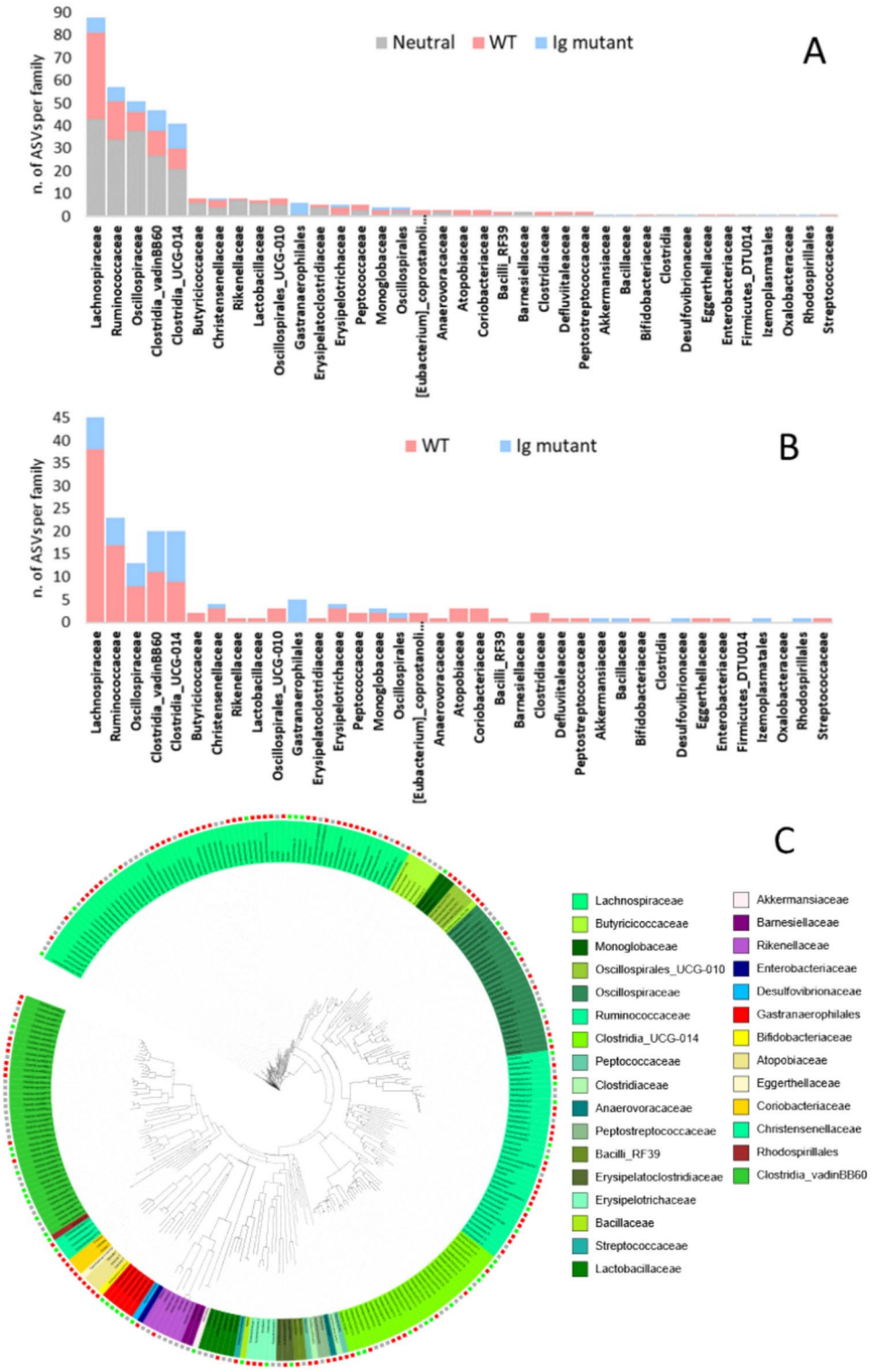


**Fig. 5**. Differently abundant ASVs in wild-type and immunoglobulin-deficient chickens. Panel **A**, distribution of individual ASVs belonging to particular families. Red columns, ASVs more abundant in wild-type chickens, blue columns, ASVs more abundant in Ig-deficient chickens. Grey columns, ASVs with similar distribution in the wild-type and Ig-deficient chickens. If an ASV cannot be classified to a family, higher taxonomic designation is shown. Panel **B**, the same as in panel A except for the fact that only differentially abundant ASVs are shown. Panel **C**, all ASVs with differential abundance in wild-type or immunoglobulin-deficient chickens together with an additional 100 of the most abundant ASVs without any preference were Clustal aligned using their partial 16S rRNA sequences. Red squares, ASVs with higher abundance in wild-type chicken, green squares, ASVs with higher abundance in immunoglobulin-deficient chickens. Grey squares, ASVs abundance which was not significantly affected by immunoglobulin expression.

