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ROLA ARCHEONÓW METANOGENNYCH W NIESWOISTYCH CHOROBACH ZAPALNYCH JELIT U DZIECI

Rozprawa na stopień doktora nauk medycznych

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Spis treści

Streszczenie w języku polskim

ROLA ARCHEONÓW METANOGENNYCH W NIESWOISTYCH CHOROBACH ZAPALNYCH JELIT U DZIECI

Wstęp: Archeony metanogenne są istotnym składnikiem mikrobioty jelitowej, a zasiedlanie przez nie jelit człowieka rozpoczyna się we wczesnej ontogenezie. Zmiany w zakresie składu archeonów metanogennych i/lub ich liczebności mogą mieć związek z rozwojem różnych jednostek chorobowych, w tym nieswoistych chorób zapalnych jelit (IBD). Dysbioza bakteryjna jest znanym zjawiskiem powiązanym z rozwojem IBD u dzieci i dorosłych, niewiele jednak wiadomo o roli dysbiozy archeonów, szczególnie u dzieci. Znaczenie archeonów metanogennych w IBD zostało przedstawione w pracy poglądowej pt. ***The Role of Methanogenic Archaea in Inflammatory Bowel Disease—A Review*** *(Cisek i wsp., Journal of Personalized Medicine, 2024)*

Cele: Głównym celem rozprawy doktorskiej była ocena znaczenia archeonów metanogennych w IBD u dzieci. Cel ten został osiągnięty poprzez realizację celów szczegółowych, które zostały opisane w poszczególnych artykułach oryginalnych. Cele szczegółowe obejmowały: 1) opracowanie autorskiego protokołu detekcji archeonów metanogennych w próbkach kału, walidację protokołu z użyciem próbek kałomoczu kur; 2) ocenę w badaniach przedklinicznych wpływu czynników środowiskowych na populację metanogenów w jelitach kur; 3) analizę liczebności archeonów metanogennych w kale dzieci z IBD w porównaniu z dziećmi z grupy kontrolnej oraz ocena zależności pomiędzy liczebnością tych drobnoustrojów a typem i stopniem aktywności choroby oraz wiekiem dzieci.

Grupy badawcze, materiał i metody: W badaniach wzięło udział 124 pacjentów w wieku od 3 do 18 lat, w tym 45 dzieci z rozpoznaniem choroby Leśniowskiego-Crohna (CD; ang. *Crohn's disease*), 52 dzieci z rozpoznaniem wrzodziejącego zapalenia jelita grubego (UC; ang. *ulcerative colitis*) oraz 27 dzieci z grupy kontrolnej. Grupę tę stanowiły dzieci bez rozpoznania IBD. Aktywność procesu chorobowego została oceniona przy pomocy następujących indeksów: indeks PUCAI (ang. *Pediatric ulcerative colitis activity index*) oraz indeks PCDAI (ang. *Pediatric Crohn's disease activity index*) a także oceny stężenia kalprotektyny w kale (FCP; ang. *fecal calprotectin*). Stężenie FCP oznaczano w technologii chemiluminescencji. Do badań przedklinicznych włączono 174 kury hodowane w trzech systemach hodowlanych (wiejskim, fermowym i eksperymentalnym), zróżnicowane pod względem dostępu do

środowiska naturalnego, podaży antybiotyków i diety. Badano także wpływ wieku i rodzaju pobranej próbki (kałomocz *vs* treść jelitowa) na zawartość metanogenów. Z kału pacjentów, a także kałomoczu i treści jelit ślepych pobranych od kur wyizolowano DNA. Dodatkowo, jako kontrole użyto próbek DNA pochodzących od 3 gatunków archeonów metanogennych i jednego konstruktu plazmidowego oraz próbek DNA wyizolowanych z 21 szczepów bakteryjnych. Oznaczenia ilościowe populacji metanogenów w jelitach ludzi i zwierząt wykonano techniką real-time PCR, celując m.in. w swoisty dla archeonów metanogennych gen *mcrA* kodujący białko enzymu zaangażowanego w ostatni etap metanogenezy u tych mikroorganizmów.

Wyniki: Wyniki badań przedstawiono w 3 pracach oryginalnych. W pracy pt. ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek i wsp., Microorganisms, 2023)* opisano protokół detekcji archeonów metanogennych z wykorzystaniem genu *mcrA*. Protokół ten cechował się lepszymi parametrami walidacyjnymi od dotychczas powszechnie stosowanej metody. Charakteryzował się on bowiem zwiększoną swoistością i czułością, odtwarzalnością oraz szerszym zakresem detekcji liniowej (pozwalał on oznaczyć 7 zamiast maksymalnie 6 rzędów wielkości kopii genu *mcrA*). Autorski protokół pozwolił zatem na obniżenie koniecznej do uzyskania pozytywnego wyniku liczby komórek (genomów) metanogenów w badanym materiale nawet o jeden rząd wielkości (np. z 571 do 57 kopii genu *mcrA* gatunku *Methanomicrobium mobile* w mieszaninie reakcyjnej). Najniższa, oznaczalna ilościowo przy częstotliwości 100% liczba kopii genu *mcrA* wynosiła 21 kopii na reakcję. Autorski protokół obniżył ponadto ryzyko fałszywie dodatniego wyniku w próbkach niezawierających metanogenów. Protokół ten pozwolił bowiem zminimalizować negatywny wpływ dimeryzacji starterów oraz innych reakcji krzyżowych (których źródłem może być DNA bakteryjne izolowane z kału) na odczyt wyników real-time PCR. Ponadto, przy zastosowaniu nowego protokołu obecność metanogenów stwierdzona została we wszystkich 20 badanych próbkach kałomoczu, natomiast aż 7 wypadło ujemnie z użyciem protokołu innych autorów.

Przeprowadzone badania przedkliniczne opisane w artykule pt. ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek i wsp., International Journal of Molecular Sciences, 2023)* potwierdziły skuteczność opracowanej metodyki. Badania eksperymentalne pokazały, że w grupie kur eksperymentalnych, hodowanych w ściśle kontrolowanych, izolowanych od środowiska naturalnego warunkach, nie wykryto obecności

archeonów metanogennych, natomiast jelita ślepe kur wiejskich i fermowych były zasiedlone na porównywalnym poziomie, tj. 10^4 do 10^5 komórek/gram badanej próbki. Ponadto wykazano, że liczebność metanogenów jest różna w zależności od rodzaju próbki (kałomocz zawierał istotnie więcej metanogenów niż treść jelitowa; $p<0.001$), oraz od wieku zwierzęcia (w grupie 1-tygodniowych kur fermowych obserwowano istotnie mniej metanogenów niż u kur fermowych w wieku 3-4 oraz 5-6 tygodni; $p<0,01$). Wykazano zatem, że czynniki środowiskowe, wiek kury oraz rodzaj pobranej do badań próbki okazały się istotnymi czynnikami warunkującymi obecność archeonów metanogennych w badanym materiale.

W pracy pt. ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)* pokazano, że u dzieci chorujących na IBD zaobserwowano istotny statystycznie spadek w liczbie populacji całkowitej metanogenów, zarówno w kale dzieci chorujących na UC ($p<0,001$) jak i CD ($p<0,05$), w stosunku do dzieci z grupy kontrolnej. Ponadto, liczebność metanogenów była niższa w UC niż w CD ($p<0,05$). Częstość występowania tych mikroorganizmów także była znacząco niższa, szczególnie w grupie pacjentów z UC ($p<0,05$) i wynosiła 83% w porównaniu do grupy kontrolnej, w której częstość wstępowania ogólnej populacji metanogenów wynosiła 100%. W tej samej grupie chorych stwierdzono także dodatnią korelację pomiędzy liczebnością *Methanosphaera stadtmanae* a stężeniem kalprotektyny w kale ($Rs = 0,41$; $p<0.05$). Z kolei w grupie pacjentów z aktywną postacią CD zaobserwowano statystycznie istotny spadek liczebności zarówno całkowitej populacji metanogenów ($Rs = -0,56$; $p<0,01$) oraz *Methanobrevibacter smithii* ($Rs = -0,53$; $p<0,05$) wraz z wiekiem.

Podsumowanie wyników i wnioski: W pracy wykazano, że 1. liczebność i częstość występowania archeonów metanogennych w kale podlega zmianom w zależności od czynników środowiskowych i wieku gospodarza; 2. U dzieci z IBD występuje dysbioza w populacji archeonów metanogennych, która zależy od postaci IBD (CD vs UC) oraz aktywności procesu chorobowego; 3. Uzyskane wyniki oraz analiza literatury pokazują, że dysbioza w populacji archeonów metanogennych może być następstwem procesu zapalnego towarzyszącemu IBD, a nie czynnikiem wywołującym chorobę.

Streszczenie w języku angielskim

THE ROLE OF METHANOGENIC ARCHAEA IN THE INFLAMMATORY BOWEL DISEASE IN CHILDREN

Introduction: Methanogenic archaea are an important component of the intestinal microbiota, and their colonization of the human intestines begins in early ontogeny. Changes in the composition of methanogenic archaea and/or their numbers may be related to the development of various diseases, including inflammatory bowel disease (IBD). Bacterial dysbiosis is a known phenomenon associated with the development of IBD in children and adults, but little is known about the role of archaeal dysbiosis, particularly in children. The importance of methanogenic archaea in IBD was presented in a review article entitled: ***The Role of Methanogenic Archaea in Inflammatory Bowel Disease—A Review*** *(Cisek et al., Journal of Personalized Medicine, 2024).*

Objectives: The main aim of the doctoral dissertation was to assess the importance of methanogenic archaea in children with IBD. It was achieved by implementing detailed objectives that were described in individual original articles. Detailed objectives included: 1) development of an original protocol for the detection of methanogenic archaea in fecal samples, validation of the protocol using chicken droppings samples; 2) assessment of the impact of environmental factors on the population of methanogens in the chicken intestines in preclinical studies; 3) analysis of the number of methanogenic archaea in the feces of children with IBD compared to children from the control group, and assessment of the relationship between the number of these microorganisms and the type and degree of disease activity and the age of the children.

Research groups, materials and methods: The study involved 124 patients aged 3 to 18 years, including 45 children diagnosed with Crohn's disease (CD), 52 children diagnosed with ulcerative colitis (UC), and 27 children from the control group. This group consisted of children without a diagnosis of IBD. The activity of the disease was assessed using the following indices: the PUCAI index (Pediatric ulcerative colitis activity index) and the PCDAI index (Pediatric Crohn's disease activity index) and the assessment of fecal calprotectin (FCP) concentration. The FCP concentration was determined using chemiluminescence technology. Preclinical studies included 174 chickens bred in three breeding systems (free-range, farm and experimental), differing in terms of access to the natural environment, antibiotic

supplementation and diet. The influence of age and the type of sample (stool vs cecal content) on the abundance of methanogens was also examined. DNA was isolated from feces of patients, droppings and cecal contents collected from chickens. Additionally, DNA samples from 3 species of methanogenic archaea and one plasmid construct, as well as DNA samples isolated from 21 bacterial strains, were used as controls. Quantitative assessment of methanogen populations in the intestines of humans and animals was performed using the real-time PCR technique, targeting, among others, a methanogen-specific *mcrA* gene, i.e. the gene encoding an enzyme involved in the last stage of methanogenesis occurring in these microorganisms.

Results: The results were presented in 3 original articles. The work entitled ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek et al., Microorganisms, 2023)* described a development of protocol for the detection of methanogenic archaea with the use of *mcrA* gene. It resulted in better validation parameters than the previous, commonly used method. It was characterized by increased specificity and sensitivity, reproducibility and a wider linear detection range (as it allowed for determination of 7 instead of 6 orders of magnitude of the *mcrA* gene copies). Therefore, the author's protocol allowed to reduce the number of methanogen cells (genomes) in the tested material necessary to obtain a positive result by as much as one order of magnitude (e.g. from 571 down to 57 copies of the *mcrA* gene copies of the *Methanomicrobium mobile* species in reaction mixture). The lowest quantifiable copy number of the *mcrA* gene at a frequency of 100% was 21 copies per reaction. The new protocol also reduced the risk of false positive results in samples not containing methanogens. The developed protocol allowed to minimize the negative impact of primer dimerization and other cross-reactions (the source of which may be bacterial DNA isolated from stool) on the real-time PCR results. Moreover, when the new protocol was used, the presence of methanogens was detected in all 20 droppings samples tested, while as many as 7 samples tested negative when using the protocol of other authors.

The conducted preclinical studies described in the article entitled ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek et al., International Journal of Molecular Sciences, 2023)* confirmed the effectiveness of the developed methodology. The study showed that in a group of experimental chickens bred in strictly controlled conditions, isolated from the natural environment, the methanogenic archaea were not detected, while the cecum of the free-range and farm chickens was inhabited at a similar level, i.e. 10^4 to 10^5

cells/gram of the tested sample. Moreover, it was shown that the number of methanogens varies depending on the type of sample (droppings contained significantly more methanogens than intestinal content; $p<0.001$), and on the age of the animal (in the group of 1-week-old farm chickens, significantly fewer methanogens were observed than in older farm chickens at 3-4 and 5-6 weeks of life; $p<0.01$). It was therefore shown that environmental factors, the age of the chicken and the type of sample collected for testing were important factors determining the presence of methanogenic archaea in the tested material.

The work entitled ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)* demonstrated that in children suffering from IBD, a statistically significant decrease was observed in the number of the total population of methanogens, both in the stool of children suffering from UC ($p<0.001$) and CD ($p<0.05$), compared to children from the control group. Moreover, the abundance of methanogens was lower in UC than in CD ($p<0.05$). The prevalence of these microorganisms was also significantly lower, especially in the group of patients with UC ($p<0.05$) where it was 83% compared to the control group, in which the prevalence of the total population of methanogens was 100%. In the same group of patients, a positive correlation was also found between the number of *Methanosphaera stadtmanae* and the concentration of fecal calprotectin ($Rs = 0.41$; $p<0.05$). On the other hand, in the group of patients with active form of CD, a statistically significant decrease in the number of total methanogens ($Rs = -0.56$; $p<0.01$) and *Methanobrevibacter smithii* was observed with age ($Rs = -0.53$; $p<0.05$).

Summary of the results and conclusions: The study showed that 1. the abundance and prevalence of methanogenic archaea in feces change depending on environmental factors and the age of the host; 2. Archaeal dysbiosis occurs in children with IBD, and it depends on the form of IBD (CD vs UC) and the activity of the disease; 3. The results obtained and the literature review demonstrate that archaeal dysbiosis may be a consequence of the inflammatory process accompanying IBD, and not a disease causing factor.

Słowa kluczowe w języku polskim i angielskim

Słowa kluczowe: archeony metanogenne, choroba Leśniowskiego-Crohna, mikrobiota jelitowa, wrzodziejące zapalenie jelita grubego, nieswoiste choroby zapalne jelit

Keywords: Crohn's disease, inflammatory bowel disease, intestinal microbiota, methanogenic archaea, ulcerative colitis

Wykaz zastosowanych skrótów

CD – choroba Leśniowskiego-Crohna (ang. *Crohn's disease*)

FCP – kalprotektyna w kale (ang. *fecal calprotectin*)

IBD – nieswoista choroba zapalna jelit (ang. *inflammatory bowel disease*)

Mb. – Methanobrevibacter

mcrA – gen kodujący podjednostkę alfa reduktazy metylo-koenzymu M

Ms. – Methanosphaera

NCBI – baza przechowująca genetyczne sekwencje nukleotydowe oraz artykuły biomedyczne (ang. *National Center for Biotechnology Information*)

PCDAI – pediatryczny wskaźnik aktywności choroby Leśniowskiego-Crohna (ang. *Pediatric Crohn's disease activity index*)

PCR – reakcja łańcuchowa polimerazy (ang. *polymerase chain reaction*)

PUCAI – pediatryczny wskaźnik aktywności wrzodziejącego zapalenia jelita grubego (ang. *Paediatric ulcerative colitis activity index*)

pz – pary zasad

Rs – współczynnik korelacji rang Spearmana

T_{an} – temperatura przyłączania starterów (ang. *annealing temperature*)

UC – wrzodziejące zapalenie jelita grubego (ang. *ulcerative colitis*)

1. Wstęp

Nieswoista choroba zapalna jelit (IBD; ang. *inflammatory bowel disease*) charakteryzuje się nawracającym, chronicznym stanem zapalanym tkanek przewodu pokarmowego, a termin IBD odnosi się do dwóch jednostek chorobowych: choroby Leśniowskiego-Crohna (CD; ang. *Crohn's disease*) oraz wrzodziejącego zapalenia jelita grubego (UC; ang. *ulcerative colitis*). Szacuje się, że na IBD choruje nawet 0,3% ludzi żyjących w krajach rozwiniętych [1], przy czym jedna czwarta przypadków diagnozowana jest u dzieci poniżej 10. roku życia [2]. W Polsce wykrywalność roczna wynosi 2,8 przypadków na każde 100 tys. dzieci do 15. roku życia [2]. Ponieważ dziecięcy IBD różni się od tego, który obserwowany jest u dorosłych, zarówno w kwestii progresji, lokalizacji anatomicznej jak i wyników leczenia [3], niezwykle ważnym jest poznanie mechanizmów związanych z rozwojem IBD w obu grupach wiekowych. Ponadto, dzieci wydają się być lepszym modelem niż dorośli do badań nad patomechanizmami IBD ze względu na to, iż niezwykle rzadko obarczone są innymi chorobami, przez co możliwym wydaje się być poznanie pierwotnych przyczyn leżących u podstaw inicjacji i rozwoju IBD [4].

Etiopatogeneza IBD nie jest do końca poznana [5]. Uważa się, że mechanizmów powiązanych z wystąpieniem IBD u pacjentów jest kilka, m.in. wrodzona podatność uwarunkowana genetycznie, czynniki związane z układem immunologicznym, a także niekorzystne uwarunkowania środowiskowe oraz zaburzenia w składzie mikrobiomu jelitowego człowieka [6,7]. Znany jest wpływ pewnych grup mikroorganizmów bezpośrednio zaangażowanych w etiopatogenezę IBD, szczególnie obecność adherentno-inwazyjnych szczepów *Escherichia coli* (AIEC; ang. *adherent-invasive E. coli*), także wzrost liczebności szczepów *Candida* spp. i *Malassezia* spp. oraz niektórych wirusów [8–10]. Z kolei obniżenie liczebności bakterii produkujących maślan jest łączona z uruchomieniem kaskady zdarzeń, która w dużym uproszczeniu powoduje zależną od niedoborów maślanu deregulację aktywności mitochondrialnej komórek jelitowych, czego efektem jest wzrost przepuszczalności bariery jelitowej, także na skutek uszkodzeń powodowanych reaktywnymi formami tlenu. Zwiększona przepuszczalność bariery jelitowej umożliwia migrację mikroorganizmów ze światła jelita przez nabłonek jelitowy, co w konsekwencji potęguje procesy zapalne [4]. Sugeruje się więc, że dysbioza bakteryjna oraz zaburzona komunikacja pomiędzy komórkami gospodarza a bakteriami, stanowią istotny mechanizm inicjacji i progresji IBD [4,11]. Niewiele jednak wiadomo o roli innego składnika mikrobioty jelitowej człowieka jakim są archeony.

