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im. Jana i Jędrzeja Śniadeckich

RADA NAUKOWA DYSCYPLINY ZOOTECHNIKA I RYBACTWO

ROZPRAWA DOKTORSKA

w formie zbioru opublikowanych i powiązanych tematycznie artykułów naukowych w dyscyplinie zootechnika i rybactwo

mgr inż. Aleksandra Bełdowska

Odpowiedź jelitowa na stymulację prebiotykiem i postbiotykiem podanym *in ovo* w trakcie rozwoju embrionalnego

Intestinal response to prebiotic and postbiotic in ovo stimulation during embryonic development

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Spis treści

1. WSTĘP

Zdrowie oraz prawidłowe funkcjonowanie przewodu pokarmowego, w tym jelit ma fundamentalne znaczenie dla wzrostu i produktywności drobiu. Zdrowe jelita ptaków są niezbędne do optymalizacji procesu trawienia oraz minimalizacji wydalania składników odżywczych u drobiu (Oviedo-Rondón, 2019). Nieprawidłowe funkcjonowanie jelit najczęściej spowodowane jest uszkodzeniem, chorobą lub stanem zapalnym, i niekorzystnie wpływa na procesy wchłaniania składników odżywczych oraz trawienie, niosąc ze sobą konsekwencje dla zdrowia i wzrostu ptaków (Elnesr i wsp., 2020). W tym aspekcie istotnym problemem może być nadmiar składników odżywczych w jelicie grubym, który prowadzi do nadmiernej proliferacji drobnoustrojów, takich jak *Clostridium perfringens* oraz *Escherichia coli* (Brown i wsp., 2012; Chan i wsp., 2013). Nadmierna obfitość bakterii patogennych może być spowodowana zbyt wysokim poziomem składników odżywczych w diecie albo suboptymalnym trawieniem (Brown i wsp., 2012). Pomimo selekcji genetycznej oraz wprowadzania zmian w składzie paszy, wydajność w obrębie stada, a także pomiędzy stadami, nadal uznawana jest za problem w produkcji drobiarskiej (Van Limbergen i wsp., 2020). Dla przemysłu drobiarskiego poprawa wydajności stada jest kluczowa w kontekście maksymalizacji zysków. Za istotny czynnik wpływający na wydajność uważany jest stan zdrowia jelit, określany jako stan równowagi symbiotycznej pomiędzy mikrobiotą, a przewodem pokarmowym, w którym zdrowie i dobrostan zwierząt pozostają niezmienione (Celi i wsp., 2019).

Błona śluzowa jelit odgrywa kluczową rolę w procesach trawienia oraz działa jako bariera chroniąca głębiej położone tkanki przed patogenami i uszkodzeniami mechanicznymi (Zhang i wsp., 2015). Warstwa błony śluzowej, wzbogacona ochronnym śluzem, stanowi pierwszą linię obrony przed zagrożeniami zewnętrznymi (Duangnumsawang i wsp., 2021). Żelowa struktura śluzu jelitowego wychwytuje i blokuje patogeny, zapobiegając ich wnikaniu i zapewniając integralność układu pokarmowego (Forder i wsp., 2007). Pokarm spożywany przez ptaki przemieszcza się przez ich układ pokarmowy w czasie od około dwóch do trzech godzin, co wymaga zaangażowania mikrobioty o wysokiej wydajności w proces pozyskiwania energii z pożywienia (Wilkinson, 2016).

Ptaki charakteryzują się wysokim zapotrzebowaniem energetycznym, a ich układ trawienny jest przystosowany do efektywnego wykorzystania składników pokarmowych. W wyniku intensywnej selekcji u kur domowych wykształciły się mechanizmy pozwalające na efektywne wykorzystywanie składników pokarmowych do budowy mięśni szkieletowych. Pozyskiwanie energii i składników odżywczych z pożywienia wymaga zaangażowania procesów biochemicznych, takich jak fermentacja włókna pokarmowego i synteza witamin (Stanley i wsp., 2014; Singh i Kim, 2021). Obecnie produkcja drobiarska obejmuje wykorzystanie zautomatyzowanych wylęgarni, które eliminują kontakt piskląt z matkami (kwokami), w związku z czym po wykluciu są one wystawiane na bezpośredni kontakt z bakteriami znajdującymi się w otaczającym je środowisku. Aby uniknąć niepożądanego, losowego składu mikrobioty u kurcząt, możliwe jest jej ukierunkowanie za pomocą bioaktywnych substancji, np. prebiotyków i probiotyków, podawanych bezpośrednio do jaja w czasie rozwoju embrionalnego (Siwek i wsp., 2018). Jedną z wykorzystywanych metod podawania substancji bioaktywnych jest technologia *in ovo*. Polega ona na iniekcji pojedynczej dawki substancji bioaktywnej w 12 dobie inkubacji jaj. Miejscem wstrzyknięcia substancji jest komora powietrzna, wyścielona dwiema warstwami błony pergaminowej, które połączone są z silnie unaczynioną błoną kosmówkową (Dunisławska i wsp., 2017). Badania przeprowadzone przez Villaluenga i wsp. (2004) wykazały, że 12 doba inkubacji jaj jest najbardziej optymalnym terminem iniekcji ze względu na najwyższy poziom występowania