| | | ASVs in WT and Ig deficient chickens | | | |
|---|---|---|---|---|---|
| | | Enriched in WT chicks | Enriched in mutant chicks | Neutral to Ig expression | Not detected |
| ASVs sorting | High Ig binding | 10 | 1 | 27 | 21 |
| | Low Ig binding | 9 | 2 | 20 | 17 |
| | Neutral Ig binding | 88 | 39 | 199 | 139 |
| | Not detected | 13 | 10 | 31 | 0 |

**Table 1**. Presence or absence of ASVs in sorting and Ig-deficient chicken experiments.

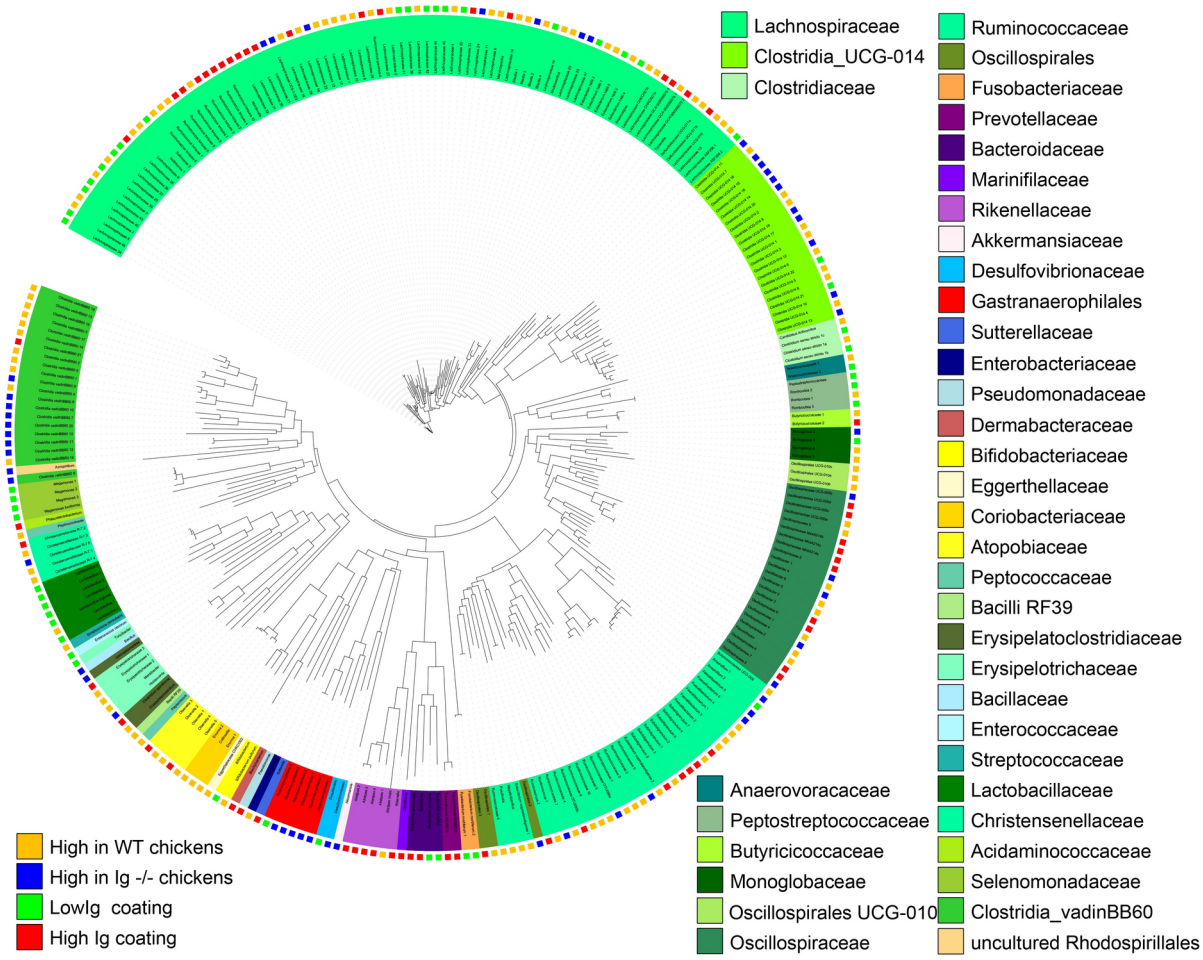


**Fig. 6**. ASVs with significantly higher or lower immunoglobulin coating, and ASVs differently abundant in microbiota of wild-type and Ig-deficient chickens. Red squares, ASVs with high immunoglobulin binding in sorting experiment; green squares, ASVs with low immunoglobulin binding in sorting experiment. Orange squares, ASVs of high abundance in microbiota of wild-type chickens; blue squares, ASVs of high abundance in microbiota of Ig-deficient chickens.