1.1 Archenony metanogenne – ważny składnik mikrobioty jelitowej

Archeony, chociaż podobne budową do bakterii, reprezentują odrębną gałąź filogenetyczną pośród organizmów prokariotycznych [12]. Pierwotnie, mikroorganizmy te znane były głównie z kolonizacji środowisk ekstremalnych, jednak w późnych latach 60' ubiegłego wieku odkryte zostały po raz pierwszy w jelitach ludzi [13–15]. Od tamtej pory, pomimo stale podejmowanych prób, dopiero ostatnie lata pozwoliły uzyskać lepszą świadomość odnośnie roli, jaką pełnić mogą archeony w zdrowiu i chorobie u człowieka. Wcześniejsze analizy nie były możliwe, gdyż dopiero od niedawna istnieją narzędzia umożliwiające skuteczną detekcję tych drobnoustrojów. Brak możliwości oceny archeonów był dotychczas związany z ich opornością na lizę lizozymem, tj. enzymem używanym w tradycyjnych protokołach do izolacji DNA [16]. Wprowadzenie lizy mechanicznej podczas tego procesu, a także ulepszenie technik opierających się na reakcji amplifikacji (PCR; ang. *polymerase chain reaction*) pozwala coraz lepiej poznać tę grupę mikrobiontów jelitowych. Opracowanie i walidacja metody wykrywania archeonów metanogennych za pomocą techniki real-time PCR stanowi znaczącą część rozprawy doktorskiej i została dokładnie opisana w artykule pt. ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek i wsp., Microorganisms, 2023).*

U zdrowych dorosłych archeony metanogenne stanowią nawet 10% wszystkich beztlenowców jelitowych [17]. Częstość ich występowania w populacji ludzkiej wzrasta wraz z wiekiem [18–20]. Z analizy wczesnych danych literaturowych wynika, że kolonizacja przewodu pokarmowego dzieci przez archeony metanogenne następuje w okresie niemowlęcym lub – najpóźniej – wczesnoszkolnym [19]. Ostatnio dostępne jednak są badania świadczące o obecności metanogenów nawet w ponad 90% próbek smółki czy soku żołądkowego jednodniowych noworodków [21,22]. Uważa się zatem, że do kolonizacji jelit dzieci przez metanogeny może dochodzić nawet na etapie życia płodowego [22]. Co ciekawe, w okolicach drugiego miesiąca życia u dzieci karmionych piersią zaobserwowano obniżenie, a nawet eliminację populacji metanogenów w jelitach, co jest prawdopodobnie wynikiem przejściowej redukcji różnorodności mikrobioty jelitowej będącej następstwem zastosowanej diety [23,24]. Po wprowadzeniu mieszanek i pokarmów stałych do diety niemowląt, wykrywalność metanogenów ponownie wzrasta, osiągając u zdrowych dzieci w wieku pomiędzy 6. miesiącem a 2. rokiem życia częstość występowania na poziomie 96% [19].

W przeciwieństwie do częstości występowania metanogenów, która wzrasta wraz z wiekiem, dane na temat różnorodności archeonów są trudne w interpretacji, gdyż zależą od zespołu badawczego i zastosowanych technik badawczych. Analiza dostępnej literatury pokazuje, że niezależnie od wieku, w przewodzie pokarmowym człowieka obecne są trzy taksony: *Methanobrevibacter (Mb.) smithii, Methanosphaera (Ms.) stadtmanae,* a także metanogeny z rzędu *Methanomassiliicoccales* [25]. Zaobserwowano, że u noworodków występuje niemal wyłącznie *Mb. smithii*, a u dzieci w wieku szkolnym gatunek ten obecny jest u 78% do 88% tych dzieci, *Ms. stadtmanae* u 8% do 11%, natomiast *Methanomassiliicoccus luminyensis* u 1% dzieci [19,26,27]. U dorosłych częstotliwość występowania *Mb. smithii* osiąga wartości w zakresie między 64% a 100%, *Ms. stadtmanae* – między 30% a 90%, natomiast *Methanomassiliicoccus spp.* – między 1% a 26% [28–32]. Ostatnia z grup archeonów osiąga wraz z wiekiem wyższe częstości występowania, zarówno u dzieci, jak i u dorosłych, natomiast dane dotyczące pozostałych taksonów stoją ze sobą niekiedy w sprzeczności [29,33]. Na podstawie przytoczonych przykładów, można stwierdzić, że dieta oraz wiek są istotnymi czynnikami wpływającymi na występowanie archeonów w jelitach ludzi. Badania przeprowadzone na zwierzętach, będące składową tej pracy doktorskiej, pozwoliły na ustalenie kolejnych takich czynników, m.in. dostępu do środowiska naturalnego, podaży antybiotyków oraz innych składowych obranego modelu hodowli. Wyniki tych badań przedklinicznych zostały opisane w artykule pt. ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek i wsp., International Journal of Molecular Sciences, 2023)*.

Korzystny wpływ archeonów metanogennych na zdrowie człowieka związany jest z wykorzystaniem wodoru w ich własnym metabolizmie, tj. w procesie metanogenezy [34]. Wodór, jako produkt uboczny bakteryjnej fermentacji jelitowej, zwrotnie hamuje ten proces, upośledzając wchłanianie elementów pokarmowych i odzysk energii z pożywienia. Powstający w wyniku działalności bakterii maślan, a także inne krótkołańcuchowe kwasy tłuszczowe oraz witaminy, to substancje niezbędne do prawidłowego funkcjonowania organizmu człowieka, a archeony metanogenne pośrednio umożliwiają ich produkcję [35,36]. Ponadto, niedawne badania sugerują możliwość wykorzystania *Methanomassiliicoccus luminyensis* jako mikroorganizmu probiotycznego w zwalczaniu trimetyloaminurii (zespół odoru rybiego) i miażdżycy [37]. Gatunek ten posiada bowiem naturalną zdolność do wykorzystywania i usuwania trimetyloaminy, będącej prekursorem tlenku trimetyloaminy, źródła nieprzyjemnego zapachu, oraz induktora produkcji blaszek miażdżycowych [37]. Udowodniono także

przeciwzapalną rolę metanogenów podczas badań nad celiakią [38]. Z drugiej strony, istnieją dowody wiążące występowanie archeonów metanogennych z wystąpieniem zapalenia przyzębia, pochwy, ropniami mózgu, stwardnieniem rozsianym, otyłością, zespołem jelita drażliwego, a także IBD u dorosłych [28,39,40].

Wpływ trzech najbardziej istotnych, opisanych powyżej taksonów archeonów metanogennych na zdrowie człowieka, jest różnorodny. Przykładowo, u dorosłych *Mb. smithii* wydaje się być gatunkiem komensalnym, choć w pewnych sytuacjach może na przykład powodować wyczerpanie zasobów maślanu w jelicie, przyczyniając się do zaistnienia dogodnych warunków do translokacji bakterii pierwotnie komensalnych przez barierę jelitową i nasilenia w ten sposób procesów zapalnych [41–43]; natomiast *Ms. stadtmanae* silnie indukuje odpowiedź immunologiczną zarówno u osób zdrowych, jak i u pacjentów z IBD [17,44,45]. Więcej informacji na temat potencjalnych mechanizmów wiążących wystąpienie IBD z archeonami metanogennymi zostało zamieszczonych w artykule pt. ***The Role of Methanogenic Archaea in Inflammatory Bowel Disease—A Review*** *(Cisek i wsp., Journal of Personalized Medicine, 2024)* wraz z ilustrującymi je schematami.

1.2 Znaczenie archenów metanogennych w patogenezie IBD

Już w latach 80’ ubiegłego wieku zaobserwowano, że dorośli chorujący na IBD rzadko uwalniają metan w wydychanym powietrzu [46,47]. Dopiero rozwój technik biologii molekularnej, ponad dwadzieścia lat później, pozwolił na ustalenie, że u dorosłych pacjentów z IBD stwierdza się zarówno obniżoną częstość występowania, jak i obniżoną ogólną liczebność archeonów metanogennych w jelitach [48]. Ponieważ, aby osiągnąć mierzalną ilość metanu w powietrzu wydychanym niezbędne jest zasiedlenie jelit przez metanogeny w liczbie co najmniej 10^8 komórek na gram suchej masy kału, brak tej cząsteczki oceniany w testach oddechowych u pacjentów z IBD jest konsekwencją niskiego poziomu zasiedlenia jelit przez te drobnoustroje [49], gdyż archeony metanogenne są jedyną składową ludzkiego mikrobiomu jelitowego zdolną do produkcji metanu [34].

U dorosłych chorujących na IBD opisano zmiany w zakresie częstości występowania i liczebności archeonów metanogennych. Wykazano, że częstość wykrywania *Mb. smithii* pozostaje na podobnym lub nieco obniżonym poziomie w stosunku do osób zdrowych, jednak wyraźne różnice są już widoczne w zakresie liczebności tego gatunku [44]. Chorzy charakteryzują się bowiem niższą liczbą *Mb. smithii* w kale, osiągając wartości w zakresie 10^4

do 10^8 komórek na gram kału, w porównaniu z 10^5 do 10^9 komórek na gram kału u zdrowych dorosłych [50]. Jednocześnie u dorosłych z IBD został udowodniony wyraźny wzrost udziału *Ms. stadtmanae*, zarówno w kwestii częstości występowania, jak i liczebności [44]. Częstość występowania *Ms. stadtmanae* była trzykrotnie wyższa u chorych na IBD, a liczebność wynosiła około 10^7 komórek na gram kału w stosunku do zdrowych dorosłych, u których stwierdzono maksymalnie 10^4 komórek *Ms. stadtmanae* na gram kału [44]. Udział metanogenów z rodzaju *Methanomassiliicoccales* nie był dotychczas badany w kontekście IBD, zarówno u dorosłych jak i u dzieci.

U dzieci badania dotyczące roli archeonów metanogennych w IBD są bardzo ograniczone. Zgodnie z aktualną wiedzą badania dotyczące udziału *Mb. smithii* i *Ms. stadtmanae* w UC nie były w ogóle prowadzone w grupie dzieci z UC. Natomiast do tej pory tylko jeden zespół, i to zaledwie w ostatnich miesiącach, zbadał dokładnie populację archeonów jelitowych w przebiegu CD u dzieci [51,52]. Badania te wykazały co prawda niewielki (nieistotny statystycznie) spadek częstości występowania oraz liczebności metanogenów u dzieci z CD, jednak zauważono, że ta grupa pacjentów różni się od dzieci zdrowych w zakresie różnorodności archeonów w kale w zależności od długości trwania CD [52]. Podsumowując, brak danych dotyczących występowania archeonów metanogennych u dzieci cierpiących na UC, oraz ograniczone dane na temat dziecięcej CD stały się przyczynkiem do przeprowadzenia badań opisanych w artykule pt. ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)*, będącym zasadniczą częścią tej pracy doktorskiej.

2. Cel rozprawy doktorskiej

Głównym celem rozprawy doktorskiej była ocena znaczenia archeonów metanogennych w nieswoistych chorobach zapalnych jelit u dzieci. Cel ten został osiągnięty poprzez realizację celów szczegółowych, które były opisane w poszczególnych artykułach oryginalnych

Cele szczegółowe

- Ustalenie i zoptymalizowanie protokołu analizy ilościowej archeonów metanogennych w próbkach kału – artykuł pt. ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek i wsp., Microorganisms, 2023)*
- Ocena występowania archeonów metanogennych w treści jelitowej i próbkach kałomoczu kury domowej oraz ocena wpływu czynników środowiskowych na liczebność tych drobnoustrojów – artykuł pt. ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek i wsp., International Journal of Molecular Sciences, 2023)*
- Analiza liczebności archeonów metanogennych w kale dzieci z IBD w porównaniu z dziećmi z grupy kontrolnej oraz ocena zależności pomiędzy liczebnością tych drobnoustrojów a typem i stopniem aktywności choroby oraz wiekiem dzieci – artykuł pt. Celem publikacji ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)*

3. Materiał

3.1 Zwierzęta doświadczalne

Do badań włączono kury, które ze względu na typ hodowli podzielono na 3 grupy badawcze: kury wiejskie, fermowe i eksperymentalne. Kury wiejskie pochodziły z trzech gospodarstw przydomowych z zachowanym swobodnym dostępem do środowiska zewnętrznego oraz innych zwierząt (chów wolno wybiegowy), gdzie nie podawano im żadnych antybiotyków. Kury fermowe pochodzące łącznie z 8 ferm miały bardziej ograniczony dostęp do środowiska zewnętrznego (zgodnie z zasadami bioasekuracji), a część z nich była poddana antybiotykoterapii. Z kolei pochodzące z jednego źródła kury eksperymentalne hodowane były w pełnej izolacji od środowiska zewnętrznego, ludzi i innych zwierząt oraz antybiotyków, a ich mikrobiota jelitowa rozwijała się jedynie w wyniku zasiedlania drobnoustrojami obecnymi w paszy i wodzie kranowej. U zwierząt eksperymentalnych i fermowych znany był wiek w czasie pobrania próbek materiału do badań. Dokładny opis badanych grup i warunki hodowli, w tym rodzaj diety i stosowanych antybiotyków, a także wiek zwierząt w momencie pobrania próbki znajduje się w pracy pt. ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek i wsp., Microorganisms, 2023),* w której wykorzystano 20 próbek kałomoczu kur wiejskich z jednego gospodarstwa oraz w pracy pt. ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek i wsp., International Journal of Molecular Sciences, 2023),* w której użyto 50 próbek kałomoczu pochodzących od kur wiejskich, 50 próbek treści jelit ślepych i 2 próbki kałomoczu pobrane od kur fermowych oraz 54 próbki treści jelit ślepych pochodzące od kur eksperymentalnych.

3.2 Pacjenci

Do badań włączono dzieci w wieku od 3 do 18 lat, chorujące na IBD, będące pacjentami Kliniki Gastroenterologii, Hepatologii, Zaburzeń Odżywiania i Pediatrii Instytutu „Pomnik – Centrum Zdrowia Dziecka" w Warszawie. Dzieci z IBD zostały podzielone na 2 grupy badawcze: dzieci z aktywna postacią choroby (UC=25, CD=21) oraz dzieci z postacią nieaktywną (UC=27, CD=24). Aktywność procesu chorobowego została oceniona przy pomocy następujących indeksów: indeks PUCAI (ang. *Pediatric ulcerative colitis activity index*) oraz indeks PCDAI (ang. *Pediatric Crohn's disease activity index*), a także na podstawie oceny stężenia kalprotektyny w kale. Przyjęto następujące wartości PUCAI oceny aktywności UC <10 –

remisja, 10-30 – łagodna postać choroby, >30 – zaostrzenie. Wartość PCDAI wynosiła odpowiednio: <12,5 – postać nieaktywna CD, >20 – postać aktywna CD. Przyjęto, że pacjenci z grupy UC z łagodnym przebiegiem choroby oraz będący w zaostrzeniu (PUCAI ≥10) będą przypisani łącznie do grupy z aktywnym przebiegiem UC, natomiast tych będących w remisji (PUCAI <10) – włączono do grupy z nieaktywnym UC. Grupę kontrolną stanowiły dzieci bez rozpoznania IBD w wywiadzie. Z grupy kontrolnej wykluczono dzieci z chorobami o podłożu autoimmunizacyjnym lub innymi chorobami, które mogłyby potencjalnie wpływać na stan mikrobioty jelitowej. Dokładna charakterystyka pacjentów włączonych do badań znajduje się w pracy pt. ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)*. Dodatkowo, w materiałach uzupełniających (str. 102) zamieszczono tabelę, w której opisano pacjentów z grupy kontrolnej.

Do badań łącznie włączono 124 próbki kału pobrane od pacjentów z UC (n=52), CD (n=45) i od dzieci z grupy kontrolnej (n=27).

4. Metody

4.1 Izolacja DNA z próbek

Izolacja DNA z kałomoczu i treści jelitowej kury oraz kału dzieci dokonano za pomocą procedury uwzględniającej lizę enzymatyczną, chemiczną oraz mechaniczną, a izolaty oczyszczano na kolumienkach ze złożem krzemionkowym (Genomic Mini AX Bacteria+) zgodnie z zaleceniami producenta (A&A Biotechnology, Gdynia). Metoda izolacji została opisana w pracy pt. ***Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens*** *(Cisek i wsp., International Journal of Molecular Sciences, 2023)* oraz ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)*.

4.2 Analiza ilościowa archeonów metanogennych

We wszystkich trzech pracach oryginalnych do pomiaru ogólnej liczby komórek archeonów metanogennych zastosowano metodę łańcuchowej reakcji polimerazy w czasie rzeczywistym (ang. *real-time PCR*), w której sekwencją poszukiwaną był fragment genu *mcrA* kodującego podjednostkę alfa reduktazy metylo-koenzymu M, tj. enzymu zaangażowanego w ostatni etap metanogenezy przebiegającej wyłącznie u archeonów metanogennych. Opracowanie i optymalizacja metody detekcji ogólnej populacji metanogenów została opisana szczegółowo w pracy pt. ***Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples*** *(Cisek i wsp., Microorganisms, 2023)*. W skrócie, do uzyskania odpowiedniej czułości i swoistości reakcji real-time PCR jako kontroli pozytywnej reakcji użyto genomowego DNA trzech referencyjnych gatunków archeonów metanogennych (*Methanobrevibacter woesei* DSM 11979, *Methanococcus maripaludis* DSM 2067 oraz *Methanomicrobium mobile* DSM 1539) oraz jednego konstruktu plazmidowego zawierającego fragment genu *mcrA* z *Methanobrevibacter* sp. D5 w formie insertu. Kontrole negatywne reakcji stanowiły izolaty DNA 21 gatunków bakterii, w tym z 11 gatunków referencyjnych. Startery zostały zaprojektowane w obrębie najbardziej konserwatywnych fragmentów sekwencji genu *mcrA*, ustalonych na podstawie analizy sekwencji pozyskanych z bazy NCBI. Optymalna temperatura przyłączania starterów (T_{an}) ustalona została w oparciu o PCR z gradientem T_{an}. Temperaturę odczytu poziomu fluorescencji ustalono doświadczalnie na podstawie analizy krzywych topnienia amplikonów uzyskanych z próbek DNA genomów

referencyjnych. Czasy trwania poszczególnych etapów real-time PCR dobrano w oparciu o długości przewidywanych amplikonów, po czym dopasowano je eksperymentalnie.

Ponadto, w pracy pt. ***Methanogenic Archaea in the Pediatric Inflammatory Bowel Disease in Relation to Disease Type and Activity*** *(Cisek i wsp., International Journal of Molecular Sciences, 2024)* do oceny ilościowej trzech badanych podgrup metanogenów użyto także innych genów w roli sekwencji docelowych. Były to kolejno: gen *nifH* dla reakcji w której oznaczany był *Mb. smithii*, *mtaB* – dla *Ms. stadtmanae* oraz gen kodujący 16S rRNA – dla Methanomassiliicoccales.

Wszystkie analizy ilościowe odbyły się w oparciu o krzywe standardowe, w których użyto DNA w/w genomów referencyjnych odpowiadających poszczególnym grupom metanogenów, rozcieńczone 10-krotnie w zakresie od 10^0 do 10^6 kopii na mieszaninę reakcyjną. Uzyskany wynik dla każdej próbki był następnie przeliczany do wartości x kopii genomów archeonów w jednym gramie kału człowieka lub kałomoczu i treści jelitowej kury.

4.3 Oznaczenie stężenia kalprotektyny w kale

Oznaczenie stężenia kalprotektyny w kale wykonano w Zakładzie Biochemii, Radioimmunologii i Medycyny Doświadczalnej. Pomiarów dokonano używając technologii chemiluminescencji w analizatorze Liaison XL (DiaSorin, Saluggia, Włochy).

4.4 Analiza statystyczna

Analiza statystyczna odbyła się z zastosowaniem programu TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, USA). Analizę rozkładu wyników wykonano testem Shapiro-Wilka, a homogenność wariancji badano testem Levene'a. Z uwagi na fakt, że uzyskane wyniki nie miały charakteru rozkładu normalnego, dalsze analizy zostały wykonane testem nieparametrycznym Kruskala-Wallisa. Do oceny korelacji zastosowano test korelacji rang Spearmana. Do oceny występowania metanogenów u pacjentów zastosowano test dokładny Fishera. Dokładny opis analiz statystycznych został przedstawiony w pracach oryginalnych.

