Animal Microbiome

https://doi.org/10.1186/s42523-026-00536-x

Article in Press

Composition of microbiota from chicken caecal mucosa considerably differs from microbiota in the caecal lumen

Received: 9 July 2025

Accepted: 16 February 2026

Published online: 25 February 2026

Cite this article as: Volf J., Karasova D., Crhanova M. *et al.* Composition of microbiota from chicken caecal mucosa considerably differs from microbiota in the caecal lumen. *anim microbiome* (2026). https://doi.org/10.1186/s42523-026-00536-x

Jiri Volf, Daniela Karasova, Magdalena Crhanova, Marcela Faldynova, Hana Prikrylova, Michal Zeman, Vladimir Babak, Jana Rajova, Pavel Kulich & Ivan Rychlik

Composition of microbiota from chicken caecal mucosa considerably differs from microbiota in the caecal lumen

Jiri Volf, Daniela Karasova, Magdalena Crhanova, Marcela Faldynova, Hana Prikrylova, Michal Zeman, Vladimir Babak, Jana Rajova, Pavel Kulich, Ivan Rychlik*

Veterinary Research Institute, Brno, Czech Republic

JV: volf@vri.cz, DK: karasova@vri.cz, MC: crhanova@vri.cz, MF: faldynova@vri.cz, HP: prikrylova@vri.cz, MZ: zeman@vri.cz, VB: babak@vri.cz, JR: rajova@vri.cz, PK: kulich@vri.cz, IR: rychlik@vri.cz

* corresponding author

Veterinary Research Institute

Hudcova 70

621 00 Brno

Czech Republic

email: rychlik@vri.cz

tel. +420 533331201

Abstract

Background

Despite the importance of microbiota at mucosal surfaces of the gut and their different composition in comparison to those colonising the gut lumen, only a few papers specifically addressed this topic. In this study, we therefore defined mucosa-associated and lumen-associated microbiota from the chicken caecum.

Results

To identify mucosa-adapted microbiota members, caeca from adult hens instead of chicks should be analysed. Mucosa-adapted microbiota included *Treponema*-like species, *Brachyspira innocens*, *Mucispirillum* sp., *Helicobacter pullorum*, *Desulfovibrio* sp. and *Anaerobiospirillum* sp. On the other hand, representatives of Bacteroidota were enriched in the caecal lumen. Reduced colonization of the mucosal surface in one-week-old chicks was a consequence of the lack of appropriate microbiota source in chicks obtained from hatcheries since when the chicks were raised in the presence of adult hens, they were efficiently colonised by mucosa-adapted microbiota within the first week of life. The identified mucosa-associated bacteria were of helical shape, chemotactic and motile, and expressed type VI secretion system *in vivo*. *Brachyspira innocens* and *Anaerobiospirillum* fermented carbohydrates while *Mucispirillum* sp. and *Helicobacter pullorum* preferred amino-acid fermentation.

Conclusions

Here, we defined mucosal microbiota of chicken caecum, microbiota which are in closer contact with chicken host than the luminal microbiota and may therefore considerably affect the behaviour of chickens.

Keywords

chicken; microbiota; caecum; mucus; metabolism; mass spectrometry; type VI secretion system

Background

Microbial communities inhabiting the intestinal tract of warm-blooded animals have a profound effect on gut health and the overall performance of their hosts [1,2]. Gut microbiota of warm-blooded omnivorous species consists of representatives of two major phyla; Bacteriodota (previously Bacteroidetes) and Bacillota (previously Firmicutes), two minor phyla; Pseudomonadota (previously Proteobacteria) and Actinomycetota (previously Actinobacteria), and additional rare phyla present only in some host species or individuals. However, the designation of some phyla as rare in the gut microbiota might be misleading as this can be affected by their specific function or location within intestinal tract. Some taxa might be present either only at a particular time during the host's life or in a particular part of the intestinal tract. Concerning the former, age-dependent development of gut microbiota is most pronounced in mammals as considerably different microbiota colonise individuals before and after weaning, *e.g.* genus *Bacteroides*, though common in nursed piglets, is underrepresented in gut microbiota of adult pigs [3]. Age-dependent development of gut microbiota has been described also in chickens but in this case it is a consequence of the fact that most of the studies start with chicks from hatcheries free from any contact with adult chickens [4]. Concerning spatial distribution, gut microbiota composition extensively differs in the small and large intestine [5]. Besides well-established changes in gut microbiota along the intestinal tract, there might be differences in gut microbiota composition also along the

transverse axis of the gut, *i.e.* microbiota in the gut lumen may differ from microbiota at mucosal surfaces.

There are papers stressing the importance of microbiota at mucosal surfaces of the gut and their different composition in comparison to those colonising the gut lumen [6]. Luminal microbiota can interact with their host mostly by soluble by-products of their metabolism since a two-staged mucus layer at the surface of epithelial cells almost excludes any direct interaction [7]. On the other hand, mucosa-adapted microbiota may come into much closer contact with epithelial cells including direct physical contact. Despite this, only a few original papers specifically addressed this topic. Mucosa-associated microbial communities have been characterised in mouse, rat, pig, macaque and humans [8,9] but little is known about those of the chicken although there might be differences due to different physiology of digestion. The majority of digesta in chickens passes from the small intestine directly to the colon. Besides this, a small amount of digesta is diverted from ileum to the caecum usually twice a day, the caecum is then closed and digesta is fermented for 8-12 hours before being ejected from the caecum into the colon. The discontinuous nature of digestion in the chicken caecum thus creates a specific ecological niche with the potential for mucosal microbiota development.