within Lachnospiraceae, Ruminococcaceae and Oscillospiraceae characterised by high immunoglobulin binding belonged to the same clades as those requiring immunoglobulin production for their colonisation. Erysipelotrichaceae, Rikenellaceae and genus *Alistipes*, family Atopobiaceae (phylum Actinobacteria) and genera *Olsenella*, *Collinsella* and *Enorma* belonged also among bacteria with high immunoglobulin coating and requiring Ig expression for their maximal colonisation (Fig. 6). Gastranaerophilales did not bind immunoglobulins and immunoglobulin production interfered with their colonisation. Two clades of unclassified Lachnospiraceae, *Eubacterium hallii*, *Romboutsia*, *Clostridium paraputrificum*, *Clostridium* sensu stricto, Peptostreptococcaceae, Peptococcaceae and lactobacilli exhibited low immunoglobulin binding but required immunoglobulin production for their colonisation (Fig. 6).

## Discussion

In this study, we tested interactions between chicken immunoglobulins and caecal microbiota in two different experiments. First, we determined which chicken caecal microbiota was coated by immunoglobulins and next, using Ig-deficient chickens, we identified taxa, colonisation of which was dependent on immunoglobulin expression. Unfortunately, an attempt in the introduction of adult type microbiota in the experiment with Ig-deficient chickens with spreading faecal material from adult hens failed and direct comparison of these two experiments was therefore restricted mainly to globally distributed spore forming Firmicutes[35,36].

Around 35% of caecal bacteria were coated with immunoglobulins which is approximately two times more than in mice in which between 7 to 20% of bacterial cells are coated[15,37]. High representation of immunoglobulin coated bacteria can be a consequence of discontinuous filling and emptying of the chicken caecum providing time for immunoglobulin binding to bacterial surface structures. The fact that immunoglobulin production is important for shaping chicken caecal microbiota is documented also by the fact that 120 ASVs were more abundant in wild-type chickens while only 52 were more abundant in Ig-deficient chickens. Together with higher microbiota diversity in wild type chickens this indicates that immunoglobulin production in the chicken caecum is more important for fixing desired microbiota rather than for removal of undesired microbiota members (which can overgrow in the absence of immunoglobulin secretion). Out of the most frequent families, only Lachnospiraceae had a similar number of ASVs with high and low immunoglobulin coating. Nearly all gut anaerobes from Lachnospiraceae encode genes for spore formation[6]which results in their wide distribution in different hosts[35,36]. Representatives of Lachnospiraceae also belong among the first colonisers of caeca of newly hatched chickens, likely for the same reason, *i.e.*ubiquitous distribution in the environment in the form of spores[38,39]. The Lachnospiraceae ASVs with low immunoglobulin coating may represent ASVs just passing through the chicken intestinal tract, ASVs of environmental origin, from hosts different from chickens, and therefore expressing surface antigens different from those of chicken adapted taxa.

Despite changes in caecal microbiota, only a mild compensatory response in chicken gene expression to the absence of immunoglobulins was observed. This consisted of activation of the mucosal innate immune system by induction of IL17, IL22 or TLR4 but the absence of extensive inflammation which would be accompanied by induction of IL1β, MMP7, AVD or ES1[16,23]. This is similar to the moderate difference of gut functions observed in humans with immunodeficiency in IgA secretion[17,26]. Although we focused on prokaryotic component of total gut microbiota, the observed compensatory response could be causes also by interactions with viruses, fungi or any other non-prokaryotic member of caecal microbiota of chickens.

Immunoglobulin coating can be antigen specific[2]as well as non-specific[26,40,41]. Non-specific immunoglobulin binding is mediated by carbohydrate interactions between highly glycosylated immunoglobulins and their secretory component, and LPS or peptidoglycans from bacterial cells[40,42]. *Lactobacillus*, *Blautia*, *Faecalibacterium*, [*Eubacterium*] *hallii*, *Megamonas*, *Fusobacterium* and *Desulfovibrio* represented taxa with reduced immunoglobulin coating. Interestingly, all of them harbour genes for S-layer proteins in their genomes[6,43] and we reported on in vivo expression of S-layer protein in *Megamonas* earlier[9]. Expression of the inert protein layer at the cell surface may therefore decrease immunoglobulin binding dependent on non-specific carbohydrate interactions, despite the fact the genes for S-layer protein can be found also in *Alistipes*[6], which exhibited an increase in immunoglobulin coating.