5. Kopie opublikowanych prac

Journal of *Personalized Medicine*

MDPI

Review

The Role of Methanogenic Archaea in Inflammatory Bowel Disease—A Review

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Abstract: Methanogenic archaea are a part of the commensal gut microbiota responsible for hydrogen sink and the efficient production of short-chain fatty acids. Dysbiosis of methanogens is suspected to play a role in pathogenesis of variety of diseases, including inflammatory bowel disease (IBD). Unlike bacteria, the diversity of archaea seems to be higher in IBD patients compared to healthy subjects, whereas the prevalence and abundance of gut methanogens declines in IBD, especially in ulcerative colitis. To date, studies focusing on methanogens in pediatric IBD are very limited; nevertheless, the preliminary results provide some evidence that methanogens may be influenced by the chronic inflammatory process in IBD. In this review, we demonstrated the development and diversity of the methanogenic community in IBD, both in adults and children.

Keywords: methanogenic archaea; methanogens; IBD; inflammatory bowel disease; Crohn's disease; ulcerative colitis; pediatric diseases

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1. Introduction

Inflammatory bowel disease (IBD) is a term that describes disorders involving chronic inflammation of gastrointestinal tissues. Its importance has been increasing in the recent years, both due to its higher prevalence worldwide [1] and higher treatment costs. It is estimated that over 0.3% of the human population of developed counties suffers from IBD, and there is an upward trend in the incidence rates in various regions of the world [2]. This is related to economic development, dietary changes, and improving economic status [3]. Moreover, the diagnostic possibilities are much better in developed countries than in developing countries, which may also result in better diagnosis of IBD. In the United States alone, the number of people affected by this disease is over 1.6 million, and the annual medical and sick leave expenses is reaching more than $1 billion [4]. The incidence in Europe and North America is estimated at 40–50 and 3.1–14.6 cases per 100,000 inhabitants per year, respectively [5,6].

IBD includes ulcerative colitis (UC) and Crohn's disease (CD). CD is characterized by an extensive inflammation throughout the entire intestinal cross-section and is usually located in a distal part of the small intestine [7], but may also affect the colon or small intestine and colon together [4]. UC mainly affects the rectal mucosa, where it usually begins [7], and may spread proximally into the colon [8].

The etiopathogenesis of IBD is not fully understood [9], although several links to this disease are well-documented. These include a combination of factors: human genetics, immunology, the environment, and microbiology [10,11]. Commensal microorganisms can be a source of antigens triggering IBD [12], but there are reports directly linking some evidently detrimental bacteria with development of IBD, of which Fusobacteria

and *Enterobacteriaceae*, particularly adherent-invasive *Escherichia coli* (AIEC), seem to be among the most important taxa [13–15]. Increased amounts of *Candida* spp. and *Malassezia* spp. have also been reported in IBD patients [16–18], as have some viruses from the Caudovirales, *Hepadnaviridae*, and *Herpesviridae* clades, such as cytomegalovirus, whose presence contributes to some life-threatening complications [19].

On the other hand, there are bacteria such as *Lachnospiraceae*, *Roseburia*, *Eubacterium rectale*, *Ruminococcus*, *Clostridium*, *Faecalibacterium* and other butyrate producers that may play a protective role, and whose abundance is usually decreased in IBD [20]. However, it should be noted that the role of these bacteria may be strain-specific [21]. In general, the loss of microorganisms producing butyrate (a signaling molecule in the mitochondrial gene expression) and subsequent reduction in this compound in the gut triggers a cascade of adverse events, including deregulation of host mitochondrial activity and an increased production of reactive oxygen species, which eventually allows for translocation of microorganisms and toxins across the epithelial barrier [20]. Moreover, the oxidative stress in the gut prompts a functional adaptation of microorganisms, while leading to microbial dysbiosis, which in turn triggers mucosal inflammation [21]. In conclusion, the abnormal host–microbiota crosstalk and microbial dysbiosis have been demonstrated to play a key role in the pathogenesis of IBD [20,21].

The role of bacteria, fungi, helminths, and viruses in the development of IBD has been extensively reviewed elsewhere [22]. Here, we focus on a forgotten and underestimated member of the gut microbiota, i.e., the methanogenic archaea. Moreover, since children are believed to be the best model for studying the pathogenesis of IBD [20], our work aims to provide as much detail as possible on this age group.

2. Methanogenic Archaea in Health and Disease

Archaea were originally known as inhabitants of extreme environments of high salinity, high temperature, and acidity [23], such as thermal springs and deep-sea hydrothermal vents [24]; only later, around 1968, were single species isolated from the human gastrointestinal tract [25]. A few decades later, archaea became known as a part of the physiological intestinal microbiota [26]. Archaea have also been reported in other compartments of human body, such as the oral cavity, nose, respiratory tract, vagina, and skin [27,28]. Currently, archaea are reported in 42 to 100% of fecal samples of the human population, depending on the individuals examined [29–31]. Archaea are estimated to account for between 0.1 and 21.3% of the total gut microbiota and are collectively known as the gut archaeome [9,31]. High-throughput sequencing analyses of intestinal samples indicated the presence of the following methanogenic orders: Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales, Methanosarcinales, Methanococcales, and Methanopyrales [26,32]. The non-methanogenic archaeal taxa have also been reported in far fewer cases, and included *Caldisphaera* (order Acidilobales), *Thermogymnomonas* (order Thermoplasmatales), and the orders Archaeoglobales, Desulfurococcales, Natrialbales, Nitrososphaerales, Sulfolobales, Thermococcales, Thermoproteales, Haloferacales, and Halobacteriales [19,26,32,33]. Interestingly, some of these include halophilic archaea, whose abundance in Koreans has been related to the consumption of fermented seafood [31,34,35].

Typically, ca. 90–99% of detected gut archaea are methanogens [27,36]. The methanogenic archaea constitute about 10% of the total anaerobic community [37]. There are three key methanogenic taxa in the human intestine: *Methanobrevibacter smithii* (*Mb. smithii*), *Methanosphaera stadtmanae* (*Ms. stadtmanae*), and Methanomassiliicoccales [32,38]. The prevalence of methanogens increases with the age of humans, with the diversity of methanogens believed to be most pronounced in extreme age groups, i.e., children and the elderly [29,33,39].

For a long time, it was assumed that methanogens begin in children not younger than 2–3 years old [40], but we now know that actual colonization begins much earlier, immediately after birth or even in the fetal period [41,42]. A study in India reported a substantial colonization of neonates in post-weaning children by *Methanobrevibacter* spp.

with a frequency of up to 98% for newborns (of which 88.7% were positive specifically for *Mb. smithii*) and 96% for young children aged 6 months to 2 years [33]. Moreover, methanogens (mostly *Mb. smithii*) were reported in the gastric juice of all one-day-old newborns (n = 50) from France [41] and in 90.9% of meconium samples collected from 33 preterm neonates before the first feeding, most of whom were born by cesarean section from mothers who had not been exposed to any antibiotics during pregnancy [43]. The specificity of delivery and lack of feeding prior to sampling challenges the common belief that colonization by methanogens occurs during vaginal birth or the breastfeeding period [41,44]. These new findings may suggest an in utero colonization, possibly through the placenta, amniotic fluid, or blood [43]. Interestingly, one study involving children from birth to one year of age demonstrated that methanogens were detected in the first days of life and disappeared by the second month of life [45]. This transient occurrence of methanogens may be related to the fact that the gut microbial diversity of young infants reduces as a result of breastfeeding [46]. Data are lacking on taxa other than *Mb. smithii* colonizing the intestines of neonates.

In older age groups, in addition to *Mb. smithii* (which was detected in 78 to 88% of school-aged children) other taxa start to emerge. *Ms. stadtmanaea* was found in 8 to 11% and *Methanomassiliicoccus luminyensis* (*Mm. luminyensis*) in 1% of European school-aged children [47–49]. More recent data from our research group report slightly different percentages in children aged from 4 to 10 years. In these cases, the prevalence of *Mb. smithii*, *Ms. stadtmanae* and Methanomassiliicoccales were 80%, 47%, and 13%, respectively [50]. The order Methanomassiliicoccales is a broad taxon that includes *Mm. luminyensis* among others, hence the differences between reported numbers [51]. Interestingly, both children and adults demonstrate a marked increase in the prevalence of Methanomassiliicoccales with age [47,50,52]. Moreover, in individual cases Methanomassiliicoccales may dominate quantitatively over Methanobacteriales (including *Mb. smithii*) [50,51].

In adults, *Mb. smithii* was found in 64%, 75–89%, 96%, and even in 100% of the population depending on the individuals studied [26,52]. Data on the prevalence of *Ms. stadtmanae* are also conflicting, as this methanogenic species is estimated to be present in 30 to 90% of adults [30,53]. Notably, the study reporting the highest prevalence of *Mb. smithii* also showed that only 17% of adults and 25% of elderly people aged 70 to 90 tested positive for *Ms. stadtmanae* [52]. Another important methanogen species, *Mm. luminyensis*, was shown to inhabit 4% of adults [47], whereas at the genus level, the prevalence of *Methanomassiliicoccus* ranged from 1 to 25.7% [54]. Interestingly, a small study involving only 10 individuals between the ages of 25–50 showed a 100% prevalence of Methanomassiliicoccales [30], an order that comprises two key genera, i.e., the free-living *Methanomassiliicoccus* and the host-associated "Candidatus *Methanomethylophilus*". The latter were found to occur in humans even more frequently than *Methanomassiliicoccus*, with a prevalence ranging from 0.5 to 41.7% [54]. In the elderly, the prevalence of Methanomassiliicoccales has reached 40% [52], and despite obvious differences in publications, the general trend is that the prevalence of Methanomassiliicoccales increases with age [47,52], which has not been observed in other methanogenic taxa.

As for absolute values, the abundance of total methanogens, their numbers are also increasing with age. Children at the age of 30 months have 10^3–10^4 methanogens per gram of fecal dry matter, whereas in 5-year-olds, this value increases to 10^4–10^8 cells per gram [29,50]. In adults, the methanogens can account over 10^{10} cells per gram [55].

The presence of methanogens is strongly related to measured levels of methane in the breath. It is estimated that 15% of Japanese citizens and up to 70% of rural Africans exhale methane, whereas in the Western countries ca. 40–60% of adults are methane producers [56]. The detection rate of methane is related to diet, lifestyle [56], and age [57]. Children as young as 3 years old do not produce methane. About 6% of children aged 3 to 4 produce methane, as do 13 to 18% of children aged 7 to 14, 39 to 46% of teenagers aged 14 to 18, and 49% of adults [50,57]. This correlation is not surprising, given that an individual must be colonized by at least 10^8 methanogen cells per gram of fecal dry weight for methane in

breath to be detectable [58]. Non-methane-producing adults are typically colonized by 10^2 to 10^6 methanogen cells per gram of feces [59].

The presence of archaea has been shown to exert a bilateral effect on human health—either positive or negative. On the one hand, methanogens participate in the circulation of matter and energy inside the intestine. By lowering the partial pressure of hydrogen, they assure the continuity of intestinal fermentation, indirectly contributing to the production of specific fermentation products such as short-chain fatty acids or vitamins by the intestinal bacteria [60]. Furthermore, there is growing evidence that the archaeal strain *Mm. luminyensis* B10 can be used as a probiotic, as it has the potential to treat metabolic disorders such as atherosclerosis and trimethylaminuria (TMAU; fishy odor syndrome) [61]. This archaeon naturally depletes trimethylamine with hydrogen in the process of methanogenesis [61]. Archaea have been shown to alleviate yet another unpleasant condition: they can reduce odor by oxidizing ammonia secreted through the skin [36,62]. Moreover, celiac disease studies have shown that Euryarchaeota may play a positive role and act as an anti-inflammatory factor in the healthy guts of children [63]. In pediatric patients aged 6 to 10 years, the presence of *Ms. stadtmanae* was also associated with a lower likelihood of asthma, indicating the tolerogenicity of this species in young patients, a slightly lower (though not statistically proven) risk of eczema, sensitization to aeroallergens and food allergens [49]. On the other hand, the presence of certain methanogenic archaea has been linked to periodontitis, brain abscess, vaginosis, diverticulosis, multiple sclerosis, obesity, colorectal cancer, irritable bowel syndrome, and IBD [26,28,64], but whether the presence of methanogens was a cause or consequence of these conditions needs further investigation. Importantly, not a single study has suggested the existence of a clearly pathogenic archaeal strain in humans [36]. So far, only one study describes them as emerging opportunistic pathogens [65].

3. The Occurrence of Methanogenic Archaea in IBD

The first reports indirectly linking methanogens to the incidence of IBD date back to the 1980s [57,66]. At that time, it was observed that adults suffering from IBD rarely excrete methane [57,66–69]. More than 20 years later, with the development of molecular biology techniques [70,71], this link was confirmed more directly, as Scanlan et al. demonstrated a reduced prevalence of total methanogens in adults suffering from IBD. In their study, the prevalence of methanogens (mostly *Mb. smithii*) in UC and CD was 24% and 30%, respectively, compared to 48% of healthy individuals [59]. A more recent study by our research group performed on children aged 3 to 18 years showed a similar tendency [50]. Of the two IBD entities, the lowest prevalence of methanogens was determined in UC, where 83% of children were colonized by total methanogenic archaea, ca. 52% by *Mb. smithii*, 15% by *Ms. Stadtmanae*, and 15% by Methanomassiliicoccales, compared to 100%, 74%, 37%, and 30%, respectively, as reported in control non-IBD children [50]. In contrast, 89% of CD patients were colonized by total methanogenic archaea, about 69% tested positive for *Mb. smithii*, 27% for *Ms. Stadtmanae*, and 13% for Methanomassiliicoccales. Therefore, the incidence of methanogens recorded in the CD and control groups did not differ significantly, which is consistent with the studies of Krawczyk et al. and Chehoud et al. [18,72].

Apart from the lower prevalence of methanogens, there is also a clear link between IBD and a higher diversity of intestinal archaea. The feces of healthy individuals usually have a homogenous composition of archaea comprising a single predominant taxon (Figure 1), such as *Methanobrevibacter* sp. [73]. In contrast, pediatric patients with chronic CD are characterized by an increased archaeal diversity, as non-methanogenic archaea such as Halobacteria have been shown to achieve considerable numbers [73]. Similar results have been observed in adult patients with IBD [74]. Adult CD is also characterized by an increased abundance of Nitrosophaerales and Thermococcales detected in the ileal aspirate [19]. In contrast, in adult UC, most of the detected archaeal lineages of the ileum belonged to Methanococcales, Methanobacteriales, Methanomicrobiales, and Methanosarcinales, therefore UC seemed to be more "methanogenic" than CD [9,19]. In the case of UC, a

difference in the archaeal composition between different parts of the intestines was also noted, as the colon was more enriched by the Methanomicrobiales order than the ileum. In CD, no such phenomenon has ever been observed [19].

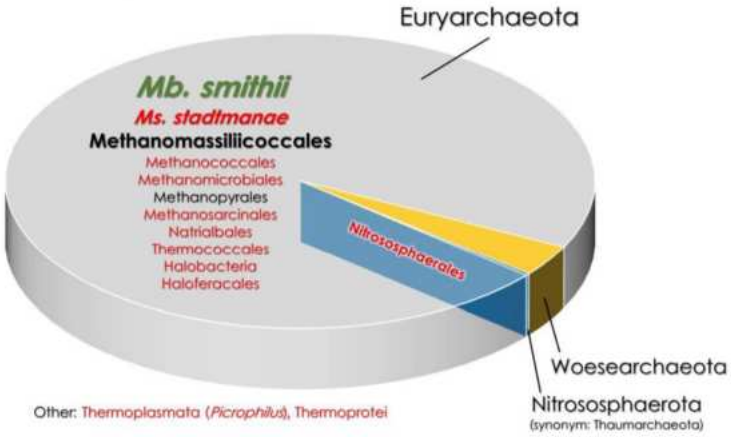

Figure 1. The comparison of gut archaeome in healthy and IBD-affected patients [9,27,73]. The font size indicates the percentage of a given taxon in the gut archaeome. The font color represents taxa that are either increased (red font) or decreased (green font) in IBD patients compared to heathy subjects. *Mb.—Methanobrevibacter*; *Ms.—Methanosphaera*.

The discoveries made over the last decade have established that not all archaeal species affect human health in the same way. For example, *Mb. smithii* is considered a commensal species [75], whereas *Ms. stadtmanae* is accused of exerting a rather detrimental effect on its host [12]. *Mb. smithii* accounts for 11.5% of the total intestinal microbiota [12] and constitutes from 94 to 100% of the gut methanogenic population [39,59,76]. *Mb. smithii* tends to be almost as prevalent in adults with IBD as in healthy adults, but in terms of its abundance, these two patient groups differ significantly [12,77]. In healthy adults, the amount of *Mb. smithii* ranged from 10^5 to 10^9 cells per gram of dried stool, and only 10^4 to 10^8 cells per gram in the IBD patients [12]. Another study reported a decreased prevalence of *Mb. smithii* in the adult IBD patients [59]. In children with CD, the prevalence and abundance values of *Mb. smithii* were not statistically different compared to the control groups [18].

On the other hand, *Ms. stadtmanae* was three times more frequent and numerous in adult IBD patients compared to healthy controls. It was shown that the quantity of *Ms. stadtmanae* in IBD patients was as high as 10^7 cells per gram of dried stool, compared to a maximum of 10^4 in healthy adults [12]. Interestingly, a recent study on children with CD reported quite a different trend. There, the prevalence and concentration of *Ms. standtmanae* DNA in stool samples were similar between children with active CD and healthy children, whereas the percentage of carriers and the amount of DNA detected were lowest in CD patients in remission [18]. It should be noted that the latter correlations were not statistically significant.

Methanomassiliicoccales, the last key methanogenic group, is greatly understudied in terms of its role in IBD. As far as we know, its representative—*Mm. luminyensis*—was first described in 2014 [78] and, since then, the role of *Mm. luminyensis* in relation to IBD has yet to be documented. So far, we know that *Mm. luminyensis* is a commensal microorganism exhibiting only low immunogenicity tested in vitro on peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (moDCs) [79]. Moreover, its sensitivity profile to the human-derived antimicrobial peptides suggests some adaptation to the intestinal environment [38,79].

4. Methanogens in the Pathogenesis of IBD

In the previous chapter we described a higher diversity of archaea occurring in IBD patients compared to healthy subjects, but recent evidence suggests an even higher archaeal diversity the longer the disease lasts. Krawczyk et al. demonstrated that in newly diagnosed pediatric CD, the percentage composition of archaea resembled that observed in healthy children [73]. Over time, both active and inactive forms of long-term CD showed substantial changes in the archaeal composition towards a percentage decrease in predominant Methanobacteria and an increase in other archaea, such as Thermoplasmata, Halobacteria, and Thermoprotei. Similarly, our recent data demonstrated a decrease in the total methanogens in pediatric IBD (particularly in active CD) in older children, i.e., those with longer duration of chronic intestinal inflammation [50]. These two studies may suggest that methanogens were not involved in the induction of IBD, but were influenced by the persistent inflammatory process in the course of IBD [50,73]. It is therefore presumed that prolonged inflammation, chronic diarrhea, and accelerated intestinal transit may (1) contribute to the loss of slow-growing, usually non-motile methanogens [4] and (2) create more favorable conditions for less typical, adventitious groups of archaea [73]. In excess, archaea can deplete large amounts of butyrate from the biofilm lining the intestinal wall. As a result, the intestinal epithelial barrier becomes more permeable, and any commensal microorganisms coexisting in the biofilm can easily enter the intestinal tissues, becoming, as some suggest, endoparasitic [80,81]. As a consequence, inflammation increases, further worsening microbial dysbiosis. Therefore, archaeal dysbiosis and gut inflammation progress in a vicious cycle, especially in the course of IBD (Figure 2). To break this cycle, any measures that reduce inflammation should be of paramount importance. These certainly include maintaining the proper bacterial–archaeal balance and the intestinal production of butyrate, a key regulator of syntrophism between the two groups of organisms [9,20]. As an aside, to date, there has been no clinical trial evaluating the impact of archaeal dysbiosis and IBD.