There are reports on chicken mucosal microbiota but these were performed in chickens within the first few weeks of life [10,11]. Since

these chickens were obtained from hatcheries, *i.e.* with interrupted transfer of microbiota from parents to offspring [12], the recorded results need not characterise real chicken-adapted mucosal microbiota characteristic for adult birds. In this study, we therefore focused on the characterisation of mucosa-associated microbiota in caeca from adult hens. The tested hypotheses included whether; i) there are any bacterial species adapted to such an ecological niche; ii) these bacteria may colonise all age categories of chickens and iii) whether colonisation of the mucosa requires any specific adaptations. We concluded that there are several bacterial species highly specialised for mucosa colonisation, and helical shape, motility and expression of type VI secretion system are common characteristics that facilitated their persistence in such an environment.

Methods

Chicken samples

Sixteen one-week-old male ISA Brown chicks and 19 completely different adult egg-laying hens (30 to 50 weeks of age) from local commercial egg producing farms, reproductive flocks or even private small-scale producers were included in this study. Chickens were humanely euthanized by carbon dioxide inhalation followed by cervical dislocation and the whole caecum was collected during post mortem analysis. Luminal content was squeezed from the caecum. Approx. 1 cm of the caecum was then opened longitudinally and washed 3 times always with fresh 5 ml of sterile PBS in 10ml plastic tubes on rotatory shaker. After a

final washing step, the mucosal samples were scraped from the inner side of the caecum with plastic scalpel. Caecal contents and mucosal scrapings were stored at -20 °C prior to DNA purification.

Transfer of mucosal microbiota by contact

Two groups of newly hatched male ISA Brown chicks were used. Chicks in the experimental group were reared in a cage together with an adult hen for the first 24 hours of life of chicks and then the donor hen was removed from the cage. Control group of chicks was reared in a separate room without any contact with the hen. Since the microbiota composition of the contact hen could influence the output of the whole experiment, four independent experiments with 4 different donor hens were conducted with 9, 4, 10 and 22 contact chicks, and 3, 5, 10 and 22 chicks in control groups, respectively. Microbiota of chicks was characterised by 16S rRNA sequencing on day 8 of life in all experiments. Additional time points were included in experiment 3 (day 11) and 4 (day 11, 25 and 42). Since these experiments were part of other projects running in laboratory, only the caecal content samples of the donor hens and all chicks were analysed.

DNA purification and 16S rRNA sequencing

Each sample was homogenised using a MagnaLyser (Roche) and the homogenate was subjected to DNA purification using QIAmp DNA Stool kit (Qiagen). Purified DNA was stored at -20 °C. The 16S rRNA sequencing was performed following the protocol described previously

[13]. Briefly, DNA samples were used as a template in polymerase chain reaction (PCR) with forward primer 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-MID-GTC CTA CGG GNG GCW GCA G-3′ and reverse primer 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-MID-GTG ACT ACH VGG GTA TCT AAT CC-3′. PCR amplification was performed using a HotStarTaq Plus Master Mix kit (Qiagen) and the resulting PCR products were purified using AMPure beads (Beckman Coulter). In the next step, groups of PCR products with different MID sequences were indexed with the same indices using a Nextera XT Index Kit (Illumina). Sequencing was performed using a MiSeq Reagent Kit v3 and MiSeq apparatus (Illumina).

Microbiota analysis was performed with QIIME 2 [14]. Raw sequence data were demultiplexed, quality filtered, and sequencing primers were removed using Je [15] and fastp [16]. Resulting sequences were denoised with DADA2 [17]. Taxonomy was assigned to amplicon sequence variants (ASVs) using the q2-feature-classifier [18] and classify-sklearn naïve Bayes taxonomy classifier against the Silva 138 database [19]. ASVs were further clustered by CD-HIT at 98.6% sequence similarity to operational taxonomic units (OTUs) representing individual species [20]. Since the size of amplified part of 16S rRNA genes ranged from 400 to 427 bp depending on bacterial species, used 98.6% sequence similarity resulted in clustering of sequences differing in 5 or less nucleotides. This threshold was adopted to sum up ASVs originating either from closely related strains of the same species or from multiple copies of 16S rRNA

gene present in the genome of the same bacterium but differing in individual nucleotides. All the software tools were used with default settings.

Metaproteome analysis of caecal samples

Data from the samples collected previously [21] were used to determine protein expression of the mucosa-associated bacterial species. The original mass spectra were newly searched against protein databases of *H. pullorum* (accession number GCF_000155495.1), *B. innocens* (SAMN36685032), *Mucispirillum* sp. (SAMN36685125) and *Anaerobiospirillum* sp. (SAMN36685126).

Scanning Electron Microscopy

Bacterial cultures isolated during our continuous projects focused on culture of gut anaerobes were grown *in vitro* on blood agar in anaerobic cabinet [13, 22]. Bacterial mass collected from blood agar was resuspended and fixed in 3% glutaraldehyde in Millonig's phosphate-buffered solution, post-fixed in 2% osmium tetroxide in Millonig's phosphate-buffered solution, dehydrated in 50, 70, 90, and 100% acetone and dried in hexamethyldisilazane. Then the samples were placed on the carbon tabs attached to the aluminium holder and coated with platinum/palladium (Cressington sputter coater 208 HR). The samples were observed under a scanning electron microscope Hitachi SU 8010 (Hitachi High Technologies).

Statistical analysis

All OTUs classified as mitochondria or assigned to domains other than bacteria were removed. Since the aim of this study was to identify common mucosa colonisers and not all possible OTUs distributed among all the samples, only the OTUs present in at least 4 chicken or 4 adult hen samples and forming more than 1 % of total microbiota in at least one sample were selected for downstream analyses. Wilcoxon matched-pairs signed-ranks test was used to determine differently abundant OTUs in mucosal and luminal samples from the same chickens. Differences were considered as significant when $p<0.05$. The test was performed separately on chick and hen samples. Linear discriminant analysis Effect Size (LEfSe) was used to determine taxa which most likely explained the differences between mucosal and luminal microbiota [23]. Because of used protocols, mucosa or lumen associated microbiota members are defined like this only based on their differential abundance in caecal mucosa or caecal digesta. Experimental design of the whole study is summarised in Fig. 1.