Though some of the ASVs were not coated extensively by immunoglobulins, these required immunoglobulin production for their colonisation. This can be explained by cross dependence on other microbiota members. Alternatively, some low immunoglobulin coated bacteria requiring immunoglobulin expression may bind and immediately degrade and utilise immunoglobulins, as shown for *Bacteroides thetaiotaomicron*[4]. This would explain their dependence but low presence of immunoglobulins on their surface.

Since common caecal microbiota members were coated by immunoglobulins, the coating likely contributed to stabilisation of caecal microbiota. Taxa which overgrew in immunoglobulin-deficient chickens belonged to those less frequently reported in chicken microbiota. Of these, *Akkermansia* is common to humans[44] but not in chickens[35] although *Akkermansia*can colonise newly hatched chickens[34] in which immunoglobulin expression is below detection until approximately 2 weeks of age[20–22]. Since *Akkermansia* is not common in adult hens, this collectively indicates that in this case, immunoglobulin production acts against the presence of *Akkermansia* in the chicken caecum.

Here we have shown that members of chicken caecal microbiota are commonly coated by immunoglobulins and require immunoglobulin production for their colonisation. This can be affected by discontinuous anaerobic fermentation in the chicken caecum providing time for interaction between immunoglobulins and microbiota. There were several taxa, mostly characteristic for the small intestine[30], with low immunoglobulin binding but still requiring immunoglobulin production for their colonisation.

## Material and methods

### Bacterial cell sorting

In total, samples from 16 chickens were subjected to the cell sorting experiment on 3 different occasions with chickens originating from 2 different flocks. Six 16-week-old reproductive flock Bovans Brown chickens were analysed first. Next, a commercial egg laying flock of ISA Brown egg layers was sampled twice. At the first visit, four 4-week-old chickens were analysed and an additional 6 chickens from the same flock were analysed when 16-week-old. The caecal contents (approx. 500 mg) was resuspended in 2% paraformaldehyde in PBS (0.4 M $Na_2HPO_4/NaH_2PO_4$, 150 mM NaCl, pH 7) and fixed at 4 °C overnight. Then, the samples were washed 5 times (centrifugation at 4,000 g for 10 min) in PBS with 0.1% Tween 80 and to discriminate the bacteria from debris, propidium iodide was added to 4 mg/L final concentration followed by vortexing for 10 s. An aliquot of this suspension was saved for determination of initial microbiota composition. In the next step, fluorescein

labelled mouse anti-chicken IgA antibodies (Southern Biotech) were added to the suspensions and incubated for 20 min at room temperature. The suspension was washed twice with PBS by centrifugation to remove unbound antibodies and immediately subjected to cell sorting. In total, 10 million bacterial cells defined as particles positive for propidium iodide fluorescence were cell sorted. Sorting was performed using BD FACS Aria Fusion operated by Diva software (BD Biosciences) under the following conditions: nozzle size 70 µm, sheath pressure 70 psi and four-way purity sort mask. IgA positive (high fluorescence) and IgA negative (low fluorescence) bacterial cells were sorted and collected into two 15 mL tubes with PBS. Gates for positive populations were set based on "fluorescence minus one" controls to eliminate background fluorescence. Bacteria were pelleted by centrifugation and DNA was purified from the pellets by QIAamp DNA Stool Mini Kit (Qiagen).

## Ig-deficient chickens

Ig-deficient chickens, originally named as JH-KO + /- and JH-KO-/-, have been described previously[31]. Maternal heterozygous males and females[31] were crossed and fertilised eggs were hatched. Altogether, 22 chicks were hatched, of these 11 were homozygous knockouts, 6 were heterozygous and 5 chicks were of wild-type genotype. Two days before chicken hatching, faecal material from adult hens was spread over the floor of animal rooms to which newly hatched chicks were placed. All the chicks were kept in the same aviary as a single flock throughout the whole experiment. The chicks were sacrificed at 4 weeks of age and caecal contents were collected and frozen at -20 °C until DNA purification. In addition, caecal tissue was collected and placed in RNALater for RNA purification and blood serum was collected to verify the presence or absence of IgY and IgA by an in-house ELISA as described previously[45].