Apart from the convincing link between IBD and the archaeal dysbiosis, other possibilities of the archaeal involvement in the pathogenesis of IBD have also been the subject of scientific research. Many of these studies were performed on specific species of methanogens. For instance, it was shown that *Mb. smithii* forms biofilms on the surface of intestinal epithelium [82], produces adhesin-like proteins and hyaluronan, all of which generally allows this archaeal species to easily persist in the gut [37]. However, some surface glycans produced by this species have been shown to be immunogenic and induce hyperactive immune responses. This, in turn, may lead in some cases to the development of IBD and other autoimmune-related diseases. This association has been detailed in the case of *Mb. smithii*, whose pseudomurein glycan structures, isolated from a few different strains, revealed the diverse immunogenic potential of these structures tested using monoclonal antibodies [83]. However, as we increasingly understand the interactions between methanogens and bacteria in the gut, it is important to note that there also may be an indirect link between methanogens and the development of IBD. Methanogens produce signaling molecules, e.g., methane or acyl-homoserine lactones. The latter have been shown to be responsible for cell communication [84]. Methanogens have also been proven to promote the growth of fermentative bacteria by decreasing the partial pressure of hydrogen in the gut in the process of hydrogen sink and through symbiotic cross-feeding [85]. It is highly likely that some alteration in the microbiome initiated by methanogens may ultimately affect interactions between the microbiota and the intestinal mucosa or immune cells, as well as the entire inflammatory profile of the gut. For instance, the presence of *Mb. smithi* has been associated with an increased acetogenesis [85,86] and acetate may exert a pro-inflammatory effects [87]. Methanogens may also interact with potentially pathogenic microorganisms, promoting their growth [88]. Moreover, it has been proven that the presence of methanogens and the associated decrease in gut motility (since methane is a signaling molecule) increases mucosal contact time with some toxic metabolic end products, including H_2S, and therefore

promotes their absorption by the intestinal epithelium or induces the intestinal epithelial barrier damage and increased transfer of pathogens [20].

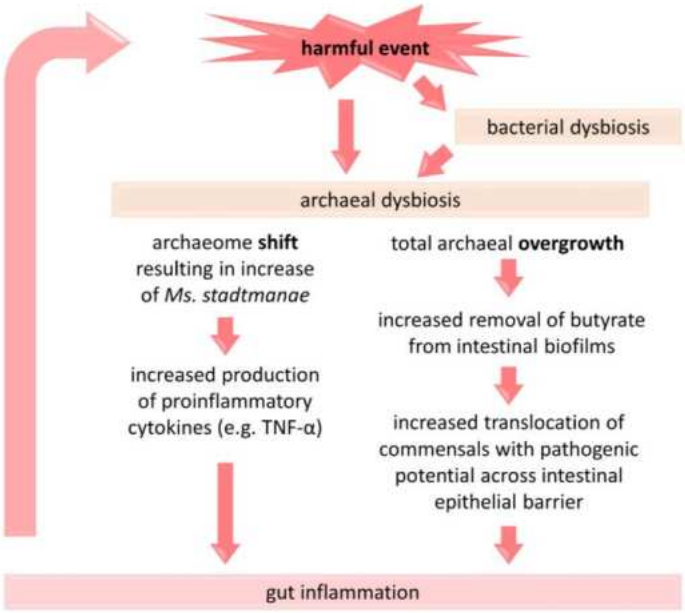

Figure 2. A brief presentation of the proposed link between dysbiosis of methanogenic archaea and the pathogenesis of IBD. A change in the composition of archaea and/or their overgrowth under the influence of detrimental factors (e.g., antibiotic administration, Western-type diet) is associated with an intensification of inflammatory processes in the gut, which may consecutively affect the gut microbiota [9,48,80,81]. TNF-α—tumor necrosis factor alpha.

In contrast to *Mb. smithii*, *Ms. stadtmanae* is accused of exerting rather strictly detrimental effect on its host. It strongly induced some inflammatory responses even in the non-IBD healthy individuals [12], whereas in the IBD patients, this species is associated with the persistence of disease [73]. *Ms. stadtmanae* has been shown to be immunogenic to human immune cells such as PBMCs [12] and dendritic cells [36,49], but not to epithelial cells such as Caco-2/BBe, suggesting a pronounced adaptation of this species to the intestinal environment [83]. *Ms. stadtmanae* has been shown to be rapidly phagocytosed by moDC and degraded by endosomal acidification [83]. The release of RNA from *Ms. stadtmanae* (more specifically the ssRNA), acting as a microbe-associated molecular pattern (MAMP), has been shown to induce the secretion of high levels of proinflammatory cytokines (Figure 2) such as interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), type-I and type-III interferons via the Toll-like receptors—TLR8 and, to some extent, TLR7. Notably, the secretion of IL-1β depended exclusively on TLR8 [89]. In contrast, DNA from *Ms. stadtmanae* did not yield the same result as its RNA [89].

The RNA-sensing TLRs have been shown to be crucial in various autoimmune and inflammatory diseases, including IBD [90]. In fact, TLR8 mRNA was reported to be upregulated 350-fold and 45-fold in the mucosal inflammatory epithelial cells in UC and CD patients, respectively, compared to controls. However, no differences were observed in TLR8 mRNA levels in lamina propria mononuclear cells between IBD patients and controls [91]. In general, the exposure to *Ms. stadtmanae* contributes to maturation of moDCs and activation of B-cells and T-cells, meaning that both innate and adaptive immune responses are initiated [4]. In contrast, stimulation of PBMCs by *Mb. smithii* has also led to

synthesis of TNF-α, but four times lower than stimulation by *Ms. stadtmanae* [12]. It has been hypothesized that both species are specifically recognized by the human immune system, but to different degrees [83]. In summary, individual methanogenic species influence the host's immunity in different ways. All of the above data should, however, be interpreted with caution as many studies have been conducted exclusively in vitro.

5. Conclusions

Methanogenic archaea are part of the physiological intestinal microbiota. The last few years have brought new data on the origins of methanogen colonization in the human intestines and their role in the proper imprinting of the immune system. There is a high probability that methanogens may play a role in non-communicable diseases, but the evidence collected so far regarding the involvement of methanogens in the pathogenesis of IBD is circumstantial. The only certainty is that archaeal dysbiosis occurs in IBD patients, but there is still no convincing evidence on whether methanogens induce pathological mechanisms or are affected by the chronic inflammatory processes that take place in IBD. To date, little is known about the possible role of methanogenic archaea in the initiation and progression of IBD, therefore more research is needed.

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Article

Improved Quantitative Real-Time PCR Protocol for Detection and Quantification of Methanogenic Archaea in Stool Samples

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Abstract: Methanogenic archaea are an important component of the human and animal intestinal microbiota, and yet their presence is rarely reported in publications describing the subject. One of the methods of quantifying the prevalence of methanogens is quantitative real-time PCR (qPCR) of the methanogen-specific *mcrA* gene, and one of the possible reasons for detection failure is usually a methodology bias. Here, we refined the existing protocol by changing one of the primers and improving the conditions of the qPCR reaction. As a result, at the expense of a slightly lower yet acceptable PCR efficiency, the new assay was characterized by increased specificity and sensitivity and a wider linear detection range of 7 orders of magnitude. The lowest copy number of *mcrA* quantified at a frequency of 100% was 21 copies per reaction. The other validation parameters tested, such as reproducibility and linearity, also gave satisfactory results. Overall, we were able to minimize the negative impacts of primer dimerization and other cross-reactions on qPCR and increase the number of not only detectable but also quantifiable stool samples—or in this case, chicken droppings.

Keywords: archaea; *mcrA*; methanogens; *Methanobrevibacter*; qPCR; real-time PCR

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1. Introduction

Methanogenic archaea are one of the many natural residents of animal and human intestines [1,2]. They partake in hydrogen sink, a process of the utilization of hydrogen, a byproduct of intestinal microbial fermentation. It is known that, without hydrogen sink, the fermentation itself would slow down, thus depriving the host of various useful nutritious compounds [3]. Moreover, methanogens interact closely with other intestinal microbionts, and their absence may be an indicator of intestinal dysbiosis [1]. Methanogens may also influence their hosts in other ways. On the one hand, some archaea were reported to have a probiotic potential, such as *Methanomassiliicoccus luminyensis* B10 in treating trimethylaminuria (TMAU), i.e., the fish-odor syndrome [4], and *Methanobrevibacter smithii* in treating severe acute malnutrition [5]; on the other hand, species such as *Methanosphaera stadtmanae* may promote inflammation [6].

Of all methanogenic archaea, species of the genus *Methanobrevibacter* are known to be predominant methanogen taxa in the gastrointestinal tracts of animals and humans. In non-rumen animals, they are followed by *Methanosphaera*, *Methanosarcina*, *Methanomassiliicoccus*, and *Methanimicrococcus*. In chickens, *Methanobrevibacter woesei* is the only dominating genus. The rumen microbiota is significantly more diverse in terms of archaeal prevalence, with four main orders, i.e., Methanomicrobiales, Methanosarcinales, Methanobacteriales, and Methanomassiliicoccales [7].

It is estimated that methanogenic archaea account for 0.05–0.8% of the intestinal microbiota in humans, 4% of the rumen microbiota, and 1–2% of the chicken cecal microbiota [7–9]. However, since the detection of archaea is challenging, those data may not be precise. For instance, to date, many studies do not report the presence of archaea in

the chicken ceca at all [10,11], whilst others say otherwise [12,13]. For this reason, chicken dropping samples were chosen as a model in this study.

It is not known whether the lack of reported archaea in the chicken intestinal microbiome is caused by their actual absence or the methodology limitations of those studies. Such limitations may include insufficient cell lysis during DNA isolation from archaea or an inadequate PCR protocol. The first issue was addressed in our previous study [14]; therefore, here, we decided to take a closer look at the subject of the 'detection' of methanogens via real-time PCR.

The detection of methanogenic archaea using real-time PCR is usually performed with the use of primers targeting either the 16S rDNA or the genes involved in methanogenesis, uniquely specific to methanogenic archaea, usually the *mcrA* gene encoding the alpha subunit of methyl-coenzyme M reductase [15]. The first target has a few major drawbacks. The 16S rDNA may not be selective enough to allow for the identification of only the methanogenic archaea and, therefore, for the undertaking of PCR on templates isolated directly from feces. The other drawback is related to the high scattering of the 16S rDNA copy number between the different taxa of archaea [16], which, as a result, does not allow for the quantification of these microbes in samples. Putting these facts together, the 16S rDNA seems the least preferred target for the quantification of methanogens in dropping or stool samples.

The *mcrA* gene, however, is a single-copy gene, which makes it ideal for quantification purposes [15]. The main difficulty in application comes from the fact that methanogenic archaea are very genetically diverse, and, therefore, finding a good location for primers along the *mcrA* gene and making sure that they comply with the rules of proper primer design can be challenging. Here, we addressed this issue and undertook exhaustive efforts in refining the existing protocol of the quantification of methanogenic archaea.

2. Materials and Methods

2.1. Primer Design

A total of 47 *mcrA* sequences of methanogenic archaea were selected from the NCBI GenBank database. The sequences were aligned by using the webPRANK tool [17] in order to identify the conserved regions (Table S1). Several potential forward primers were created, and they were checked together with the reverse primer *mcrA*-rev [18] (Table 1) in the Oligo Analyzer [19] and Primer-BLAST [20] tools with regard to their specificity and tendency to form secondary structures.

Table 1. The list of oligos used for the *mcrA* gene detection.

Oligo Name	Sequence 5′–3′	Oligo Binding Site 5′–3′ *	Tm [°C]	Product Size with Primer *mcrA*-Rev [bp]	References
mcrA-rev	CGTTCATBGCGTAGTTVGGRTAGT	446–467	62–68	n/a	[21]
mlas	GGTGGTGTMGGDTTCACMCARTA	1–23	62–70	469	[21]
*mcrA*_F3	CTTGAARMTCACTTCGGTGGWTC	199–221	62–66	271	This study and [14]

* Primer binding site refers to the nucleotide position in the *mcrA* gene of *Methanobrevibacter woesei* (acc. EU919432.1).

2.2. DNA Templates

The total genomic DNA of 3 genetically and taxonomically diverse reference strains of methanogens, i.e., *Methanobrevibacter woesei* DSM 11979, *Methanococcus maripaludis* DSM 2067, and *Methanomicrobium mobile* DSM 1539, was chosen as positive controls and standards. Moreover, since the BLAST analysis of the *mcrA* sequence demonstrated that *Methanobrevibacter* sp. D5 was the closest to the consensus of 26 methanogens (*Methanobrevibacter* spp. and environmental species; Table S2) in the binding site of the F3 primer, its 472 bp long *mcrA* gene fragment was picked out as a group representative and the

fourth positive control. It was used in the form of a plasmid construct (*mcrA*_MB) carrying the 472 bp long mlas/*mcrA*-rev primer amplicon. For quantification purposes, the *mcrA*-positive (*mcrA*+) plasmid was linearized with the use of a single cutting VspI restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Then, in order to select only the linearized form, it was subjected to agarose gel electrophoresis. A band representing the linearized form was cut out, purified with a Basic DNA Purification Kit (EURx, Gdańsk, Poland), and diluted in a Tris buffer (10 mM Tris HCl, pH 8.5), as were all the other DNAs used in this study. In order to create 4 standard curves, all 3 archaeal DNAs and one *mcrA*+ plasmid were serially diluted 10-fold, from approx. 10^6 to 10^{-1} genome copies/µL.

The genomic DNA of 21 bacterial strains, i.e., *Enterococcus avium* ATCC 14025, *Enterococcus casseliflavus* ATCC 700327, *Enterococcus raffinosus* ATCC 49464 (courtesy of Dr. habil. Beata Dolka, Institute of Veterinary Medicine, Warsaw University of Life Sciences—SGGW), *Lactobacillus sakei* ATCC 15521 (courtesy of Dr. Ilona Stefańska, Institute of Veterinary Medicine, Warsaw University of Life Sciences—SGGW), *Escherichia coli* ATCC 8739, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Clostridium septicum* ATCC 12464, *Blautia obeum* DSM 25238, *Ruminococcus gauvreauii* DSM 19829, *Helicobacter cinaedi* DSM 5359, *Desulfovibrio piger* DSM 749, *Proteus* sp., *Streptococcus* sp., *Streptococcus* sp. (beta-hemolytic), *Corynebacterium* sp., *Pseudomonas aeruginosa*, *Pasteurella* sp., *Klebsiella* sp., *Staphylococcus* sp. (coagulase-negative), *Porphyromonas* sp., and Bacteroidetes bacterium, was the non-target control of PCR. The strains were purchased or received either in the form of DNA or a bacterial pellet, from which the DNA was isolated with the use of a Genomic Bacteria+ kit (A&A Biotechnology, Warsaw, Poland) according to the producer's instructions. The concentrations of the controls were measured with a Quantus fluorometer and the QuantiFluor dsDNA System (Promega Corporation, Madison, WI, USA), and they were converted into the number of genome copies per µL by using the Science Primer web tool [22].

Lastly, 20 dropping samples collected from free-range chickens were subjected to DNA isolation with the use of a Genomic Mini AX Bacteria+ kit (A&A Biotechnology, Gdynia, Poland) and to mechanical lysis via sonication in accordance with the protocol described previously [14].

2.3. Initial Comparison of the Mlas and mcrA_F3 forward Primers

For unification purposes, the *mcrA*_F3/*mcrA*-rev and mlas/*mcrA*-rev primer pairs were compared with the use of the same reagents routinely used in our lab. The reaction mixture included 10 µL of RT HS-PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 0.5 µM of each primer (Table 1), 1 µL of *mcrA*+ plasmid in 10-fold dilutions, and water with BSA (3.5 µg per reaction) to reach a final volume of 20 µL. The reaction conditions closely resembled the conditions from the original study [18], and they were as follows: initial denaturation at 95 °C for 3 min; 45 cycles comprising denaturation at 95 °C for 30 s; annealing at 55 °C for 45 s; extension at 72 °C for 30 s; and fluorescence acquisition either at 81 °C or 83 °C after 20 s for mcrA_F3/*mcrA*-rev and mlas/*mcrA*-rev primer pairs, respectively. In contrast to the original publication, no additional preincubation at 37 °C or postincubation at 72 °C was implemented.

2.4. Gradient PCR

The reaction mixture included 15 µL of RT HS-PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 0.5 µM *mcrA*_F3 and *mcrA*-rev primers (Table 1), 6.24×10^4 of *mcrA*+ plasmid, and water with BSA (3.5 µg per reaction) to reach a final volume of 30 µL. A gradient PCR with an annealing temperature between 54 and 62 °C was performed. The results were visualized using agarose gel electrophoresis.

2.5. Selected Validation Parameters of the Assays

The sensitivity and specificity of the assays were evaluated for standards from approx. 10^{-1} to 10^6 copies per reaction and for at least 7 runs. The intra- and inter-assay variability

was tested in triplicate. Melting analyses were performed after every run to check the specificity of the assays.

In the SYBR Green detection approach, the reaction mixture included 10 µL of RT HS-PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 0.5 µM primers (Table 1), 1 µL of DNA, and water with BSA (3.5 µg per reaction) to reach a final volume of 20 µL. A real-time PCR was performed in which the slopes after denaturation and after annealing were set up to the universal value of 2.2 °C/s. The reaction conditions for the mlas/*mcrA*-rev primer pair were as follows: initial denaturation at 95 °C for 3 min, 45 cycles comprising denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 30 s, and fluorescence acquisition at 83 °C after 20 s. The reaction conditions for the *mcrA*_F3/*mcrA*-rev primer pair were as follows: initial denaturation at 95 °C for 3 min, 45 cycles comprising denaturation at 94 °C for 20 s, annealing at 60 °C for 20 s, extension at 72 °C for 20 s, and fluorescence acquisition at 81 °C after 20 s.

2.6. Detection of Methanogens in Chicken Dropping Samples

The DNA samples isolated from the chicken droppings were tested with the use of the *mcrA*_F3/*mcrA*-rev and mlas/*mcrA*-rev primer pairs as described above, with 100 to 200 ng of DNA used for the tests. The products were separated by using agarose gel electrophoresis in order to double check the specificity of the assays.

3. Results

3.1. Primer Design

A new forward primer (*mcrA*_F3) was designed approx. 176 bases downstream of the original forward primer (mlas), making future amplicons shorter and, therefore, presumably better for quantification purposes (Table 2).

Table 2. Excerpt of the *mcrA* gene sequence alignment of methanogens in the binding sites of the newly designed *mcrA*_F3 primer.