Results

Microbiota composition and diversity

Altogether 1,680,962 reads were obtained by 16S rRNA sequencing of 16 luminal and 16 mucosal samples from the caecum of 1-week-old chicks and 19 luminal and 19 mucosal samples from the caecum of adult hens with an average of 24,014 ± 14,317 reads per sample. Obtained reads were classified to 1,613 OTUs but only 176 OTUs (43 in chicks and 142 in

adult hens, partially overlapping) formed more than 1 % of total microbiota in at least one of the chickens and were present at least in 4 chicks or 4 adult hens. These passed through selection thresholds and were used in subsequent statistical analyses. Such OTUs formed around 99 % of total microbiota in the samples of one-week-old chicks and around 80 % of total microbiota in the samples from adult hens (Fig. 2A and Supplementary Table 1). When considering β diversity using PCoA analysis and weighted Unifrac distances, luminal and mucosal samples of one-week-old chicks overlapped while luminal and mucosal samples from adult hens formed visually separated clusters (Fig. 2B).

Luminal and mucosal microbiota in the caecum of one-week-old chicks

In one-week-old chicks, Bacillota (previously Firmicutes) (57.4 ± 17.3 %) (mean ± SD) and Pseudomonadota (previously Proteobacteria) (40.1 ± 17.1 %) dominated in luminal microbiota at the phylum level. Individual chicks were colonised also with representatives of Bacteroidota (previously Bacteroidetes). The composition of mucosal microbiota in one-week-old chicks was similar to luminal microbiota with Bacillota forming 64.4 ± 17.0 % and Pseudomonadota forming 29.6 ± 16.4 % of total mucosal microbiota.

At the OTU level, three OTUs were significantly enriched in mucosal samples of one-week-old chicks. These included *B. plebeius*, *B. caecicola* and *Janthinobacterium* forming 0.52 ± 1.30 %, 0.77 ± 2.69% and 0.40 ± 0.73 % of total mucosal microbiota, respectively. On the other hand, *E.*

coli comprising 36.12 ± 14.37 % of total luminal microbiota and 26.72 ± 16.33 % of mucosal microbiota was the only OTU significantly more associated with luminal samples than with mucosal samples in one-week-old chicks (Supplementary Table 1).

Luminal and mucosal microbiota in the caecum of adult hens

In adult hens, microbiota in the caecal lumen consisted of Bacteriodota (51.4 ± 10.9 %), Bacillota (9.9 ± 3.7 %), Verrucomicrobiota (4.85 ± 6.5 %), Fusobacteriota (3.9 ± 6.1 %), Spirochaetota (3.1 ± 4.0 %), Desulfobacterota (2.1 ± 2.6 %) Pseudomonadota (1.4 ± 1.2 %) and Defferibacterota (1.1 ± 1.1 %). Synergistota, Campylobacterota, WPS-2 and Actinomycetota (previously Actinobacteria) each formed less than 1 % of total microbiota across all samples. On the other hand, microbiota from the caecal mucosa consisted of Bacteriodota (30.3 ± 11.5 %), Bacillota (12.4 ± 4.7 %), Spirochaetota (10.6 ± 13.2 %), Deferribacterota (10.3 ± 8.9 %), Desulfobacterota (5.5 ± 2.8 %), Fusobacteriota (4.3 ± 5.4 %), Campylobacterota (3.7 ± 3.2 %), Pseudomonadota (2.8 ± 2.9%) and Verrucomicrobiota (1.3 ± 1.6 %). Significant differences between luminal and mucosal microbiota at the phylum level were recorded for Deferribacterota, Spirochaetota, Desulfobacterota and Campylobacterota, all being more abundant in the mucosa than in the lumen, and Bacteriodota dominating in the caecal lumen (Fig. 3A).

Seventeen OTUs were significantly enriched in the mucosal layer of adult hens compared to in the caecal lumen. The top 10 OTUs according to

abundance included *Treponema* sp., three members of genus *Mucispirillum*, *Helicobacter pullorum*, two members of genus *Desulfovibrio*, unclassified Lachnospiraceae, *Brachyspira innocens* (partial 16S rRNA sequence showed 100 % identity to *Brachyspira innocens* and *Brachyspira murdochii* but this OTU will be called as *B. innocens* in the rest of this study) and *Anaerobiospirillum* sp. The highest enrichments in the mucosa compared to lumen abundance were observed for *Mucispirillum*, *Desulfovibrio*, *Brachyspira* and *Helicobacter*. Lower levels of enrichment were recorded for *Treponema* and *Anaerobiospirillum* (Tab. 1, Fig 3B). All these OTUs were highly prevalent in adult hens being present in 79 to 100 % of samples (Tab. 1) and altogether mucosa-specific bacterial species formed between 10 and 60 % of total mucosal microbiota (Fig. 3C).

There were also 56 OTUs significantly enriched in luminal samples compared to the mucosal ones. This group consisted almost exclusively of members of phylum Bacteriodota (53 OTUs) and the remaining three OTUs belonged to Bacillota.

INSERT TABLE 1 HERE.

Identification of mucosal and luminal microbiota by LEfSe analysis

Independently performed LEfSe analysis confirmed conclusions based on Wilcoxon matched-pairs signed-ranks test, *i.e.* that representatives of phyla Deferribacterota, Spirochaetota, Desulfobacterota and

Campylobacterota were associated with the mucosa while representatives of Bacteriodota belonged among lumen colonisers (Fig. 4).