bifidobakterii po stymulacji. Za tym terminem przemawia również szybki wzrost zarodka kurzego, podczas którego drastycznie zwiększa się zużywanie tłuszczu żółtka, intensywnie wzrasta przyswajalność składników odżywczych, a jednocześnie zmniejsza się pęcherzyk żółtkowy (Kpodo i Proszkowiec-Weglarz, 2023). Badania wykazały, że już w 13. dobie rozwoju embrionalnego zarodki mają wykształconą rurkę neuronową i zaczynają odczuwać ból (Aleksandrowicz i Herr, 2015). Stymulacja *in ovo* w 12 dniu inkubacji jaj opiera się przede wszystkim na zwiększeniu populacji mikrobioty obecnej w zarodku przez zastosowanie prebiotyków lub synbiotyków (Siwek i wsp. 2018). Technologia *in ovo* ze względu na swoją skuteczność znalazła zastosowanie w stymulacji kurzych zarodków przede wszystkim w celu warunkowania odpowiedzi immunologicznej i stymulowania mikrobioty jelitowej (El-Kholy i wsp., 2021).

Mikrobiota jelitowa składająca się z licznych mikroorganizmów, w tym bakterii komensalnych, patogennych, drożdży, wirusów i pierwotniaków, odgrywa znaczącą rolę w utrzymaniu zdrowia jelit i wpływa na ogólny dobrostan drobiu (Karl i wsp., 2018). Na skład i funkcję mikrobioty wpływa kilka czynników, w tym dieta i warunki środowiskowe. Bakterie *Lactobacillus* są dominującą grupą w górnym odcinku przewodu pokarmowego kurcząt, podczas gdy *Clostridium* i *Enterococcus* są bardziej powszechne w jelicie cienkim i ślepym (Khan i wsp., 2020). Wykazano, że obecność pożytecznych bakterii, takich jak *Bifidobacterium* i *Lactobacillus*, wywiera działanie przeciwzapalne i stymuluje metabolizm lipidów w wątrobie, przyczyniając się do poprawy ogólnego stanu zdrowia organizmu gospodarza (Deng i wsp., 2023). Mikrobiota jelitowa reguluje odpowiedź immunologiczną, procesy metaboliczne i trawienne, a także wchłanianie składników odżywczych (Wickramasuriya i wsp., 2022). Bakterie jelitowe dostarczają niezbędnych bodźców do stymulowania odpowiedzi immunologicznej piskląt, dzięki czemu dorosłe osobniki są mniej podatne na infekcje i stany zapalne (Tiku i wsp., 2020).

Migdałki jelita ślepego i śledziona są głównymi narządami zaangażowanymi w odpowiedź immunologiczną. Migdałki są częścią tkanki limfatycznej związanej z jelitami (ang. *gut-associated lymphoid tissue*, GALT), znajdują się więc w bliskiej relacji z mikrobiotą jelit. GALT rozwinął mechanizmy regulacyjne i przeciwzapalne, które mogą eliminować lub tolerować dany skład mikrobioty (Dunisławska i wsp., 2023). Migdałki jelitowe mają zdolność rozpoznawania bakterii komensalnych i patogennych, dzięki której, w razie zagrożenia, mogą aktywować odpowiedź immunologiczną gospodarza (Bavananthasivam i wsp., 2021).

Dane literaturowe wskazują na duże znaczenie mikrobioty jelitowej drobiu dla funkcjonowania mitochondriów. Bakterie i mitochondria mają podobne cechy strukturalne i funkcjonalne. Wykazano, że metabolity takie jak krótkołańcuchowe kwasy tłuszczowe (ang. *short-chain fatty acids*, SCFA), gazy fermentacyjne i modyfikowane kwasy tłuszczowe mogą wpływać na funkcje mitochondriów (Chen i Vitetta, 2020). Według dostępnych danych, korzystny profil mikrobioty jelitowej może poprawić funkcjonowanie mitochondriów. Mikrobiota jelitowa wysyła sygnały do mitochondriów. Te sygnały trafiają do mitochondriów w komórkach tworzących wyściółkę jelit (komórki nabłonkowe) oraz w komórkach układu immunologicznego. Dzięki temu mikrobiota pomaga regulować pracę mitochondriów. Sygnalizacja mikrobioty jelitowej z mitochondriami zmienia metabolizm mitochondriów, aktywuje komórki odpornościowe, indukuje sygnalizację inflamasomu i zmienia funkcję bariery nabłonkowej (Jackson i Theiss, 2020).

Aby poprawić zdrowie jelit u drobiu, jednocześnie przestrzegając unijnych regulacji dotyczących zakazu profilaktycznego stosowania antybiotyków w produkcji drobiarskiej, wprowadzono dedykowane suplementy diety oraz dodatki do pasz (Elnesr i wsp., 2020).