Acc. No.	Species	*mcrA*_F3 Sequence CTTGAARMTCACTTCGGTGGWTC	Product Size with Primer *mcrA*-Rev (bp) *
KC618341.1	*Methanopyrus* sp.	ATGGAGACCCACTTCGGTGGATC	271
HQ896500.1	*Methanomassiliicoccus luminyensis*	ATGGAAACCCACTTCGGTGGTTC	271
AF414042.1	*Methanopyrus kandleri*	ATGGAGACGCACTTCGGAGGTTC	271
MH004454.1	*Methanosarcina mazei*	CTTGAAGACCACTTCGGTGGGTC	271
AB288266.1	*Methanosarcina horonobensis*	CTTGAAGACCACTTCGGTGGATC	271
AB288268.1	*Methanosarcina subterranea*	CTTGAAGACCACTTCGGTGGGTC	271
U22242.1	*Methanolobus oregonensis*	CTTGAGGACCACTTCGGTGGATC	n/a
EU715818.1	*Methanolobus zinderi*	CTCGAAGACCACTTCGGTGGATC	271
U22243.1	*Methanolobus taylorii*	CTCGAAGACCACTTTGGTGGATC	n/a
AB703629.1	*Methanolobus profundi*	CTCGAAGACCACTTCGGTGGATC	n/a
U22245.1	*Methanolobus vulcani*	CTCGAAGACCACTTCGGTGGATC	n/a
JN081865.1	*Methanocella conradii*	CTCGAGGACCACTTCGGCGGCTC	n/a
KJ441441.1	*Methanocella* sp.	CTCGAGGACCACTTCGGTGGCTC	n/a
AB300467.1	*Methanocella paludicola*	CTCGAGGACCACTTCGGTGGCTC	n/a
AF414044.1	*Methanomicrobium mobile*	ATGGAAGACCACTTCGGCGGTTC	n/a
MH004450.1	*Methanoculleus marisnigri*	ATGGAGGACCACTTCGGCGGTTC	271
AB300784.1	*Methanoculleus palmolei*	ATGGAGGACCACTTCGGCGGGTC	n/a
AB300787.1	*Methanoculleus bourgensis*	ATGGAAGACCACTTCGGCGGGTC	271
AB300779.1	*Methanoculleus chikugoensis*	ATGGAGGACCACTTCGGCGGTTC	271
DQ229160.1	*Methanogenium organophilum*	ATGGAAGACCACTTCGGTGGTTC	271
DQ229161.1	*Methanogenium boonei*	ATGGAAGACCACTTTGGCGGGTC	n/a
JQ917190.1	*Methanobacterium* sp.	CTGGAAGACCACTTTGGCGGTTC	n/a

Table 2. *Cont.*

Acc. No.	Species	*mcrA*_F3 Sequence CTTGAARMTCACTTCGGTGGWTC	Product Size with Primer *mcrA*-Rev (bp) *
GU385700.1	*Methanobrevibacter smithii*	CTTGAAACTCACTTCGGTGGATC	271
LK054628.1	*Methanobrevibacter oralis*	CTCGAAACTCACTTCGGTGGATC	n/a
CP001719.1	*Methanobrevibacter ruminantium*	CTTGAAACTCACTTCGGTGGTTC	271
KF214818.1	*Methanobrevibacter* sp. D5/*mcrA*+ plasmid	CTTGAAACTCACTTCGGTGGATC	271
EU919431.1	*Methanobrevibacter gottschalkii*	CTTGAAACTCACTTCGGTGGATC	271
KC865050.1	*Methanobrevibacter boviskoreani*	CTTGAAACCCAATTCGGTGGATC	271
EU919432.1	*Methanobrevibacter woesei*	CTTGAAACTCAATTCGGTGGATC	271
KC865051.1	*Methanobrevibacter wolinii*	CTTGAAACTCAATTCGGTGGATC	271
AF414035.1	*Methanobrevibacter arboriphilus*	CTTGAAACCCAATTCGGTGGTTC	n/a
HM802934.1	*Methanobacterium movens*	TTAGAAACCCTCTTCGGTGGATC	n/a
AY289750.1	*Methanobacterium thermaggregans*	CTCGAGGACCAGTTTGGTGGATC	n/a
AB300780.1	*Methanothermobacter wolfeii*	CTGGAGGACCAGTTCGGAGGATC	271
AB523786.1	*Methanothermobacter tenebrarum*	CTCGAGACACAATTTGGAGGATC	271
HQ283274.1	*Methanothermobacter crinale*	CTTGAGACACAGTTCGGCGGATC	n/a
KP006500.1	*Methanobacterium aggregans*	CTCGAGACCCAGTTCGGTGGATC	n/a
AY386125.1	*Methanobacterium aarhusense*	TTAGAAACACAGTTCGGTGGATC	n/a
AF313806.1	*Methanobacterium bryantii*	CTTGAAGATCAGTTCGGTGGATC	n/a
X07793.1	*Methanococcus voltae*	TTAGAAGACCACTTCGGTGGCTC	271
AF414048.1	*Methanothermococcus thermolithotrophicus*	TTAGAAGACCACTTCGGAGGTTC	271
AB703637.1	*Methanococcus maripaludis*	TTAGAAGACCACTTCGGTGGATC	271
M16893.1	*Methanococcus vannielii*	TTAGAAGACCACTTTGGTGGATC	271
AY354034.1	*Methanococcus aeolicus*	TTGGAAGACCACTTTGGAGGTTC	271
FJ982887.1	*Methanosphaera* sp.	TTAGAAGACCACTTCGGTGGATC	271
AF414047.1	*Methanosphaera stadtmanae*	TTAGAAGACCACTTTGGTGGATC	271

* n/a—not available due to incomplete *mcrA* sequence.

3.2. Initial Comparison of the Mlas and mcrA_F3 Forward Primers

Both primer pairs were compared under very similar temperature conditions and timings as presented in the original study in order to exclude any additional effects on the assay performance. Since the amplicons generated with the use of the *mcrA*_F3/*mcrA*-rev primer pair were shorter and had a slightly lower melting temperature (Tm), the temperature of fluorescence acquisition had to be adjusted—it was set to 81 °C instead of 83 °C.

At an annealing temperature of 55 °C, both primers performed similarly well in terms of Cq values for the five most concentrated dilutions of the *mcrA*+ plasmid (Figure 1). However, there was a profound difference in the shape and height of the amplification curves as the dilution ratio increased. In contrast to the original primer pair, the *mcrA*_F3/*mcrA*-rev pair generated curves that were well-pronounced until the last standard dilution, i.e., 6.24 copies of the *mcrA*+ plasmid per reaction. Since there was a minimal signal from the non-template control (NTC), an optimization of the real-time PCR conditions for the new primer pair was required to keep primer dimerization as minimal as possible.

3.3. Gradient PCR

The primer pair *mcrA*_F3/*mcrA*-rev performed best at an annealing temperature (Ta) in the range of 59–60 °C. Amplification at 61 °C and above resulted in increasing primer dimerization (Figure 2).

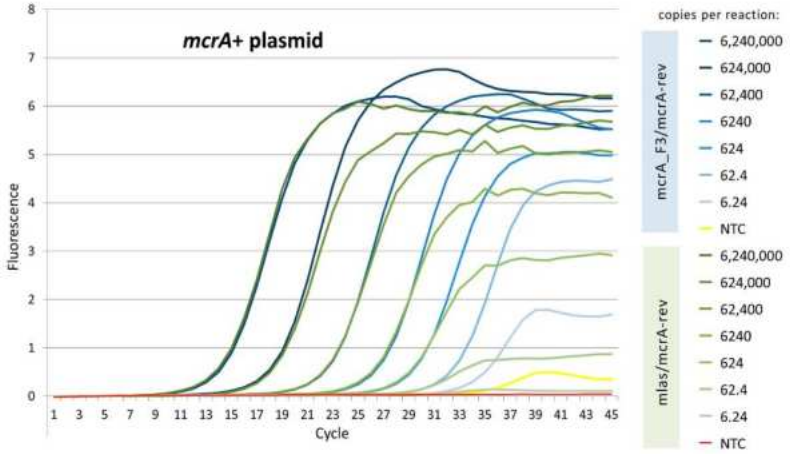

Figure 1. Comparison of amplification curves generated with annealing temperature of 55 °C.

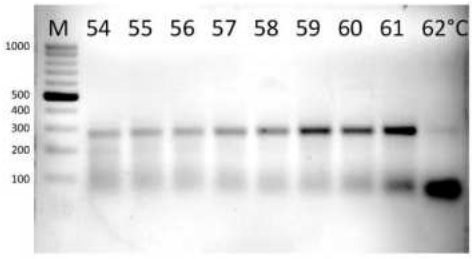

Figure 2. The results of gradient PCR. The optimal Ta was between 59 and 60 °C. The product length was 270 bp. M—DNA ladder.

3.4. Selected Validation Parameters of the Assays

At approx. 10^1 copies per reaction, 100% of the replicates of all four standard DNAs tested positive using the *mcrA*_F3/*mcrA*-rev primer pair, whilst only three standard DNAs tested positive using the mlas/*mcrA*-rev primer pair (Table 3). The only exception—*Methanomicrobium mobile*—had two limits of detection determined for each primer pair separately, i.e., 5.71×10^2 (or 2.76 log10) and 5.71×10^1 (or 1.76 log10) copies per reaction using the original and the *mcrA*_F3/*mcrA*-rev primer pair, respectively.

Moreover, the *mcrA*_F3/*mcrA*-rev primer pair demonstrated a higher amplification specificity than the mlas/*mcrA*-rev primer pair, as it favored the generation of specific products over primer dimers, even at low concentrations of the target DNAs. This was especially noticeable in the case of *Methanomicrobium mobile* and the *mcrA*+ plasmid (Table 3; Figure 3).

Table 4 presents the reproducibility and efficiency of the assays. Only the Cq values for the specific reactions are reported. Efficiency within the acceptable range of 90 to 110% was observed in all cases. Amplification with the *mcrA*_F3/*mcrA*-rev primer pair occurred with lower efficiency, and it was more variable at lower template concentrations.

Table 3. Detection of the *mcrA* gene in both assays.

Estimated Plasmid/Genome Copy No.	mlas/*mcrA*-Rev			*mcrA*_F3/*mcrA*-Rev		
	No. Positive/No. Tested		Melting Analysis *	No. Positive/No. Tested		Melting Analysis *
***mcrA*+ Plasmid**						
6.24×10^6	8/8		S	9/9		S
6.24×10^5	8/8			9/9		
6.24×10^4	8/8			9/9		
6.24×10^3	8/8			9/9		
6.24×10^2	8/8			10/10		
6.24×10^1	8/8	6/8		10/10		
		2/8	S + PD			
6.24×10^0	2/8		S + PD	3/10		S + PD
6.24×10^{-1}	0/8		PD	0/10		PD
***Methanobrevibacter woesei* DSM 11979**						
6×10^6	n/a		n/a	2/2		S
6×10^5	8/8		S	7/7		
6×10^4	8/8			7/7		
6×10^3	8/8			7/7		
6×10^2	8/8			8/8		
6×10^1	8/8	2/8		8/8	4/8	
		6/8	S + PD		4/8	S + PD
6×10^0	2/8		S + PD	2/8		S + PD
6×10^{-1}	0/8		PD	0/8		PD
***Methanococcus maripaludis* DSM 2067**						
2.11×10^6	8/8		S	9/9		S
2.11×10^5	8/8			9/9		
2.11×10^4	8/8			9/9		
2.11×10^3	8/8			9/9		
2.11×10^2	8/8			9/9		
2.11×10^1	8/8	2/8		9/9	3/9	
		6/8	S + PD		6/9	S + PD
2.11×10^0	2/8		S + PD	3/9		S + PD
2.11×10^{-1}	0/8		PD	0/9		PD
***Methanomicrobium mobile* DSM 1539**						
5.71×10^6	8/8		S	7/7		S
5.71×10^5	8/8			7/7		
5.71×10^4	9/9			7/7		
5.71×10^3	8/8			7/7		
5.71×10^2	9/9	1/9		7/7	4/7	
		8/9	S + PD		3/7	S + PD
5.71×10^1	1/8		S + PD	7/7		S + PD
5.71×10^0	0/8		PD	0/7		PD
5.71×10^{-1}	0/8		PD	0/7		PD

* S = specific amplicons (mlas/*mcrA*-rev: 83.82 for *mcrA*+ plasmid, 83.27 for *Methanobrevibacter woesei*, 84.58 for *Methanococcus maripaludis*, and 87.08 °C for *Methanomicrobium mobile* amplicons; *mcrA*_F3/*mcrA*-rev: 83.55 for *mcrA*+ plasmid, 83.08 for *Methanobrevibacter woesei*, 84.65 for *Methanococcus maripaludis*, and 86.91 °C for *Methanomicrobium mobile* amplicons); PD = primer dimers.

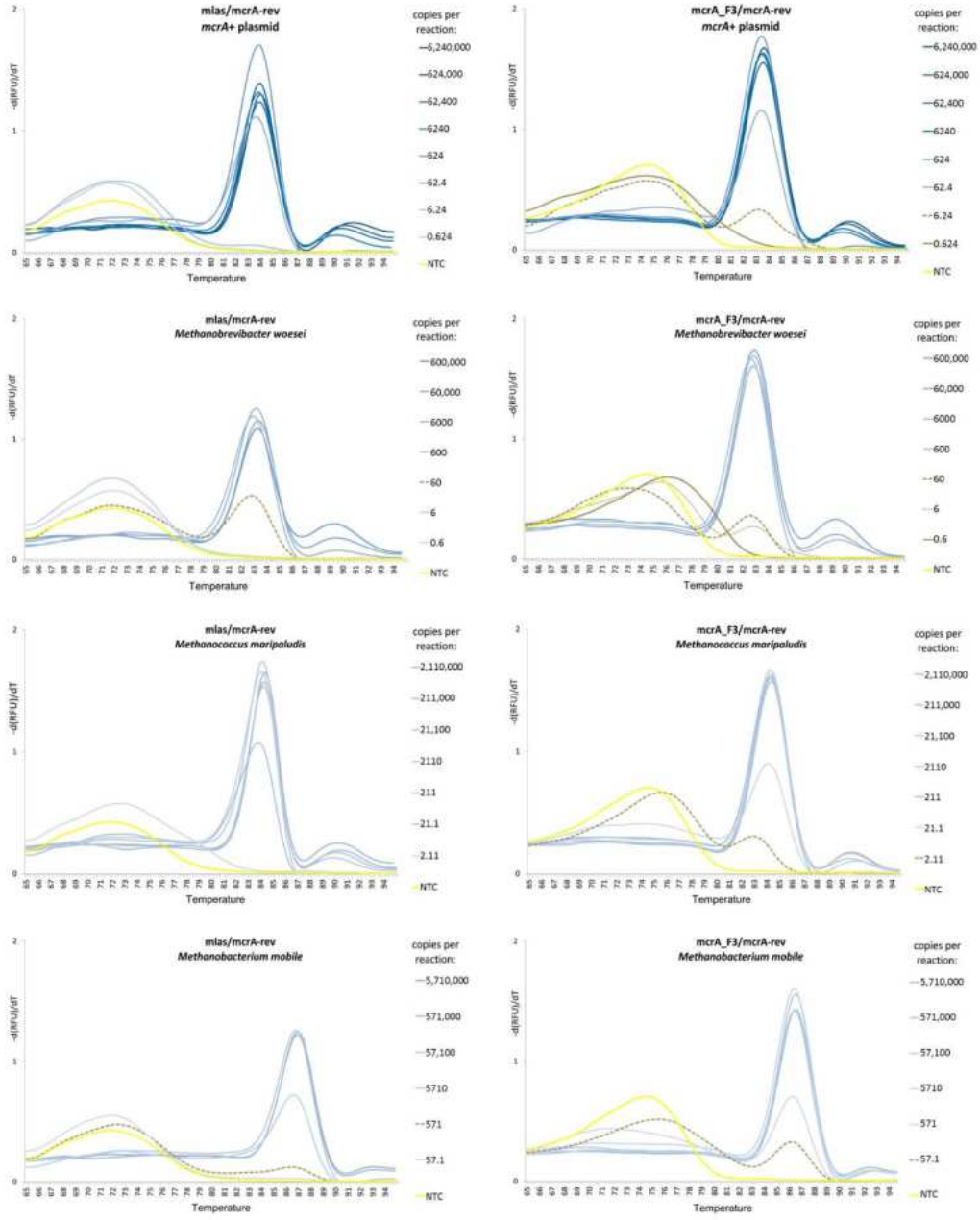

Figure 3. The melting profiles of 4 standards generated by using mlas/*mcrA*-rev and *mcrA*_F3/*mcrA*-rev primer pairs.

Table 4. Reproducibility and efficiency of the assays.

Estimated Plasmid/Genome Copy No.	Mlas/*mcrA*-Rev					*mcrA*_F3/*mcrA*-Rev				
	Intra-Assay Variability		Inter-Assay Variability		Efficiency (%)	Intra-Assay Variability		Inter-Assay Variability		Efficiency (%)
	Mean Cq ± SD	CV%	Mean Cq ± SD	CV%		Mean Cq ± SD	CV%	Mean Cq ± SD	CV%	
***mcrA*+ Plasmid**										
6.24×10^6	13.32 ± 0.02	0.11	13.20 ± 0.14	1.03		14.94 ± 0.17	1.10	15.11 ± 0.03	0.20	
6.24×10^5	17.09 ± 0.08	0.48	17.05 ± 0.35	2.05		19.16 ± 0.13	0.68	19.24 ± 0.19	0.97	
6.24×10^4	21.08 ± 0.17	0.81	21.01 ± 0.47	2.24	91.69	23.48 ± 0.28	1.18	23.01 ± 0.4	1.76	90.92
6.24×10^3	24.72 ± 0.12	0.48	24.71 ± 0.24	0.96		27.32 ± 0.38	1.40	27.10 ± 0.29	1.05	
6.24×10^2	28.26 ± 0.24	0.84	27.75 ± 0.55	1.97		30.91 ± 0.12	0.38	30.32 ± 0.8	2.64	
6.24×10^1	30.58 ± 0.04	0.13	30.81 ± 0.57	1.85		33.77 ± 0.07	0.21	32.57 ± 0.77	2.36	
6.24×10^0	31.56 * (1/3)	n/a	31.34 * (2/3) ± 0.31	0.99	n/a	34.55 * (1/3)	n/a	33.72 ± 1.05	3.11	n/a
6.24×10^{-1}	negative					negative				
***Methanobrevibacter woesei* DSM 11979**										
6×10^5	18.09 ± 0.21	1.14	18.45 ± 0.4	2.14		18.69 ± 0.34	1.81	19.62 ± 0.78	3.95	
6×10^4	21.80 ± 0.12	0.56	21.63 ± 0.41	1.89		22.95 ±0.2	0.88	23.33 ± 0.51	2.18	
6×10^3	25.38 ± 0.3	1.17	25.29 ± 0.4	1.57	109.33	26.89 ± 0.18	0.68	26.72 ± 0.08	0.29	98.96
6×10^2	28.69 ± 0.13	0.45	28.43 ± 0.33	1.15		30.87 ± 0.11	0.36	30.53 ± 0.24	0.79	
6×10^1	30.48 ± 0.15	0.50	30.63 ± 0.05	0.17		32.88 ± 0.3	0.92	32.76 ± 0.25	0.77	
6×10^0	30.82 * (2/3) ± 0.21	0.69	30.97 * (1/3)	n/a	n/a	negative		33.90 * (2/3) ± 0.7	2.07	n/a
6×10^{-1}	negative					negative				
***Methanococcus maripaludis* DSM 2067**										
2.11×10^6	14.41 ± 0.25	1.72	14.79 ± 0.05	0.35		16.09 ± 0.52	3.22	16.80 ± 0.28	1.64	
2.11×10^5	18.07 ± 0.08	0.44	18.54 ± 0.04	0.24		20.30 ± 0.33	1.61	20.61 ± 0.15	0.71	
2.11×10^4	22.53 ± 0.04	0.19	22.15 ± 0.3	1.37	103.18	24.26 ± 0.24	0.97	24.17 ± 0.31	1.29	90.84
2.11×10^3	25.98 ± 0.09	0.34	25.43 ± 0.52	2.06		28.14 ± 0.21	0.73	27.81 ± 0.63	2.27	
2.11×10^2	29.21 ± 0.13	0.46	28.50 ± 0.61	2.14		31.81 ± 0.19	0.60	31.44 ± 0.44	1.40	
2.11×10^1	30.84 ± 0.07	0.23	30.90 ± 0.17	0.55		34.87 ± 0.26	0.74	34.51 ± 0.81	2.36	
2.11×10^0	negative		31.11 * (2/3) ± 0.06	0.18	n/a	negative		34.87 ± 0.59	1.68	n/a
2.11×10^{-1}	negative					negative				
***Methanomicrobium mobile* DSM 1539**										
5.71×10^6	18.47 ± 0.05	0.29	18.31 ± 0.21	1.12		20.19 ± 0.56	2.79	19.49 ± 0.78	4.01	
5.71×10^5	22.22 ± 0.03	0.11	22.00 ± 0.22	1.00		24.22 ± 0.3	1.24	23.31 ± 0.79	3.38	
5.71×10^4	25.85 ± 0.09	0.33	25.44 ± 0.42	1.63	104	28.32 ± 0.14	0.48	27.38 ± 0.74	2.69	97.67
5.71×10^3	29.34 ± 0.2	0.69	28.68 ± 0.41	1.42		32.46 ± 0.26	0.79	30.54 ± 0.82	2.67	
5.71×10^2	30.65 ± 0.3	0.97	31.12 ± 0.16	0.52		35.21 ± 0.59	1.69	33.86 ± 1.07	3.17	
5.71×10^1	negative		31.73 * (1/3)	n/a	n/a	36.17 ± 0.79	2.18	36.19 ± 0.77	2.12	
5.71×10^0	negative					negative				
5.71×10^{-1}	negative					negative				

* instances where not all replicates tested positive; n/a—not applicable.