Transfer of mucosa-associated microbiota by contact with adult hens

Since mucosa-associated microbiota was absent or lowly abundant in one-week-old chicks, in the next experiment we tested whether mucosa-adapted bacteria can colonise newly hatched chicks. To address this hypothesis, newly hatched chicks were placed in contact with adult hens. *Mucispirillum*, *Desulfovibrio* and *Helicobacter* were efficiently transferred since contact with adult hens resulted in transfer in 25, 38 and 41 chicks out of 45 chicks used in all 4 experiments, respectively (Fig. 5A). In contrast, *Anaerobiospirillum* was transmitted to 5 chicks out of 23 chicks in three experiments in which the donor hens were positive for *Anaerobiospirillum* and *Treponema* was transmitted to 3 chicks out of 35 chicks in three experiments in which the donor hens were positive for *Treponema*. Only in a single experiment the donor hen was colonised by *Brachyspira* and not a single contact chick became *Brachyspira* positive. All control chickens in all the experiments kept without any contact with donor hens remained free of *Mucispirillum*, *Helicobacter*, *Brachyspira* or *Treponema*. *Desulfovibrio* and *Anaerobiospirillum* were recorded in 3 and 1 chicks out of 40 controls, respectively (Fig. 5B, Supplementary Table 2).

Characteristics of mucosal microbiota

Given certain bacteria are adapted to a specific ecological niche, finally we determined the characteristics of selected mucosal microbiota. First,

morphology of *Brachyspira innocens*, *Mucispirillum* sp. and *Helicobacter pullorum* was determined by scanning electron microscopy. All three strains exhibited a helical cell shape when grown *in vitro* (Fig. 6).

Next, we determined *in vivo* protein expression of mucosal bacterial species by reanalysis of earlier collected protein mass spectra against databases consisting of all predicted *H. pullorum*, *B. innocens*, *Mucispirillum* and *Anaerobiospirillum* proteins. Because of retrospective analysis, the protein expression of mucosa-associated bacteria was determined in less appropriate samples of caecal digesta. When we attempted to determine their expression directly in mucosal scrapings, minimal number of non-chicken proteins was detected. Altogether 164, 136, 51 and 229 proteins from *H. pullorum*, *Mucispirillum*, *Brachyspira* and *Anaerobiospirillum* were detected as expressed *in vivo*, respectively (Supplementary Table 3). Classification of expressed proteins into COG categories differentiated these species into carbohydrate dependent and independent. *Brachyspira innocens* and *Anaerobiospirillum* fermented carbohydrates since approximately 15 % of detected proteins were assigned to the category of carbohydrate metabolism and transport. On the other hand, *Helicobacter* and *Mucispirillum* exhibited carbohydrate independent metabolism since only 2.2 and 1.2 % of all expressed proteins were assigned to carbohydrate metabolism (Fig. 6). All mucosa colonisers also expressed flagellar and chemotactic proteins and *Helicobacter*, *Mucispirillum* and *Anaerobiospirillum* expressed proteins of type VI secretion system (T6SS).

Additional expressed proteins were specific for individual species. *Mucispirillum* expressed dimethyl sulfoxide reductase, which indicates that it used dimethyl sulfoxide as an electron acceptor in anaerobic respiration. Sulphur metabolism in *Mucispirillum* was associated with the expression of sulphide dehydrogenase consuming free H_2S, and rhodanase inactivating toxic cyanides by their conversion to isothiocyanate. Another part of *Mucispirillum* metabolism included amino acid fermentation. Amino acids were transported to the cytoplasm by L-cystine-binding ABC transporter protein, branched-chain amino acid ABC transporter and amino acid ABC transporter. Propionyl-CoA resulting from fermentation of branched amino acids was converted to succinyl-CoA and succinate by the activity of methylmalonyl-CoA mutase. *Mucispirillum* could capture free hydrogen due to the expression of two different subunits of [Ni-Fe] hydrogenase.

Helicobacter pullorum produced ATP by ATP synthases, subunits of which belonged among the highly expressed proteins. *H. pullorum* could produce ammonia *in vivo* since ammonia could be released in 4 enzymatic reactions catalysed by highly expressed asparaginase, aspartate ammonia lyase, glutamate dehydrogenase and cytochrome c nitrite reductase. *H. pullorum* also likely captured free hydrogen by expressed [Ni-Fe] hydrogenase. Fumarate was used as an electron acceptor due to the expression of fumarate reductase.

The metabolism of *Anaerobiospirillum* was dominated by metabolism of carbohydrates since 45 proteins out 229 expressed participated in mono- and oligo- saccharide transport, glycolysis, pentose-phosphate pathway, gluconeogenesis and glycogen synthesis. *Anaerobiospirillum* was highly dependent on anaerobic respiration of fumarate since fumarate reductase flavoprotein subunit FrdA and fumarate reductase iron-sulfur subunit FrdB were expressed as the 2nd and 16th most abundant proteins.

Brachyspira innocens expressed glycolytic enzymes such as phosphoglucomutase (32nd most abundant protein), glucose-6-P isomerase (14th), fructose-bisphosphate aldolase (4th) and transaldolase (17th) from the pentose cycle. Saccharide degradation was further supported by the expression 4 monosaccharide and oligosaccharide transporters. However, the expression of glycolytic enzymes downstream from fructose-bisphosphate aldolase was not recorded. *B. innocens* also metabolised fatty acids or branched amino acids since EtfA and EtfB subunits of electron bifurcating butyryl-CoA dehydrogenase, and AcaD and FadE acyl-CoA dehydrogenase were highly expressed. *B. innocens* also expressed two subunits of glycine reductase converting glycine to acetyl phosphate and ammonia.