## Sequencing of V3/V4 region of 16S rRNA genes

Following homogenization in a MagNALyzer (Roche), the DNA was extracted from caecal contents using the QIAamp DNA Stool Mini Kit (Qiagen). The DNA concentration was determined spectrophotometrically, DNA samples of were diluted to the same concentration of 5 ng/mL and were used as a template in PCR with forward primer 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-MID-GT-CCTACGGGNGGCWGCAG-3′ and reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-MID-GT GACTACHVGGGTATCTAATCC-3′.

The sequencing italics served as index and adapter ligation whereas the underlined sequences allowed for the amplification over V3/V4 region of 16S rRNA genes. MIDs represent different sequences of 5, 6, 9, or 12 base pairs in length which were used for post-sequencing differentiation of samples from different chickens. PCR amplification was performed using HotStarTaq Plus MasterMix kit (Qiagen) and the resulting PCR products were purified using AMPure beads. In the next step, the concentration of PCR products was determined spectrophotometrically, the DNA was diluted to 100 ng/µL and groups of 14 PCR products with different MID sequences were indexed with a Nextera XT Index Kit following the manufacturer's instructions (Illumina). Prior to sequencing, the concentration of differently indexed samples was determined using a KAPA Library Quantification Complete kit (Kapa Biosystems). All indexed samples were diluted to 4 ng/µL and 20% of phiX DNA was added. Sequencing was performed using MiSeq Reagent Kit v3 and MiSeq apparatus according to the manufacturer's instructions (Illumina).

## Sequencing data analysis

Microbiota analysis was performed with QIIME 2[46]. Raw sequence data were demultiplexed, quality filtered and sequencing primers were clipped using in-house scripts utilizing Je[47] and fastp[48]. Resulting sequences were denoised with DADA2[49]. Taxonomy was assigned to amplicon sequence variants (ASV) using the q2-feature-classifier[50] classify-sklearn naïve Bayes taxonomy classifier against the Silva 138 database[51] with 99.9% similarity threshold for individual ASVs.

*mRNA purification and real time reverse transcribed PCR (real time RT PCR)*

Samples of chicken caecal tissues (50–100 mg) were homogenised in TRI Reagent (MRC) and RNA was recovered from upper water phase as previously described[20]. mRNA was immediately reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primers. cDNA was diluted 10 times with sterile water prior to real-time PCR. PCR was performed in 3 µL volumes in 384-well microplates using QuantiTect SYBR Green PCR Master Mix (QIAGEN) and a NanoDrop pipetting station (Inovadyne) for PCR mix dispensing as described previously[16]. The Ct values of the genes of interest were normalised (ΔCt) to a geomean Ct value of 3 reference genes, TBP1, HMBS and ADA, and the relative expression of each gene of interest was calculated as $2^{\Delta Ct}$. All the primers are listed in Supplementary File S2.

## Statistics

Qiime calculation was performed separately for each experiment (cell sorting of immunoglobulin coated bacteria and microbiota in wild-type and Ig-deficient chickens) as well as for both experiments in a single calculation. PCoA visualisation using Bray Curtiss distance matrix was produced from separated calculations and all other analyses used an ASV table produced by calculation with data from both experiments. This allowed for exact identification of the ASVs with high or low immunoglobulin binding and dependent or independent on immunoglobulin production. Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) was used for sequence alignment and produced phylogenetic trees were edited in iTOL v6 (https://itol.embl.de/)[52].

Differences in microbiota composition at phylum, family and ASV levels were identified by Wilcoxon matched-pairs signed rank test with Pratt's modification for sorted microbial populations comparing the abundance of each taxon in the high and low immunoglobulin coated population. Microbiota abundance in wild-type and immunoglobulin-deficient chickens was compared by Mann–Whitney test. Gene expression in

wild-type and immunoglobulin-deficient chickens was compared by t test. In all tests, comparisons with $p < 0.05$ were considered as significantly different.

## Data availability

The datasets generated during the current study are available in the following repositories: The raw sequence reads have been deposited in the NCBI Short Read Archive under the BioProject ID PRJNA1111757.

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## Author contributions

BK and BS performed animal experiments with Ig -/- chickens, collected samples and determined genotypes of individual chickens. IR and JV wrote the manuscript. HS and JV performed cell sorting experiment. MC and DK were responsible for 16S rRNA sequencing. VB was responsible for statistical analysis. All authors read and approved the final manuscript.

## Declaration

## Competing interests

The authors declare no competing interests.

## Additional information

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**Correspondence** and requests for materials should be addressed to I.R.