The dynamic range, referred to as the range of template input generating a linear curve ($R^2 \geq 0.980$) with acceptable efficiency, was larger in the case of the new primer pair. Here, the dynamic range of the real-time PCR assay measured for three standard DNAs

(*mcrA*+ plasmid, *Methanococcus maripaludis*, and *Methanomicrobium mobile*) spanned 6 to 7 orders of magnitude, and for *Methanobrevibacter woesei*, it spanned 5 orders of magnitude. In contrast, amplification with the use of the mlas/*mcrA*-rev primer pair generated only two standard curves covering a linear range of 6 orders of magnitude (of the *mcrA*+ plasmid and *Methanococcus maripaludis*). The dynamic range for the remaining two standard DNAs (*Methanobrevibacter woesei* DSM 11979 and *Methanomicrobium mobile*) covered only 5 orders of magnitude.

The R^2 value was between 0.9934 and 0.9966 for the mlas/*mcrA*-rev primer pair and between 0.9913 and 0.9992 for the *mcrA*_F3/*mcrA*-rev primer pair.

The reproducibility of the assays as defined by the values of standard deviation (SD) and the coefficient of variation (CV%) in the within-run and between-run tests are reported independently for all four tested *mcrA*+ genomes in Table 4.

The Cq values of the generated non-target controls were higher in the case of the new *mcrA*_F3/*mcrA*-rev primer pair, usually well above the value of 30 (Table 5). Moreover, by comparing the Cq values of the bacterial DNA and the Cqs of the last *mcrA*+ dilution points, the comparison favors the new primer pair, as the calculated difference between the Cqs is simply bigger. In contrast, as many as 18 non-target controls had a Cq lower than 30.8, which was the lowest reported value for the last standard dilution point for the mlas/*mcrA*-rev primer pair (Table 4). Moreover, the amplification curve generated, e.g., from *Escherichia coli* by using the original primer pair indicated a strong false-positive result (viewed as a high rate of increase), which was not observed in the case of the *mcrA*_F3/*mcrA*-rev primer pair (Figure 4).

Table 5. The amplification results of the non-target controls.

DNA Template	Estimated Genome Copy No. Per Reaction or Amount *	Cq	
		Mlas/*mcrA*-Rev	*mcrA*_F3/*mcrA*-Rev
Escherichia coli ATCC 8739	4.38×10^5 copies	26.76	34.17
Salmonella Typhimurium ATCC 14028	3.44×10^5 copies	31.58	34.06
Clostridium septicum ATCC 12464	4.48×10^5 copies	31.19	32.98
Lactobacillus sakei ATCC 15521	6.6×10^6 copies	29.59	30.41
Enterococcus avium ATCC 14025	5.86×10^6 copies	26.14	32.41
Enterococcus casseliflavus ATCC 700327	2.48×10^6 copies	26.47	33.18
Enterococcus raffinosus ATCC 49464	7.7×10^4 copies	27.65	33.20
Blautia obeum DSM 25238	2.08×10^7 copies	28.11	34.28
Ruminococcus gauvreauii DSM 19829	8.83×10^5 copies	27.82	35.21
Helicobacter cinaedi DSM 5359	1.71×10^7 copies	24.63	36.36
Desulfovibrio piger DSM 749	1.32×10^8 copies	29.06	34.49
Proteus sp.	6.1 ng	23.15	32.92
Streptococcus sp.	17 ng	26.45	34.66
Streptococcus sp. (beta-hemolytic)	0.118 ng	28.21	34.46
Corynebacterium sp.	1.15 ng	30.43	36.49
Pseudomonas aeruginosa	70 ng	26.35	32.14
Pasteurella sp.	0.377 ng	31.43	37.80
Klebsiella sp.	28 ng	26.23	32.45
Staphylococcus sp. (coagulase-negative)	3.28 ng	26.98	36.10
Porphyromonas sp.	2.43 ng	25.53	29.05
Bacteroidetes bacterium	1.13 ng	27.18	36.13

* fluorimetric measurements.

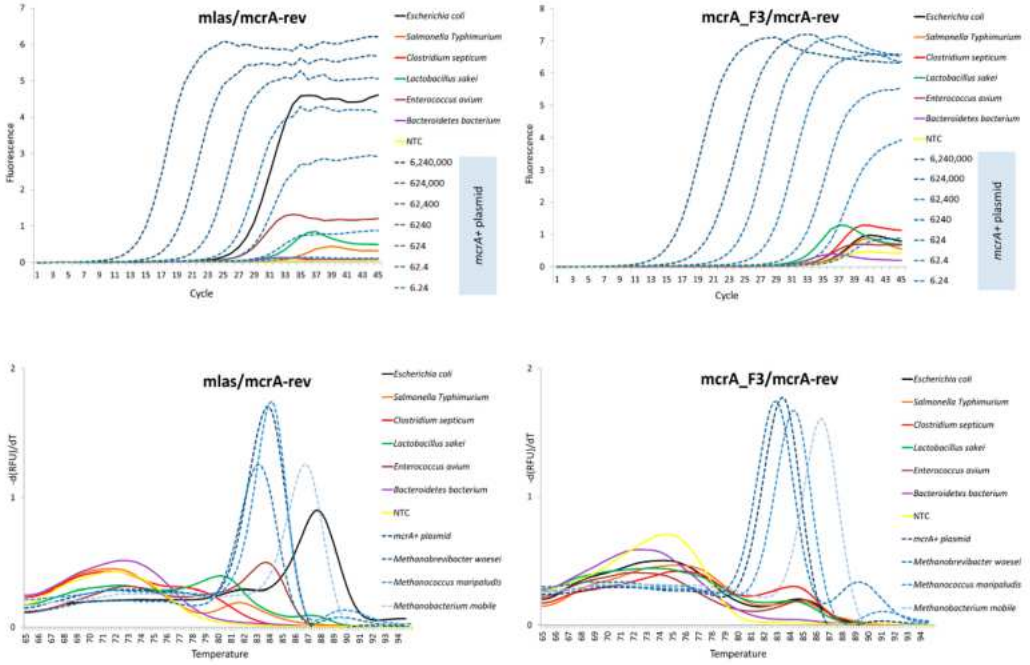

Figure 4. The melting profiles and the amplification plots of non-target controls set together with all 4 positive controls (upper charts) and with dilution series of the *mcrA*+ plasmid (lower charts) for comparison.

The melting profile of the mlas/*mcrA*-rev amplicons generated from the bacterial DNAs indicated a substantial amplification of the non-specific products from *E. coli* and *Enterococcus avium*. Those amplicons had a Tm within or above the range of specific products, making them measurable in terms of the Cq. In contrast, the tendency to amplify products with a Tm around the specific peak was marginal when the mcrA_F3/*mcrA*-rev primer pair was used (Figure 4).

3.5. Methanogen Load in Chicken Dropping Samples

Given that the temperature in the range between approx. 83 and 87 °C was determined experimentally as being *mcrA*-specific, only seven dropping samples could be considered *mcrA*+ using the original mlas/*mcrA*-rev primer pair (Table 6). In contrast, as many as 13 samples fell under the same criteria when the *mcrA*_F3/*mcrA*-rev was used, and another 7 samples were just under the lower limit.

The primer pair mlas/*mcrA*-rev did not amplify any specific product in the remaining 13 dropping samples despite generating low Cq values in all cases but one (sample 8).

Table 6. Methanogens detected in chicken dropping samples with the use of mlas/*mcrA*-rev and *mcrA*_F3/*mcrA*-rev primer pairs.

Sample	Mlas/*mcrA*-Rev		*mcrA*_F3/*mcrA*-Rev	
	Cq	Tm (°C)	Cq	Tm (°C)
1	24.82	77.84; 82.25	28.17	82.51
2	24.54	71.84	29.62	79.47; 83.69
3	24.50	77.94; 83.23	23.17	83.36
4	25.59	83.18; 88.91	27.98	82.55
5	25.16	83.43	27.63	83.59
6	28.19	74.85	31.14	80.32; 83.28
7	28.09	77.62	30.63	83.05
8	negative	75.82; 80.96	28.27	77.08; 83.67
9	27.20	83.07	25.34	83.13
10	25.57	79.63; 83.17	28.15	83.11
11	25.73	80.65	28.58	77.11; 83.31
12	27.93	77.41	30.53	83.11
13	27.67	75.94; 82.02	28.80	82.88
14	28.36	82.97	29.40	77.51; 82.87
15	30.45	79.71	31.22	76.09; 82.87
16	25.60	75.51; 81.37	29.85	82.98
17	26.47	76.13; 81.10	28.90	76.59; 81.80
18	28.14	75.72	29.86	77.92; 83.12
19	29.29	75.91; 83.01	29.93	83.12
20	26.96	74.38	28.48	82.88

4. Discussion

Publications bringing up the subject of primer and qPCR validation are sparse, with a strong emphasis being placed on pathogens [23–25]. This paper is—to the best of our knowledge—the first one that focuses on methanogenic archaea in dropping samples of chickens.

The primers first designed by Steinberg and Regan [21] were used with success by several authors, providing a great deal of knowledge on the subject of diversity and the prevalence of methanogenic archaea in various environments, i.e., in landfills, wastewater, the gastrointestinal tracts of insects, and human stool samples [26–29].

However, our very preliminary research showed that, by using these primers together with the different temperature settings reported previously for qPCR by Steinberg and Regan [18] and Lecours et al. [6], methanogens remained undetected in the majority of the tested dropping samples. In our opinion, the reason behind this was the length of the generated *mcrA* amplicons of approx. 470–490 bp. Therefore, we tried to explore the possibility of designing a new assay.

Once the new forward primer was designed, the next step was to compare its performance to that of the original one. In order to exclude the potential influence of any other conditions (such as the annealing temperature) on the primer performance, this test was performed as closely as possible to the conditions described in the original study by Steinberg and Regan [18]. By changing only one primer, we were able to obtain a significant improvement. Since there was some evidence of primer dimerization resulting in the generation of a signal in the NTC, the real-time PCR protocol had to be optimized. As a consequence, the *mcrA*_F3 forward primer—together with the original reverse primer and improved PCR conditions—was able to generate amplicons of approx. 270 bp under more stringent conditions, which, as was later proven, was enough to detect methanogens in almost all tested samples that previously underwent mechanical lysis according to the protocol we designed previously [14]. In the aforementioned study, we used a hydrolysis probe in order to reduce any unspecific signals that could have arisen from the primer dimers [14]; however, the use of this probe might have resulted in the loss of some reads from the methanogenic archaea due to potential mismatches between the probe and its target. Here, we tried a different approach—a four-step real-time PCR with fluorescence

acquisition at temperatures just under the point of melting of the specific products. Such changes induced the generation of lower Cq values of the assay.

A higher annealing temperature favored the generation of specific products over primer dimers, even at low concentrations of the target DNAs. Moreover, our assay improved specificity with respect to the cross-reactions with the non-target controls. In the original assay, even the temperature of fluorescence acquisition set to 83 °C was not high enough to prevent one from measuring the Cq of those non-specific products, since their Tm was, in some cases, as high as or even higher than the Tm of the specific ones. Of course, the non-specific reactions using the mlas/*mcrA*-rev primer pair may have occurred due to the fact that the annealing temperature was relatively low, but by analyzing the results from the initial experiment, no further optimization would have given results similar to the primer pair proposed in our study.

Although the Cq values of the standards generated using the *mcrA*_F3/*mcrA*-rev primer pair were generally higher than those generated using the previously published primer pair, the ability to differentiate between the positive and the non-target controls by comparing the Cq values was much higher using the *mcrA*_F3/*mcrA*-rev primer pair. In general, the *mcrA*_F3/*mcrA*-rev assay led to the amplification of three out of four positive controls and all dropping samples with a higher sensitivity. Both the intra- and inter-assay CVs for the standard DNAs were satisfactory low. This assay was also more specific than the mlas/*mcrA*-rev assay, and it had a reproducible limit of detection of approx. 21 to 57 copies of target DNA per reaction depending on the DNA template used.

Steinberg and Regan [18] reported that they were able to achieve a limit of detection of approx. 415 copies per reaction with the use of the mlas/*mcrA*-rev primer pair. Our results show that the limit of detection generated by the same primers is in the range of 21 to 571 copies per reaction, which is in line with the original study.

5. Conclusions

The current results present a novel qPCR approach in the detection of methanogenic archaea using the *mcrA*_F3/*mcrA*-rev primer pair. Although the proposed protocol was not able to entirely eliminate non-specific reactions (cross-amplification), most validation parameters improved significantly. Moreover, the shortening of the amplicon allowed for a more accurate quantification of methanogenic archaea in dropping and stool samples, especially those that underwent mechanical lysis during DNA isolation. In the long run, the proposed protocol may improve the detection rates of methanogens in human and animal gastrointestinal tracts and contribute to a better understanding of the role of archaea in the health and disease of their hosts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms11030660/s1, Table S1: Multiple alignments of the *mcrA* sequence fragment of 47 methanogenic archaea. Table S2: Multiple alignments of the *mcrA* sequence fragment of 26 *Methanobrevibacter* and environmental species; nucleotides in bold represent the target of the *mcrA*_F3 primer.

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Microorganisms Involved in Hydrogen Sink in the Gastrointestinal Tract of Chickens

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Abstract: Hydrogen sink is a beneficial process, which has never been properly examined in chickens. Therefore, the aim of this study was to assess the quantity and quality of microbiota involved in hydrogen uptake with the use of real-time PCR and metagenome sequencing. Analyses were carried out in 50 free-range chickens, 50 commercial broilers, and 54 experimental chickens isolated from external factors. The median values of acetogens, methanogens, sulfate-reducing bacteria (SRB), and [NiFe]-hydrogenase utilizers measured in the cecum were approx. 7.6, 0, 0, and 3.2 $\log_{10}$/gram of wet weight, respectively. For the excreta samples, these values were 5.9, 4.8, 4, and 3 $\log_{10}$/gram of wet weight, respectively. Our results showed that the acetogens were dominant over the other tested groups of hydrogen consumers. The quantities of methanogens, SRB, and the [NiFe]-hydrogenase utilizers were dependent on the overall rearing conditions, being the result of diet, environment, agrotechnical measures, and other factors combined. By sequencing of the 16S rRNA gene, archaea of the genus *Methanomassiliicoccus* (*Candidatus Methanomassiliicoccus*) were discovered in chickens for the first time. This study provides some indication that in chickens, acetogenesis may be the main metabolic pathway responsible for hydrogen sink.

Keywords: acetogens; *Campylobacter jejuni*; hydrogen uptake; methanogenic archaea; *Methanomassiliicoccus*; chicken gut microbiota; sulfate-reducing bacteria

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1. Introduction

Intestinal fermentation is essential for sustaining the host's well-being and proper functioning. It contributes to the breakdown of dietary fiber and other otherwise indigestible compounds into more accessible products, such as short-chain fatty acids (SCFAs) [1]. Hydrogen—a fermentation byproduct—when accumulated, has the ability to inhibit the regeneration of electron carriers by bacterial fermenters, and therefore adversely inhibits fermentation. The problem of hydrogen buildup appears to be solved by the process of hydrogen sink carried out by certain groups of intestinal microorganisms [2].

The production of butyrate—the most substantial energy source for colonocytes—generates large amounts of free hydrogen, larger than the formation of other SCFAs, such as propionate [1,3]. In order to ensure the continuity of this process, and to keep the proper development of intestinal villi, hydrogen sink seems essential [1]. Moreover, its role is to maintain the proper structure of the gut microbiome and homeostasis, as high concentrations of H_2 were found to affect both the hydrogen-producing and the non-hydrogen-producing microorganisms [3]. There is also a direct link found between the dysbiosis involving the methanogenic archaea (one of the hydrogen consumers) and overall dysbiosis affecting the host's health [4]. In a broader sense, hydrogen sink is indirectly related to the maintenance of the host's health.

There are only a few publications mentioning the presence of hydrogen consumers in chickens, and thus the issue is poorly understood. It is known that some species of bacteria and archaea found in the intestinal microbiome of chickens can be the providers of enzymes that ensure the utilization of hydrogen, and the continuity of intestinal fermentation [5]. For instance, bacteria of the genera *Megamonas*, *Wolinella*, *Helicobacter*, and *Campylobacter* (including *C. jejuni*) produce nickel–iron [NiFe]-hydrogenases, and bacteria of the *Lachnospiraceae* family—the acetyl-coenzyme A synthase, and methanogenic archaea—produce methyl-coenzyme M reductase. Of course, the share of individual enzymes, and thus the microbionts producing them, is diverse [5]. In addition, some publications presenting this issue stand in conflict with each other: some authors argue that certain groups of microbionts—such as methanogenic archaea—do not occur in the chicken intestines, and therefore cannot affect the metabolism of hydrogen [6,7], whilst others clearly indicate their presence [8].

Only a few publications mention the existence of methanogenic archaea in the chicken gut microbiome, highlighting the dominant role of *Methanobrevibacter woesei* [9]. Experiments on rumen microbiota point out the competition between the methanogenic archaea and acetogens, and also the sulfate-reducing bacteria (SRB) [10]. No other similar phenomenon has ever been studied in chickens. On the other hand, to the best of our knowledge, only three publications mention any correlation between the bacteria of the family *Lachnospiraceae* and *C. jejuni* in chickens, but their focus was not on hydrogen sink whatsoever [11–13]. That is why our study addresses this issue.

The necessity of elimination of *C. jejuni* from chicken rearing is a matter of public health because campylobacteriosis is the most common cause of foodborne gastrointestinal infection in humans, and has been so since at least 2007 [14]. Broiler meat (and products thereof) is considered the main source of the human campylobacteriosis [14]. Moreover, these infections may have long-term consequences, including the Guillain–Barré syndrome [15]. In poultry, *C. jejuni* is not exactly meaningless either, since it may be correlated with the impairment of the bird's well-being, and reduction in animal productivity [16]. After all, a lot of effort has been put into studying the competitive exclusion of *C. jejuni* by lactobacilli [1]. There is even an entire branch of industry that sells certain strains of lactic acid bacteria as probiotics. However, in birds we observe a kind of evolutional adaptation and a slight "tolerance" toward *C. jejuni*, which may indicate that perhaps this bacterium plays a certain role in the chicken gut [12]. Hypothetically, by providing enzymes for hydrogen sink, *C. jejuni* may even become useful for its host. For this reason, some part of our study has been directed at the detection and quantification of both *C. jejuni* and *Ligilactobacillus salivarius* (as a representative of the lactic acid bacteria), and their possible interactions.

In summary, the aim of this study was to identify microbionts involved in the hydrogen sink within the gastrointestinal tracts of chickens with the use of both real-time PCR and sequencing techniques.

2. Results

2.1. The Effect of Sample Type on the Composition of Microbiota

There was a statistically significant difference between the excreta and the cecal contents in five out of six studied microbial groups (Figure 1). The median values of the methanogenic archaea, SRB, *L. salivarius*, and *C. jejuni* were higher in the excreta samples. The acetogens, on the other hand, were more abundant in the cecal content samples than in the excreta. The differences in counts of the [NiFe]-hydrogenase utilizers were negligible.