Discussion

In this study, we defined *Helicobacter pullorum*, *Mucispirillum* sp., *Brachyspira innocens Anaerobiospirillum* sp., *Desulfovibrio* sp. and *Treponema* sp. as bacteria highly adapted to the mucosal surface of the

chicken caecum, despite the fact that their association with caecal mucosa was based only on their differential abundance in mucosa and digesta determined by DNA sequencing, i.e. we did not confirm their presence in caecal mucus by alternative protocols, e.g. light microscopy. Such bacteria have been reported as members of chicken gut microbiota. However, they were always classified as minority gut microbiota members. Such a conclusion was observationally correct because faecal or caecal material was analysed [24–26] and a small amount of mucus together with its microbiota must be continuously shed off to gut lumen. Interestingly, here we show that such conclusions were biologically incomplete since if mucosal scrapings were taken, minority species become important majority ones. Out of the common gut microbiota members. *i.e.* phyla Bacillota and Bacteroidota, most representatives of Bacillota were equally distributed in caecal lumen and mucosa. On the other hand, Bacteriodota were enriched in luminal samples, which indicates that members of Bacteriodota do not colonise mucosal surface in chicken caecum. A similar distribution of Bacteriodota was reported in humans and pigs [27,28].

Mucosal microbiota attracts attention for its likely intimate relationship with its host [8,9]. Despite this, mucosal microbiota has not been analysed extensively. Samples from healthy humans cannot be obtained and those collected from individuals subjected to surgery are biased by the health issue which was the reason for surgery. Despite this, *Helicobacter* but not *Mucispirillum* or Spirochaetae, was reported as

associated with mucosal surfaces in the human gut [29]. *Mucispirillum* was described as a mucosa-associated bacterial species in mice [29,30] and *Helicobacter* species were isolated from mucosal scrapings or colon tissue in mice [31,32] but without any mention of Spirochaeta. Data on mucosal microbiota in pigs are more confounding since neither *Helicobacter* nor *Mucispirillum* were detected at their mucosal surfaces and *Treponema* dominated in the gut lumen [27,33]. There are several reports on mucosal microbiota in chickens but these were performed in broilers with underdeveloped microbiota [8,11,34,35] and as we show in this study, testing for mucosal microbiota in chicks 1-4 weeks of age may provide confounding data. *Helicobacter* was recorded in caecal mucosa of adult hens but without comparison with its presence in caecal lumen [36].

Absence of mucosal microbiota in chickens of broiler age is not a consequence of chick immaturity. Chicks can be colonised by mucosal microbiota within the first week of their life and *Mucispirillum*, *Helicobacter*, *Desulfovibrio* and *Anaerobiospirillum* were transmitted to newly hatched chickens by contact with donor hens (Fig. 5). The fact that bacterial species from mucosal microbiota appear mostly in adult egg layers or chickens with access to the outdoors [37–39] indicates that there is a lack of sources of mucosal microbiota in hatcheries and farms in the early days of production and that these anaerobic species do not survive efficiently in the external aerobic environment. Their mode of colonisation, *i.e.* long term colonisation after a single dose administration, is therefore the same as we have shown for strict anaerobes like

Bacteroides, *Megamonas* or *Sutterella* colonising the caecal lumen [13,40] . This is in complete agreement with a report on the co-appearance of *Helicobacter* with *Bacteroides*, *Alistipes*, *Faecalibacterium*, *Sutterella*, *Megamonas* and *Barnesiella* in broilers [41], suggesting the likelihood of being colonised by *Bacteroides*, *Sutterella* or *Barnesiella* is similar to being colonised by *Helicobacter*, *Mucispirillum* or *Brachyspira*, since all are influenced by their inability to survive in the external environment [42,43].

Except for their helical cell shape facilitating swimming in the viscous mucosal environment [44], there were two other characteristics common for chicken mucosa-colonising microbiota revealed by proteomics from caecal samples, namely motility and expression of T6SS. Motility is not common among gut anaerobes and flagellar operons were recorded only in *Anaerotruncus* and some *Flavonifractor* isolates [45]. The minimal presence of motility-related proteins and flagella is likely influenced by the immunogenicity of flagellin and its recognition by TLR5 receptor [46], as well as the fact that active movement is negligible in comparison to the movements caused by peristaltic mixing in the caecal lumen. However, the structure of *Helicobacter* flagellin can evade TLR5 recognition [47] and Spirochaeta express flagella located in the periplasm, *i.e.* not exposed to the outside [48]. In addition, the mucosal layer is less influenced by peristalsis and all of this selects allow for the expression of flagella in mucosa-colonising species [49].

Helicobacter, *Mucispirillum* and *Anaerobiospirillum* expressed T6SS proteins. The T6SS is a contact-dependent structure that injects cytotoxic proteins directly to the target cells [50,51]. It was shown that the target of the machinery could be either other bacteria or the host cells [52]. T6SS is expressed also by *Bacteroides* that colonise the gut lumen [50] and the presence of these systems enables *Bacteroides* to outcompete similar species *in vitro* and *in vivo* [53,54]. Although the target of T6SS is not clearly identified, the high prevalence of this secretion system among mucosal microbiota could imply their role in mucosal colonisation [55]. Elimination of excessive microbiota from mucosal surfaces by T6SS from a few mucosa-adapted microbiota may be beneficial for the host in keeping mucosal surfaces free of extensive microbial colonisation.

Brachyspira and *Anaerobiospirillum* utilised saccharides as their main source of carbon and energy. It is quite unlikely that saccharides originating from feed may reach the caecal mucosa, so these carbohydrates likely originated from glycosylated host proteins. *Helicobacter* and *Mucispirillum* exhibited metabolism independent of saccharide fermentation, which may enable a non-competitive co-existence with carbohydrate fermenters in the mucosa. Both *Mucispirillum* and *Helicobacter* expressed Ni-Fe hydrogenase and therefore consumed free hydrogen [56]. Since *Desulfovibrio* also uses hydrogen for reduction of sulphates [25], mucosal microbiota may protect the chicken epithelial cells from excess hydrogen. One of the end products of *Desulfovibrio* metabolism is H_2S, which can be both

stimulatory and toxic to eukaryotic cells [57,58]. Metabolism of H_2S is also one of the most characteristic features of the chicken caecum [59]. Maintaining a balance in mucosal H_2S concentration is important for the host, and *Mucispirillum* expressing sulphide dehydrogenase oxidising sulphide may counteract H_2S production by *Desulfovibrio*, thus maintaining an equilibrium tolerated by the host. The potentially protective function of mucosal microbiota can be seen also in the expression of rhodanase by *Mucispirillum*, which converts highly toxic cyanide into non-toxic thiocyanates.