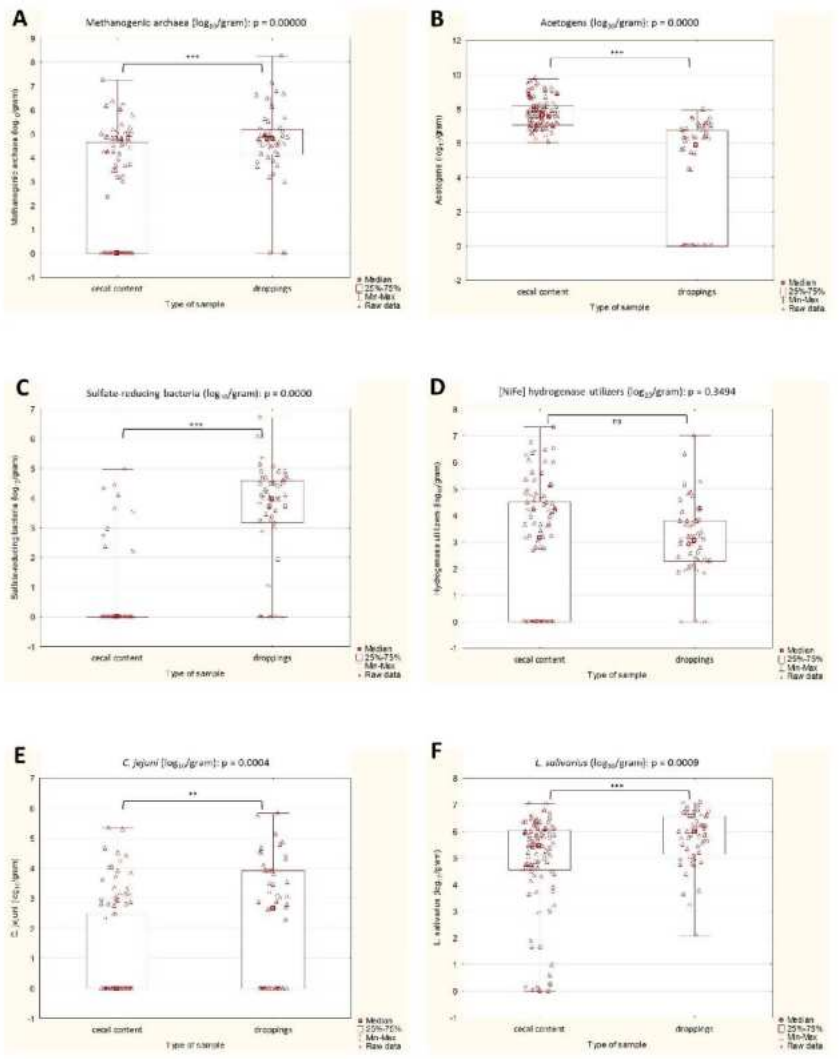

Figure 1. (**A**–**F**). Box plots representing the abundance of selected microbial groups in ceca and excreta samples. Statistical significance between cecal contents and excreta is marked by asterisks. Values of ** $p < 0.01$, and *** $p < 0.001$ were regarded as significant; ns: non-significant.

2.2. The Effect of Three Rearing Methods on the Composition of Microbiota

The amount of acetogens varied significantly among all three methods of chicken rearing (and the sample type). The highest, statistically significant counts of these microbes were observed in the cecal contents of the commercial chickens, followed by the cecal contents of the experimental chickens, and the excreta of the free-range chickens, which had the lowest median of acetogens (Figure 2).

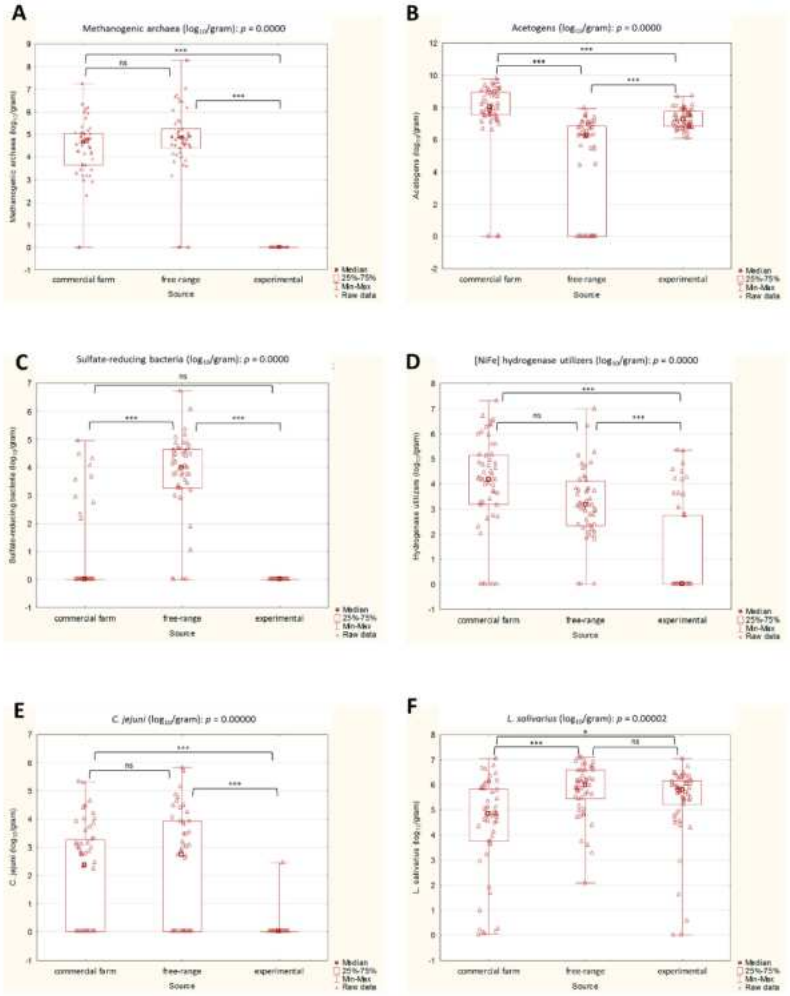

Figure 2. (**A**–**F**). Box plots representing the abundance of selected microbial groups in three rearing systems. Statistical significance among the three groups is marked by asterisks. Values of * $p < 0.05$, and *** $p < 0.001$ were regarded as significant; ns: non-significant.

When comparing the experimental vs. free-range chickens, statistically significant differences in the medians were observed in methanogenic archaea, the [NiFe]-hydrogenase utilizers, and *C. jejuni*. The abundance of these three groups was higher in the excreta of free-range chickens than in the ceca of the experimental group. Similar observations were made for the experimental vs. commercial chickens, where the counts of methanogens, [NiFe]-hydrogenase utilizers, and *C. jejuni* in the ceca of commercial chickens also exceeded those reported in the ceca of the experimental group.

As for the SRB, statistical significance was reported between the free-range and commercial farm chickens, and for the free-range vs. experimental chickens. In both of these cases, the comparison favored the excreta of the free-range, which were the only ones that had a median value above 0.

In the case of *L. salivarius*, statistical significance was observed only in the comparison of commercial chickens to experimental and free-range chickens. The lactobacilli counts reported in the ceca of the commercial chickens were lower than those observed in the other two groups.

2.3. The Effect of Age on the Composition of Microbiota

In commercial chickens, acetogens were the most varying variable among the chicken age groups (Figure 3). A statistical significance was observed between week 1 and week 3, and week 1 vs. weeks 5–6. In addition, the differences between week 3 and weeks 3–4, and weeks 3–4 vs. weeks 5–6 were statistically important.

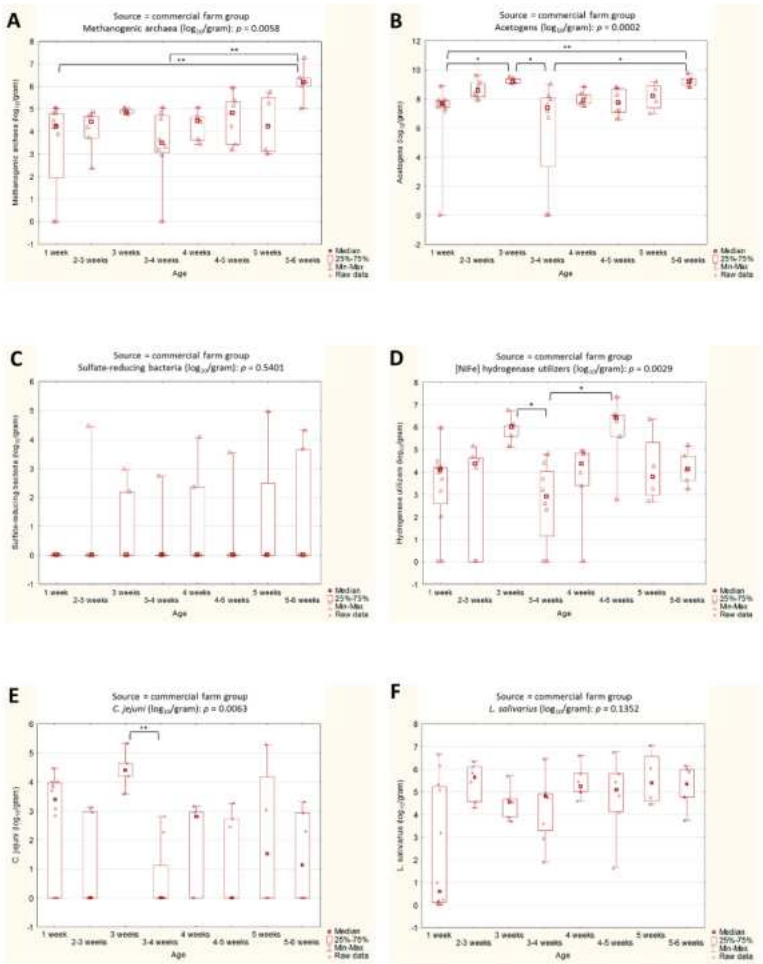

Figure 3. (**A**–**F**). Box plots representing the abundance of selected microbial groups across age groups in the commercial chickens. Statistical significance among the age groups is marked by asterisks. Values of * $p < 0.05$, and ** $p < 0.01$ were regarded as significant. The non-significant results remained unmarked.

Only two statistically significant median differences were observed in the methanogenic archaea (week 1 vs. weeks 5–6, and weeks 3–4 vs. 5–6) and the [NiFe]-hydrogenase utilizers (week 3 vs. 3–4, and weeks 3–4 vs. 4–5). Counts of *C. jejuni* differed in one case, i.e., between weeks 3 and 3–4. No statistical significance among the age groups was observed in the SRB and *L. salivarius* median counts.

By contrast, in the experimental chickens, the lactobacilli counts were the only variable that was statistically significant in relation to the chicken age (Figure 4). The median values between day 0 and days 4, 7, and 14 were significantly higher. The opposite relationship was observed between days 4 and 21. Interestingly, in this case, the younger chick had higher counts of *L. salivarius*.

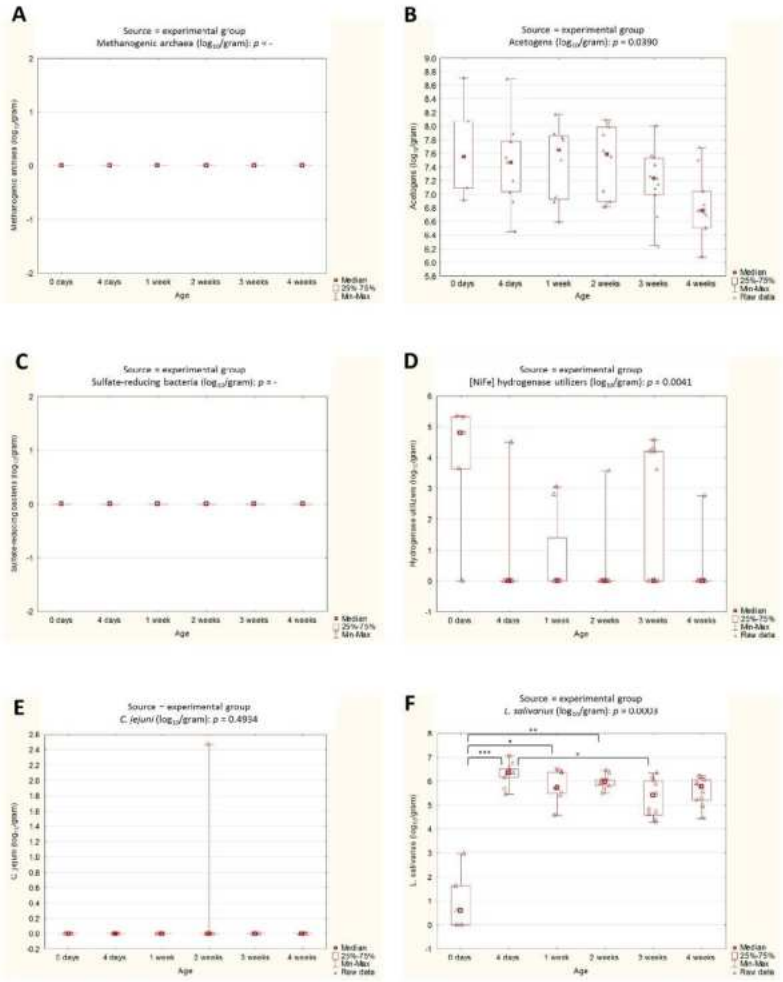

Figure 4. (**A**–**F**). Box plots representing the abundance of selected microbial groups across age groups in the experimental farm chickens. Statistical significance among age groups is marked by asterisks. Values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were regarded as significant. The non-significant results remained unmarked.

As for the acetogen counts in the ceca of experimental chickens, the general trend was to decrease with chicken age, especially by the end of the experiment; however, those differences were not statistically proven. No other linear trend was observed among the age groups in either the experimental or commercial farm chickens.

The age vs. microbial populations relationship in the excreta of the free-range chickens was impossible to establish due to technical reasons.

2.4. Interactions among Microbionts Involved in Hydrogen Sink

The analyses of associations among the microbionts involved in hydrogen sink showed negligible (R_s between 0 and 0.20) to moderate (R_s between 0.41 and 0.60) correlations among six studied microbial populations when the three rearing groups were tested together (Tables S1–S4). When correlations were analyzed separately for each of the rearing groups, the highest number of statistically significant relationship results—altogether six, weakly to moderately correlated—was observed in the free-range chickens alone: methanogens vs. acetogens (R_s 0.38), SRB vs. *C. jejuni* (R_s 0.39), *L. salivarius* vs. [NiFe]-hydrogenase utilizers (R_s 0.57), acetogens vs. SRB (R_s 0.45), and SRB vs. [NiFe]-hydrogenase utilizers (R_s 0.41). There was only one strongly correlated relationship between the [NiFe]-hydrogenase utilizers and *C. jejuni* (R_s 0.76). All these correlations were positive.

When correlations were analyzed in the commercial farm chicken group, we observed a strong and positive correlation between methanogens and acetogens (R_s 0.64). The other three relationships were weakly correlated: acetogens vs. SRB (R_s 0.36), acetogens vs. *L. salivarius* (R_s 0.28), and *L. salivarius* vs. *C. jejuni* (R_s −0.34). The last pair was the only one negatively correlated.

2.5. Sequencing Analysis

Sequencing analysis of the V3–V4 16S rRNA gene showed that the archaeal population was limited to only two methanogenic genera—*Methanobrevibacter* and *Methanomassiliicoccus*—and their presence was restricted to samples from the free-range and commercial chickens, respectively (Table 1). Analysis of the *Desulfovibrionaceae* family revealed that there were other genera than *Desulfovibrio* in the commercial farm chickens, including *Bilophila* sp. The percentage of *Lachnospiraceae* was the highest of all microbial groups potentially involved in hydrogen sink. Their abundance was the highest in the experimental chickens and lowest in the free-range chickens. A contrasting situation was observed in the *Selenomonadaceae* family, which was limited to only one genus—*Megamonas*. A relatively high abundance of *Coriobacteriia* was also observed, especially in the free-range chickens, and *Bifidobacterium* and *Enterobacterales* in the commercial chickens.

Table 1. The abundance of selected hydrogen consumers in the total microbiota of samples grouped according to the chicken rearing method.

Microorganism	Catalytic Subunits in Hydrogen Sink [6,17,18]	Experimental Chickens			Commercial Farm Chickens			Free-Range Chickens		
		No. of Hits *	Percentage of Microbiota		No. of Hits *	Percentage of Microbiota		No. of Hits *	Percentage of Microbiota	
			Min. [%]	Max. [%]		Min. [%]	Max. [%]		Min. [%]	Max. [%]
Methanobrevibacter	McrA, group 4h and 4i [NiFe]-hydrogenase, AcsB, and FrdA	0/11	0	0	0/30	0	0	4/15	0	1.038
Methanomassiliicoccus		0/11	0	0	3/30	0	0.032	0/15	0	0
Desulfovibrionaceae	group 1b and 1d [NiFe]-hydrogenase, AprA, DsrA, FrdA, and CydA	0/11	0	0	14/30	0	1.706	3/15	0	0.012
Desulfovibrio		0/11	0	0	0/30	0	0	3/15	0	0.012

Table 1. *Cont.*

Microorganism	Catalytic Subunits in Hydrogen Sink [6,17,18]	Experimental Chickens			Commercial Farm Chickens			Free-Range Chickens		
		No. of Hits *	Percentage of Microbiota		No. of Hits *	Percentage of Microbiota		No. of Hits *	Percentage of Microbiota	
			Min. [%]	Max. [%]		Min. [%]	Max. [%]		Min. [%]	Max. [%]
Lachnospirales	AcsB and HydB	11/11	2.56	74.39	30/30	1.799	53.96	12/15	0	11.34
Peptostreptococcus	AsrA, AcsB, and CydA	0/11	0	0	1/330	0	0.017	0/15	0	0
Clostridium	HydB, AprA, and AsrA	9/11	0	7.228	12/30	0	2.325	9/15	0	0.918
Eubacterium	AcsB, HydB, and AsrA	0/11	0	0	12/30	0	1.277	7/15	0	1.245
Selenomonadaceae	group 1d [NiFe]-hydrogenase, HydB, FrdA, AprA, NarG, NrfA, DmsA/Tor, and CydA	4/11	0	0.052	4/30	0	20.63	9/15	0	27.98
Megamonas	HydB and CydA	0/11	0	0	4/30	0	20.63	9/15	0	27.98
Coriobacteriia	group 1i [NiFe]-hydrogenase and DmsA/TorA	4/11	0	1.157	27/30	0	18.59	11/15	0	32.84
Actinomycetales	FrdA, NarG, DmsA/TorA, and CydA	0/11	0	0	1/330	0	0.006	10/15	0	0.059
Corynebacterium	group 1f [NiFe]-hydrogenase, FrdA, NarG, and CydA	2/11	0	0.113	4/30	0	0.714	7/15	0	7.663
Bifidobacterium	FrdA and HydB	1/11	0	0.365	20/30	0	49.79	11/15	0	19.12
Prevotella	FrdA and NrfA	0/11	0	0	0/30	0	0	1/15	0	0.041
Bacteroides	HydB, FrdA, and NrfA	0/11	0	0	16/30	0	11.1	4/15	0	0.064
Enterobacterales	group 1c and 1d [NiFe]-hydrogenase, NarG, NapA, NrfA, DmsA/TorA, and CydA	1/11	0	0.006	30/30	0.006	54.19	9/15	0	22.19
Pseudochrobactrum	CydA	0/11	0	0	1/30	0	0	0/15	0	0.156
Synergistes	HydB	0/11	0	0	0/330	0	0	2/15	0	1.788

* number of positive samples/number of samples tested.

All sequencing data have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) repository under BioProject no. PRJNA944200.

3. Discussion

The process of hydrogen sink is best described in ruminants. It is known that in the environment of the rumen, the methanogenic archaea dominate over the homoacetogens, especially at low concentrations of H_2 [2,19]. Only when methanogenesis is suppressed do acetogens take over methanogen's place [18]. The activity of the remaining hydrogen consumers, i.e., the nitrate- and the sulfate-reducing bacteria—although thermodynamically more favorable than methano- and homoacetogenesis—is usually limited by low nitrate and sulfate concentrations originating from a diet [2]. The impact of dietary shifts on the exact mechanisms responsible for hydrogen metabolism remains largely unknown, and so far has only been studied in the rumen, where methanogenesis is naturally the predominant hydrogen sink [20]. For this reason, any changes observed in ruminants in response to their diet in terms of the methanogen vs. acetogen load, may not translate into chickens, which—as was here demonstrated—rely mainly on acetogenesis.

Acetogenesis was shown to be the preferred way of hydrogen utilization in the hindgut of monogastric herbivores. This phenomenon has been verified in the ceca of rabbits, and the feces of horses [19]. In humans, methanogenesis and homoacetogenesis are the two predominating hydrogen sinks [2]. Studies focusing on hydrogen uptake in chickens are extremely limited [5], and therefore we decided to study this subject.

In this study, the acetogens were generally the most abundant group, followed by methanogenic archaea and [NiFe]-hydrogenase utilizers. The acetogens were more abundant in the cecal contents of both experimental and commercial-farm chickens than they were in the excreta of the free-range chickens. This correlation seems to be justified since *Lachnospiraceae*—probably the largest population of acetogens—has already been shown to be more abundant in the cecal contents than in the excreta in another study [21,22]. The

same authors also demonstrated that lactobacilli tend to be more abundant in the excreta than in the ceca, which is also in line with our study. On a side note, the use of excreta in the free-range group of chickens was due to technical and administrative reasons, as cecal samples were simply unavailable.

It is known that the gut microbiota evolves with age after the time of hatching, and, with time, certain taxa outcompete the others [23]. In our study, the only statistically confirmed association between age and the composition of microbiota was observed for *L. salivarius* in the experimental chicken group. Interestingly, lactobacilli were highly abundant in very young chicks, and their prevalence in the 4-day-old chicks was almost as high as the prevalence in the 4-week-old chickens. Moreover, *L. salivarius* was detected in the ceca of three out of five chicks which hatched just hours before the sample collection, which only confirms that colonization of the guts of the chicken embryos by certain bacteria, such as lactobacilli, occurs before hatching, through the egg shell [24].

We also observed the tendency (albeit not statistically significant) for a decrease in the level of acetogen counts in relation to the age of the experimental chickens. The acetogens reached their highest counts in week 2 and seemed to be less abundant with time, especially by the end of the 4-week-long experiment. By assuming that *Lachnospiraceae* are the core acetogens, our findings are in line with a study by Videnska et al. [23] that first reported that this family of bacteria accounts for approx. 90% of the chicken gut microbiota at 2 weeks of age, and is being replaced by other bacteria starting with week 3. There were no more statistically significant differences among the age groups, which was most likely caused by the low number of observations, and the fact that many samples tested negative for at least one microbial group.

The methanogenic archaea were not detected in the experimental chickens at any age. This was probably caused by the lack of sources of these microorganisms in the strictly controlled environment with standard, ready-made feed, and communal tap water. Saengkerdsub et al. [25] reported that colonization of chickens with *Methanobacteriales* starts on day 3; however, they used sawdust from the bedding of older chickens with a mature gut microbiota presumably colonized by archaea. The same authors also established the quantity of *Methanobrevibacter woesei* in 56- to 72-week-old chickens at the level of 5.50 and 7.19 $\log_{10}$/gram of wet weight of cecal content. The cecal contents of the commercial farm chickens that we studied had a mean value of 4.24 $\log_{10}$/gram of wet weight. These differences may be related both to the age of the chickens and the methodology, as we used the *mcrA* gene as a target in real-time PCR, whereas the authors mentioned above used the 16S rRNA gene and cultivation.

Methanobrevibacter woesei has been the primary species of methanogens in the chicken gut since approx. 2007, but recently two more species have been discovered—*Methanocorpusculum* and UBA71, both renamed *Candidatus Methanospyradousia* [26]. The sequencing analysis of our samples, i.e., N3, SA2, and SB5, revealed that chickens are also colonized by *Candidatus Methanomassiliicoccus*. Until now, archaea belonging to the genus *Methanomassiliicoccus* have only been found in the gastrointestinal tracts of humans and pigs [27].

It was previously proven that in the presence of excess sulfate, the SRB displace the methanogens [28]. In this study, this did not occur, as SRB were generally rarely detected—mostly in the excreta of free-range chickens—and no correlation between the two groups was ever observed in any experimental configuration. However, the sequencing analysis of our samples revealed certain amounts of bacteria belonging to the genus *Bilophila*. Interestingly, it outnumbered the most common *Desulfovibrio* species [29]. In 2022, the new *Candidatus Bilophila faecipullorum* was reported in the feces of young chickens [26]. Unfortunately, our data did not allow for a full name description of detected sequences.

Until now, as many as 26 distinct hydrogenase subgroups have been discovered, including the hydrogenases that either catalyze the production or the consumption of hydrogen [17]. The bidirectional groups of hydrogenases are also quite common, which makes any research on hydrogen sink even more confusing and difficult to follow. Some research studies report a high abundance of the uptake hydrogenases from *Megamonas*

(Selenomonadales); others also report *Wolinella*, *Helicobacter*, and *Campylobacter* to be their source in chickens [5,17,21]. Therefore, this was the starting point for designing an assay targeting the [NiFe]-hydrogenases of these genera as a representation of the H_2-utilizing hydrogenases. We were able to quantify the uptake [NiFe]-hydrogenases as the third (after acetogens and methanogens) force responsible for hydrogen sink. Studies on ruminants consuming a fiber-rich diet revealed that the amount of detected *hydB* gene was approx. 1.8 times higher in these cows than in those ingesting a starch-rich diet [20]. In our study, this correlation was not observed, as experimental chickens receiving feed consisting of fiber-rich sunflower meal demonstrated a lower abundance of *hydB* compared to the free-range chickens receiving corn as the feed's major ingredient. As for the sequencing analysis, no *Helicobacter* nor *Wolinella* was ever detected in any of the studied chicken groups. This is especially interesting since there are reports suggesting that *Helicobacter* is often found in commercial broilers [21]. *Helicobacter pullorum* is considered a pathogen, and so is *Campylobacter* spp. [15]. In the present study, *L. salivarius* was shown to negatively correlate with *C. jejuni* and the other [NiFe]-hydrogenase utilizers. The competitive exclusion of lactobacilli and *C. jejuni* in the gastrointestinal tracts has already been well established [1]; however, surprisingly the negative correlation indices reported in this study for the cecal samples were only weak to moderate.

There were also other microbes potentially involved in hydrogen sink detected by 16S rRNA gene sequencing, such as *Eubacterium*, *Enterobacterales*, and *Coriobacteriia*; however, it is difficult to say how many of those were actually hydrogen consumers. In order to fully determine the proportion of the H_2 uptake genes, further studies should be conducted, e.g., whole-metagenome sequencing would be of great importance.

One last remark of this study relates to the experimental group of chickens. Generally, animals kept in isolated conditions are the key element of many studies [16,23]. However, our results clearly indicated that these chickens were characterized by small diversity of microbionts involved in hydrogen sink, and—as an animal model—were found not optimal for studying any microbial interactions.

4. Materials and Methods

4.1. Animals

A total of 154 chickens (*Gallus gallus domesticus*) representing three different rearing methods were included in this study: 54 experimental chickens, 50 commercial farm chickens, and 50 free-range chickens. The selected rearing conditions, including the diet, the usage of antibiotics, and the type of environment are listed in Table 2.

Table 2. The selected rearing parameters.

Chicken Group	Diet	Antibiotics	Rearing Environment
Experimental	Commercial feed for chickens from 1 to 6 weeks old (DKM1; Farmer Sp. z o.o., Biskupice Oloboczne, Poland), composed of sunflower meal, wheat bran, barley, wheat, and corn	No	No contact with the natural environment, other animals, and people.
Free-range	Commercial feed (e.g., Kokoszka-Nioska; ELPOL, Osina Mala, Poland) comprising corn, wheat, barley, black sunflower seeds, gold millet, oat, red millet, yellow peas, green peas, linseed, safflower seeds, and rape seeds; kitchen waste including potatoes, carrots, and eggshells, and worms found in the paddock	No use of antibiotics in the flocks from which the samples were collected. However, the use of antibiotics in the reproductive farms from which the chicks derived cannot be ruled out.	Yes, free-ranging, having contact with other farm and wild animals.

Table 2. *Cont.*

Chicken Group	Diet	Antibiotics	Rearing Environment
Commercial	A variety of standard commercial feeds varying between farms, adapted to the type and age of chickens (intensive broiler production system).	No growth-promoting antibiotics. Chickens from groups G, H, K, M, N, and SA÷ SD were reared without access to any antibiotics. Chickens from group T received colistin and amoxicillin. No data available for the remaining groups.	Indoor broiler chicken farms with implemented biosecurity procedures.

4.1.1. The Experimental Chickens

A total of 54 white leghorn chicks were hatched from the SPF (specific pathogen-free) eggs (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) in a sanitized incubator (Heka Incubator, Przewoz, Poland) and transferred to sanitized cages where they were kept in standard (non-SPF) conditions. The chickens received water and feed ad libitum. Cages were cleaned daily. The birds did not receive any vaccinations. The chickens were sacrificed on days 0, 4, 7, 14, 21, and 28, either by cervical dislocation (days 0-4) or lethal injection with pentobarbital in a dose of 150 mg/kg (older chickens). The ceca were isolated aseptically and subjected to DNA isolation on the same day. For the metagenome sequencing analysis purposes, chickens were pooled in groups of no more than six individual DNA samples per one pooled sample, and therefore two name entries correspond to one sacrificed chicken group (Table 3).

Table 3. Groups of the experimental chickens.

Group Name	Age	Sample Type	No. of Chickens
X0	0	Cecal contents	5
XA1	4 days	Cecal contents	4
XB1	4 days	Cecal contents	5
XA2	1 week	Cecal contents	4
XB2	1 week	Cecal contents	4
XA3	2 weeks	Cecal contents	5
XB3	2 weeks	Cecal contents	5
XA4	3 weeks	Cecal contents	5
XB4	3 weeks	Cecal contents	6
XA5	4 weeks	Cecal contents	6
XB5	4 weeks	Cecal contents	5

4.1.2. The Commercial Farm Broiler Chickens

The carcasses of chickens were submitted as soon as possible after killing from the commercial farms located in the Mazovia Province of Poland to the Department of Pathology and Veterinary Diagnostics, Institute of Veterinary Medicine, Warsaw University of Life Sciences (Warsaw, Poland). The chickens were of different ages (Table 4). During necropsy, the ceca of healthy chickens were aseptically removed. No gross lesions were found in the gastrointestinal tract or in other organs.

Table 4. Groups of commercial chickens.

Group Name	Age	Coop Location	Sample Type	No. of Chickens
F	1 week	Farm I	Cecal contents	7
G	3–4 weeks	Farm II	Cecal contents	4
H	1 week	Farm III	Cecal contents (4 samples) and excreta (1 sample)	4
I	4–5 weeks	Farm IV	Cecal contents	6
K	3–4 weeks	Farm III	Cecal contents (1 sample) and excreta (1 sample)	1
M	2–3 weeks	Farm V	Cecal contents	6
N	4 weeks	Farm V	Cecal contents	3
OA	4 weeks	Farm VI	Cecal contents	3
OB	5 weeks	Farm VI	Cecal contents	2
T	3 weeks	Farm IV	Cecal contents	5
SA	5–6 weeks	Farm VII	Cecal contents	3
SB	5–6 weeks	Farm VII	Cecal contents	3
SC	5 weeks	Farm VIII	Cecal contents	2
SD	4–5 weeks	Farm III	Cecal contents	1

4.1.3. Rural Free-Range Chickens

In the free-range chickens, the collection of ceca was technically not possible, and therefore samples of excreta had to be included in the study. Samples of fresh excreta were collected from the floor of three henhouses hosting the free-range laying hens. The flocks were located in three different rural areas across Poland (Table 5). All birds had access to large outdoor runs during daytime. If the samples were from the same location, the collections were performed months apart from each other, with new chickens introduced into a flock.

Table 5. Groups of free-range chickens.

Group Name	Age	Coop Location	Sample Type	No. of Chickens
A	n/a	Henhouse I	Excreta	1
B	n/a	Henhouse I	Excreta	1
E	n/a	Henhouse I	Excreta	2
L	n/a	Henhouse I	Excreta	11
J	n/a	Henhouse II	Excreta	7
P	n/a	Henhouse IIII	Excreta	7
R	n/a	Henhouse I	Excreta	21

n/a—not available.

4.2. DNA Isolation

Ceca from the experimental and the commercial chickens were longitudinally sectioned to collect 200 mg of the cecal content together with the cecal mucosa, which was scraped off the intestinal wall with the use of sterile scalpel blades. As for the free-range chickens, a total of 200 mg of dropping samples was collected. Then, a DNA isolation procedure was performed according to the protocol described previously [30].

4.3. Quantitative Real-Time PCR

The following key functional genes were chosen as targets for the quantitative real-time PCR: the *mcrA* gene encoding methyl-coenzyme M reductase alpha subunit for methanogenic archaea, the *aprA* gene encoding adenosine 5′-phosphosulfate reductase alpha subunit for the SRB, the *acsB* gene encoding acetyl-CoA synthase beta subunit for acetogens, the *hyaB*/*hydB* gene encoding [NiFe]-hydrogenase large subunit for *Wolinella*, *Helicobacter*, and *Campylobacter*, and the *mapA* gene encoding membrane-associated protein for *C. jejuni* alone. The last target microorganism—*L. salivarius*—was detected with the use of the 16S rRNA gene (Table 6). With the exception of the latter, all target genes occur in a single copy per genome. For *L. salivarius*, the results of the real-time PCR were divided by seven (i.e., the number of operons per genome in *L. salivarius*; [31]) to achieve the number

of cells per gram of cecal/excreta content. Primers used for *hyaB* were designed de novo for the purpose of this study.

Table 6. Primers used in this study.

Microorganism	Target Gene	Forward Primer 5′–3′ Sequence	Reverse Primer 5′–3′ Sequence	Amplicon Length [bp]	Reference
Methanogenic archaea	*mcrA*	CTTGAA RMTCAC TTCGGT GGWTC	CGTTCA TBGCGT AGTTVG GRTAGT	Approx. 270	[32]
SRB	*aprA*	TGGCAG ATCATG ATYAAY GG	GGCCGT AACCGT CCTTGA A	Approx. 385	Forward primer—modified [33]; reverse primer—modified [34]
Acetogens	*acsB*	CTBTGY GGDGCI GTIWSM TGG *	AARCAW CCRCAD GADGTC ATIGG *	216	[18]
selected [NiFe]-hydrogenase utilizing bacteria	*hyaB/hydB*	ATTGAA GTTGTT GTTGAT GAWAAY AATGT	AGMCCA ATCAAG CCCRTG	300	This study
C. jejuni	*mapA*	CTATTT TATTTT TGAGTG CTTGTG	GCTTTA TTTGCC ATTTGT TTTATT A	589	[35]
L. salivarius	16S rDNA	TACACC GAATGC TTGCAT TCA	AGGATC ATGCGA TCCTTA GAGA	138	[36]

* I—inosine.

Standard curves were generated by using decimal dilutions, from approx. 10^0 to 10^6 copies per reaction of genomic reference DNA. The following reference DNAs were used: *mcrA*+ positive plasmid containing an insert of the *mcrA* sequence fragment from GenBank acc. KF214818.1:976-1447, *Desulfovibrio piger* DSM 749 (SRB), *Ruminococcus gauvreauii* DSM 19829 (acetogen), *Helicobacter cinaedi* DSM 5359 ([NiFe]-hydrogenase carrier), *C. jejuni* 405 (courtesy of Dr. Agnieszka Sałamaszyńska-Guz), and *L. salivarius* 3D (courtesy of Dr. hab. Magdalena Kizerwetter-Świda). The new primer pair targeting the [NiFe]-hydrogenase large subunit was designed in silico by comparing the *hydB/hyaB* sequences from the *Wolinella*, *Helicobacter*, and *Campylobacter* group against homologic sequences from other bacteria.

The reaction mixture included 10 µL of RT HS-PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 0.5 µM primers (Table 6), 1 µL of cecal or 0.5 µL of excreta DNA, and water to reach a final volume of 20 µL. Samples were quantified individually, in triplicate. The thermal conditions were first set experimentally in order to achieve the optimal amplification efficiency by using reference DNAs and a gradient PCR. The reaction conditions for each quantitative assay are presented in Table 7. In each reaction, the amplification comprised 45 cycles. The real-time PCR results were calculated into the number of cells in 1 g of the cecal content or excreta.

Table 7. Temperature settings and DNA standards used for the absolute quantification of each group of microorganisms.

Real-Time PCR Step	Methanogenic Archaea	SRB	Acetogens	[Nife]-Hydrogenase Utilizing Bacteria	*C. jejuni*	*L. salivarius*
Initial Denaturation	95 °C—5 min					
Denaturation	94 °C—20 s	94 °C—20 s	94 °C—20 s	94 °C—20 s	94 °C—20 s	94 °C—20 s
Annealing	60 °C—20 s	62 °C down to 60 °C after first 10 cycles with 0.1 °C/s decreasing rate—20 s (touchdown PCR)	61 °C—20 s	64 °C—20 s	58 °C—20 s	68 °C—20 s
Elongation	72 °C—20 s	72 °C—30 s	72 °C—20 s	72 °C—20 s	72 °C—20 s	72 °C—20 s
Signal acquisition *	81 °C—20 s + Acq	89°C—20 s + Acq	80 °C—20 s + Acq	81 °C—20 s + Acq	79 °C—20s + Acq	82 °C—20 s + Acq
Melt analysis *	95 °C—5 s, then 60 °C—1 min, and 95 °C—continuous Acq with ramp rate 0.11 °C/s					

* Acq—acquisition of fluorescence signal.

4.4. Statistical Analysis

The Shapiro–Wilk test was used in order to check whether the quantification results of the real-time PCR have a normal distribution. The homogeneity of variance was checked with Levene's test. Then, a non-parametric Kruskal–Wallis H test was applied to evaluate the statistical significance of variation among the hydrogen consumers and *L. salivarius* regarding the sample type, source, and age of the chickens. Spearman's rank correlation test was used to measure the strength and direction of the microbial associations grouped by the rearing methods. According to the guidelines for interpretation of Spearman's rho rank correlation by Prion and Haerling, 2014 [37], the correlations were considered very strong when the values of R_s were between 0.81 to 1, strong—0.61 to 0.80, moderate—0.41 to 0.60, weak—0.21 to 0.40, and negligible—0 to 0.20. All statistical analyses were performed in TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and Microsoft Office Excel 2016 (Redmond, WA, USA).

4.5. Sequencing Analysis

The metagenome analysis of archaea and bacteria was performed based on the hypervariable V3–V4 region of the 16S rRNA gene. Only samples with high-quality DNA were selected for sequencing. Samples from the commercial and free-range chickens were sequenced individually, whereas in the case of the experimental chickens, a total of max. six samples from each group were pooled and in this form subjected to sequencing.

The analysis was outsourced to Genomed S.A. (Warsaw, Poland). In short, the 341F and 785R primers were used together with a Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA). The sequencing was performed in the paired-end technology (PE), 2 × 300 nt with Illumina v3 kit by a MiSeq instrument (San Diego, CA, USA), which also performed an initial automatic analysis comprising of demultiplexing and generation of fastq files. The species-specific classification of the reads was performed with the use of QIIME 2 according to the Silva 138 reference sequence database. The following tools were then used: FIGARO for read quality control, Cutadapt for initial data processing, and DADA2 for the selection of ASV (amplicon sequence variant) and further steps of the analysis.

5. Conclusions

This work presents the possible routes of hydrogen disposal, pointing out the strong position of acetogenesis as the leading metabolic pathway for hydrogen sink. In this study, we have demonstrated that acetogens were dominant over the other tested groups of hydrogen consumers, whereas the numbers of methanogenic archaea, SRB, and the [NiFe]-hydrogenase utilizers depended on the sample type and rearing conditions. In order to fully determine the role of specific gut microbionts in hydrogen sink, further studies should be conducted.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24076674/s1.

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Institutional Review Board Statement: The animal study protocol involving the experimental chickens was approved by the 3rd Local Ethics Committee in Warsaw (protocol code: 58/2013, approved on 23 October 2013). The ethical review and approval for the commercial farm chickens and the free-range chickens were waived for this study because no procedures were ever performed on the living animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within this article and supplementary materials. The sequencing data have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) repository under BioProject no. PRJNA944200.

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