Conclusions

In this study, we have shown that there is a limited number of bacterial species adapted for the colonisation of mucosal surfaces in the chicken caecum. These bacteria are usually absent from microbiota of one-week-old chicks, but this is caused only by their inability to survive in aerobic conditions. When the chicks are raised in contact with adult hens, the chicks can be colonised by mucosal microbiota within the first week of their life. Efficient colonisation of the mucosa seems to be associated with a helical cell shape, motility and the ability to express type VI secretion system, which may help the host to keep mucosal surfaces free from extensive microbial colonisation.

Declarations

Ethics statement and consent to participate

The handling of animals in the study was performed in accordance with current Czech legislation (Animal Protection and Welfare Act no. 246/1992 Collection of the Government of the Czech Republic). The specific experiments were approved by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic on 15 January 2018 (permit number MZe1922).

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during the current study are available in the following repositories: Raw sequencing data have been deposited in GenBank under accession number PRJNA1190354.

Funding

This work has been supported by projects RO0523 of the Czech Ministry of Agriculture. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

IR and JV analysed data and wrote the manuscript. JR and MZ performed mass spectrometry. AS, MF and HP were responsible for chicken experiments and sample collection. DK and MC performed 16S rRNA

sequencing. PK prepared electron microscopy images. VB was responsible for statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

Authors wish to thank Peter Eggenhuizen for English language corrections.

Authors' information

Additional information on authors' research activities can be found at https://probio.vri.cz/en/main-page/.

References

1. Hou K, Wu Z-X, Chen X-Y, Wang J-Q, Zhang D, Xiao C, et al. Microbiota in health and diseases. Sig Transduct Target Ther. 2022;7:1–28.

2. Kraimi N, Dawkins M, Gebhardt-Henrich SG, Velge P, Rychlik I, Volf J, et al. Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. Physiol Behav. 2019;210:112658.

3. Karasova D, Crhanova M, Babak V, Jerabek M, Brzobohaty L, Matesova Z, et al. Development of piglet gut microbiota at the time of weaning influences development of postweaning diarrhea - A field study. Res Vet Sci. 2021;135:59–65.

4. Videnska P, Sedlar K, Lukac M, Faldynova M, Gerzova L, Cejkova D, et al. Succession and replacement of bacterial populations in the caecum of egg laying hens over their whole life. PLoS One. 2014;9:e115142.

5. Videnska P, Faldynova M, Juricova H, Babak V, Sisak F, Havlickova H, et al. Chicken faecal microbiota and disturbances induced by single or repeated therapy with tetracycline and streptomycin. BMC Vet Res. 2013;9:30.

6. Paone P, Cani PD. Mucus barrier, mucins and gut microbiota: the expected slimy partners? Gut. 2020;69:2232–43.

7. Johansson MEV, Holmén Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci USA. 2011;108 Suppl 1:4659-65.

8. Daniel N, Lécuyer E, Chassaing B. Host/microbiota interactions in health and diseases-Time for mucosal microbiology! Mucosal Immunol. 2021;14:1006–16.

9. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. Cell. 2018;174:1388-1405.e21.

10. Awad WA, Mann E, Dzieciol M, Hess C, Schmitz-Esser S, Wagner M, et al. Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with *Campylobacter jejuni* infection. Front Cell Infect Microbiol. 2016;6:154.

11. Campos PM, Miska KB, Kahl S, Jenkins MC, Shao J, Proszkowiec-Weglarz M. Effects of *Eimeria tenella* on cecal luminal and mucosal microbiota in broiler chickens. Avian Dis. 2022;66:39–52.

12. Rychlik I. Composition and function of chicken gut microbiota. Animals (Basel). 2020;10:103.

13. Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D, Sebkova A, et al. Gut anaerobes capable of chicken caecum colonisation. Microorganisms. 2019;7:597.

14. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–7.

15. Girardot C, Scholtalbers J, Sauer S, Su S-Y, Furlong EEM. Je, a versatile suite to handle multiplexed NGS libraries with unique molecular identifiers. BMC Bioinformatics. 2016;17:419.

16. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–90.

17. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581-3.

18. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2’s q2-feature-classifier plugin. Microbiome. 2018;6:90.

19. Kim M, Oh H-S, Park S-C, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol. 2014;64:346–51.

20. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590–6.

21. Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik I. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Appl Environ Microbiol. 2015;82:1569–76.

22. Crhanova M, Karasova D, Juricova H, Matiasovicova J, Jahodarova E, Kubasova T, et al. Systematic culturomics shows that half of chicken caecal microbiota members can be grown in vitro except for two lineages of Clostridiales and a single lineage of Bacteroidetes. Microorganisms. 2019;7:496.

23. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biology. 2011;12:R60.

24. Seidlerova Z, Kubasova T, Faldynova M, Crhanova M, Karasova D, Babak V, et al. Environmental impact on differential composition of gut microbiota in indoor chickens in commercial production and outdoor, backyard chickens. Microorganisms. 2020;8:767.

25. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. Extensive microbial and functional diversity within the chicken cecal microbiome. PLoS One. 2014;9:e91941.

26. Videnska P, Rahman MM, Faldynova M, Babak V, Matulova ME, Prukner-Radovcic E, et al. Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. PLoS One. 2014;9:e110076.

27. Burrough ER, Arruda BL, Plummer PJ. Comparison of the luminal and mucosa-associated microbiota in the colon of pigs with and without swine dysentery. Front Vet Sci. 2017;4:139.

28. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science. 2005;308:1635–8.

29. Galley JD, Yu Z, Kumar P, Dowd SE, Lyte M, Bailey MT. The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut Microbes. 2014;5:748–60.

30. Robertson BR, O'Rourke JL, Neilan BA, Vandamme P, On SLW, Fox JG, et al. *Mucispirillum schaedleri* gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodents. Int J Syst Evol Microbiol. 2005;55:1199–204.

31. Fox JG, Dewhirst FE, Tully JG, Paster BJ, Yan L, Taylor NS, et al. Helicobacter hepaticus sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J Clin Microbiol. 1994;32:1238–45.

32. Taylor NS, Xu S, Nambiar P, Dewhirst FE, Fox JG. Enterohepatic *Helicobacter* species are prevalent in mice from commercial and academic institutions in Asia, Europe, and North America. J Clin Microbiol. 2007;45:2166–72.

33. Adhikari B, Kim SW, Kwon YM. Characterization of microbiota associated with digesta and mucosa in different regions of gastrointestinal tract of nursery pigs. Int J Mol Sci. 2019;20:1630.

34. Hankel J, Kittler S, Chuppava B, Galvez E, Strowig T, Becker A, et al. Luminal and mucosa-associated caecal microbiota of chickens after experimental *Campylobacter jejuni* infection in the absence of *Campylobacter*-specific phages of group II and III. Microb Genom. 2022;8:mgen000874.

35. Richards P, Fothergill J, Bernardeau M, Wigley P. Development of the caecal microbiota in three broiler breeds. Front Vet Sci. 2019;6:201.

36. Abdelhamid MK, Quijada NM, Dzieciol M, Hatfaludi T, Bilic I, Selberherr E, et al. Co-infection of chicken layers with *Histomonas meleagridis* and avian pathogenic *Escherichia coli* is associated with dysbiosis, cecal colonization and translocation of the bacteria from the gut lumen. Front Microbiol. 2020;11:586437.

37. Zhou X, Jiang X, Yang C, Ma B, Lei C, Xu C, et al. Cecal microbiota of Tibetan chickens from five geographic regions were determined by 16S rRNA sequencing. Microbiologyopen. 2016;5:753–62.

38. Hess C, Zloch A, Bilic I, Hacksteiner K, Kuchling S, Hess M. High prevalence of *Brachyspira* spp. in layers kept in alternative husbandry systems associated with frequent species variations from end of rearing to slaughter. Avian Pathol. 2017;46:481–7.

39. Varriale L, Coretti L, Dipineto L, Green BD, Pace A, Lembo F, et al. An outdoor access period improves chicken cecal microbiota and potentially increases micronutrient biosynthesis. Front Vet Sci. 2022;9:904522.

40. Kubasova T, Kollarcikova M, Crhanova M, Karasova D, Cejkova D, Sebkova A, et al. Contact with adult hen affects development of caecal microbiota in newly hatched chicks. PLoS One. 2019;14:e0212446.

41. Kaakoush NO, Sodhi N, Chenu JW, Cox JM, Riordan SM, Mitchell HM. The interplay between *Campylobacter* and *Helicobacter* species and other gastrointestinal microbiota of commercial broiler chickens. Gut Pathogens. 2014;6:18.

42. Karasova D, Faldynova M, Matiasovicova J, Sebkova A, Crhanova M, Kubasova T, et al. Host species adaptation of obligate gut anaerobes is dependent on their environmental survival. Microorganisms. 2022;10:1085.

43. Kubasova T, Seidlerova Z, Rychlik I. Ecological adaptations of gut microbiota members and their consequences for use as a new generation of probiotics. Int J Mol Sci. 2021;22:5471.

44. Martínez LE, Hardcastle JM, Wang J, Pincus Z, Tsang J, Hoover TR, et al. *Helicobacter pylori* strains vary cell shape and flagellum number to maintain robust motility in viscous environments. Mol Microbiol. 2016;99:88–110.

45. Medvecky M, Cejkova D, Polansky O, Karasova D, Kubasova T, Cizek A, et al. Whole genome sequencing and function prediction of 133 gut anaerobes isolated from chicken caecum in pure cultures. BMC Genomics. 2018;19:561.

46. Cullender TC, Chassaing B, Janzon A, Kumar K, Muller CE, Werner JJ, et al. Innate and adaptive immunity interact to quench microbiome flagellar motility in the gut. Cell Host Microbe. 2013;14:571–81.

47. Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL, Peek RM. *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. J Infect Dis. 2004;189:1914–20.

48. Santecchia I, Ferrer MF, Vieira ML, Gómez RM, Werts C. Phagocyte escape of Leptospira: The role of TLRs and NLRs. Front Immunol. 2020;11:571816.

49. Lertsethtakarn P, Ottemann KM, Hendrixson DR. Motility and chemotaxis in *Campylobacter* and *Helicobacter*. Annu Rev Microbiol. 2011;65:389–410.

50. Coyne MJ, Roelofs KG, Comstock LE. Type VI secretion systems of human gut Bacteroidales segregate into three genetic architectures, two of which are contained on mobile genetic elements. BMC Genomics. 2016;17:58.

51. Hussain NAS, Kirchberger PC, Case RJ, Boucher YF. Modular molecular weaponry plays a key role in competition within an environmental *Vibrio cholerae* population. Front Microbiol. 2021;12:671092.

52. Jiang F, Waterfield NR, Yang J, Yang G, Jin Q. A *Pseudomonas aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic cells. Cell Host Microbe. 2014;15:600–10.

53. Chatzidaki-Livanis M, Geva-Zatorsky N, Comstock LE. *Bacteroides fragilis* type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. Proc Natl Acad Sci U S A. 2016;113:3627–32.

54. Verster AJ, Ross BD, Radey MC, Bao Y, Goodman AL, Mougous JD, et al. The landscape of type VI secretion across human gut microbiomes reveals its role in community composition. Cell Host Microbe. 2017;22:411-419.e4.

55. Serapio-Palacios A, Woodward SE, Vogt SL, Deng W, Creus-Cuadros A, Huus KE, et al. Type VI secretion systems of pathogenic and commensal bacteria mediate niche occupancy in the gut. Cell Rep. 2022;39:110731.

56. Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev. 2007;107:4206–72.

57. Blachier F, Davila A-M, Mimoun S, Benetti P-H, Atanasiu C, Andriamihaja M, et al. Luminal sulfide and large intestine mucosa: friend or foe? Amino Acids. 2010;39:335–47.

58. Bouillaud F, Blachier F. Mitochondria and sulfide: a very old story of poisoning, feeding, and signaling? Antioxid Redox Signal. 2011;15:379–91.

59. Volf J, Rajova J, Babak V, Seidlerova Z, Rychlik I. Detoxification, hydrogen sulphide metabolism and wound healing are the main functions that differentiate caecum protein expression from ileum of week-old chicken. Animals (Basel). 2021;11:3155.

Figure legends

Figure 1. Experimental design of the study. Mucosal and luminal microbiota in the caecum of one-week-old chicks and adult hens was analysed first. Next, ability of mucosa-adapted microbiota to colonise newly hatched chicks was tested in experiments with contact donor hen. Finally, protein expression, *i.e.* likely metabolism, of selected mucosa-adapted bacterial species was determined by protein mass spectrometry in caecal samples.

Figure 2. Microbiota diversity in mucosal and luminal contents of one-week-old chicks and adult hens. Panel A – Relative abundance at phylum level. Panel B – PCoA clustering using weighted Unifrac matrix distances. Microbiota composition of one-week-old chicks (green colour) differed from microbiota of adult hens (orange colour). The mucosal samples (open symbols) of adult hens clustered separately from luminal samples (closed symbols), in contrast to the samples of one-week-old chicks.

Figure 3. Microbiota in luminal and mucosal samples of one-week-old chicks and adult hens. Panel A - the ratio of mucosal and luminal abundances of the most abundant mucosa-associated phyla in individual hens. Panel B - the ratio of mucosal and luminal abundances of 10 the most abundant mucosa-associated microbiota OTUs in individual hens. The line represents the median value. Panel C - Abundance of all mucosa-adapted genera out of total mucosal microbiota.

Figure 4. LEfSe analysis of mucosal and luminal microbiota from the caecum of adult hens. Bacteroidota at different taxonomic levels were preferentially associated with the caecal lumen (LUM, red background). On the other hand, *Helicobacter*, *Mucispirillum*, *Desulfovibrio* and *Brachyspira* dominated in the caecal mucosa (MUC, green background).

Figure 5. Transfer of mucosa-associated microbiota from adult hens to newly hatched chicks by contact alone. Panel A, qualitative view. Pie charts indicate qualitative positivity of the donor hens for mucosal microbiota since in the absence of hen colonisation, the absence of the same bacterium in the contact chicks is of no relevant meaning. The number of tested chicks at each experiment and sampling time point is shown. Panel B, quantitative visualisation including colonisation of control chicks.

Figure 6. Scanning electron microscopy of bacterial species colonising chicken caecal mucosa. *Brachyspira innocens* ET903 (A), *Mucispirillum* sp. ET911 (B) and *Helicobacter pullorum* (C) were grown *in vitro* on blood agar and bacterial culture was subjected to electron microscopy. Bar = 2 µm. Panel D - COG classification of *in vivo* expressed proteins. Only categories exceeding 5 % in any bacteria are described.

Table

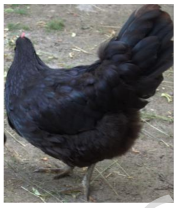
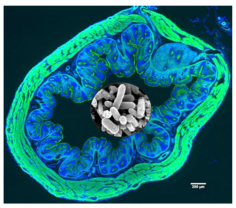
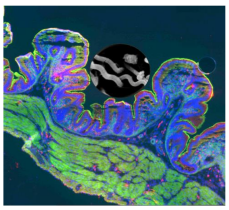
Table 1. Top 10 mucosa-associated microbiota OTUs identified in adult hens.

	Adult hen					One-week-old chicks	
Mucosa-associated OTU	**MEAN abundance (%)**	**MAX abundance (%)**	**Prevalence (%)**	**MUC/LUM ratio**	**LEfSe**	**Prevalence (%)**	**MAX abundance (%)**
Treponema OTU1	8.79	36.54	78.9	5.72		12.5	0.02
Mucispirillum OTU3	4.80	20.21	94.7	42.38	MUC *	0	0
Mucispirillum OTU8	3.58	12.04	100	4.06	MUC	0	0
Helicobacter OTU10	3.08	11.14	100	12.11	MUC	0	0
Desulfovibrio OTU30	2.27	5.53	100	17.31	MUC	0	0
Anaerobiospirillum OTU24	2.1	5.56	63.2	1.36		6.3	0.02
Brachyspira OTU28	1.86	5.61	78.9	16.68	MUC	0	0
Desulfovibrio OTU32	1.84	5.22	100	5.20	MUC	6.3	0.01
Mucispirillum OTU20	1.74	7.42	31.6	67.31		0	0
Lachnospiraceae OTU50	1.36	3.74	100	2.33		81.3	32.67

* MUC - OTU identified as significantly associated with mucosal samples by LEfSe

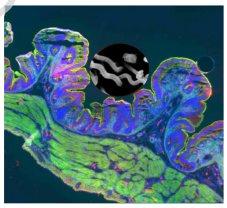
I. Identification of mucosal microbiota in the caecum of adult hens


X

II. Transfer of mucosal microbiota by contact with adult hens


X



III. Protein expression of selected mucosa specific microbiota members