Preparaty te mają na celu wspieranie naturalnej mikrobioty jelit oraz wzmacnianie układu immunologicznego u drobiu. Stosowane suplementy obejmują przede wszystkim probiotyki, prebiotyki, kwasy organiczne, a także naturalne ekstrakty roślinne (Phillips i wsp., 2023). Prebiotyki, takie jak oligosacharydy, odgrywają istotną rolę w modulowaniu mikrobioty jelitowej przez promowanie wzrostu korzystnych mikroorganizmów. Suplementacja paszy oligosacharydami lub redukcja polisacharydów do oligosacharydów to jedna ze strategii pomocnych w zmniejszaniu stymulacji układu immunologicznego i łagodzeniu niepożądanych skutków dla zdrowia jelit, wywoływanych przez formy polimerowe węglowodanów wchodzących w skład włókna pokarmowego (Tiwari i wsp., 2020). Arabinoza i ksyloza występują w postaci arabinoksylanu w składnikach paszowych. Udowodniono, że arabinoksylan pobudza odpowiedź immunologiczną u kur zakażonych pasożytem *Eimeria* (Teng i Kim, 2018). Mannany nie ulegają hydrolizie pod wpływem endogennych enzymów gospodarza u gatunków monogastrycznych, dlatego mogą wiązać się z komórkami wrodzonego układu odpornościowego, w tym makrofagami, za pomocą domen rozpoznających węglowodany, wywołując w ten sposób odpowiedź immunologiczną (Teng i Kim, 2018). Udowodniono, że mannan stymuluje makrofagi oraz komórki dendrytyczne, powodując zwiększoną produkcję cytokin prozapalnych. Aktywacja komórek dendrytycznych odgrywa istotną rolę w wywoływaniu odpowiedzi immunologicznych. Skuteczność odpowiedzi wywołanej przez te komórki zależy od stanu rozwojowego komórek dendrytycznych gospodarza (Santander i wsp., 2011). Ksylooligosacharydy (XOS) i mannooligosacharydy (MOS) to funkcjonalne oligosacharydy wytwarzane odpowiednio z ksylanu i mannanu przez enzymatyczną hydrolizę, które mają korzystne działanie immunologiczne i zdrowotne. Oba oligosacharydy zajmują pozycję pośrednią między cukrami prostymi a polisacharydami pełniąc funkcję zarówno włókna pokarmowego, jak i prebiotyków. Dodatkowo XOS i MOS są odporne na hydrolizę przez amylazę śliny oraz hydrolizę przez enzymy soku żołądkowego i trzustkowego. Nie są one wchłaniane w jelicie cienkim, zapewniając w ten sposób podłoże do fermentacji mikrobiologicznej w jelicie ślepym drobiu (Amaretti i wsp., 2013). Teng i Kim (2018) wykazali, że MOS zwiększa namnażanie się bakterii *Bifidobacterium* oraz *Lactobacillus* w jelitach brojlerów. Badania przeprowadzone przez De Maesschalck i wsp. (2015) wykazały, że XOS poprawia parametry wzrostu, zwiększa wysokość kosmków jelitowych, powoduje wzrost populacji bakterii *Lactobacillus* oraz zwiększa poziom octanu, maślanu i mleczanu w jelicie ślepym brojlerów. Wcześniejsze badania przeprowadzone przez zespół Katedry Biotechnologii i Genetyki Zwierząt wskazują, że stymulacja galaktooligosacharydem (GOS) wpływa na skład i liczebność mikrobioty jelitowej u kurcząt brojlerów. W szczególności zaobserwowano zwiększenie populacji bakterii *Bifidobacterium* spp. w jelicie ślepym. Stymulacja *in ovo* GOS spowodowała również zmiany w poziomie ekspresji genów w jelicie biodrowym i ślepym kurcząt brojlerów (Slawinska i wsp., 2019). Wpływ prebiotyków i synbiotyków podawanych *in ovo* na poziom ekspresji genów po wykluciu jest ściśle zależny od rodzaju/składu substancji bioaktywnej i terminu stymulacji. Badania opisane przez Sławinską i wsp. (2016) wskazują, że geny związane z odpornością w migdałkach jelita ślepego i śledzionie ulegają wyciszeniu po podaniu *in ovo* substancji bioaktywnych. U 42-dniowych ptaków, które poddane były stymulacji *in ovo* GOS, wykazano zmniejszenie ekspresji genów w migdałkach jelit ślepych. Sam GOS i synbiotyk na bazie GOS z dodatkiem *Lactococcus lactis* subsp. *cremoris* oraz synbiotyk zawierający inulinę i *Lactococcus lactis* spowodowały zmniejszenie ekspresji genów *IL-4*, *IL-6*, *IL-8*, *IL-12*, *IFNβ* oraz *IFNγ* odpowiedzialnych za regulację odpowiedzi immunologicznej (Sławińska i wsp., 2016). Wykazane zostało, że synbiotyki działają jako regulator zarówno transkrypcji genów, jak i ekspresji białek (Dunisławska i wsp., 2021). Udowodniono, że podawanie *Lactobacillus plantarum* z oligosacharydami z rodziny rafinozy w jednej dawce powoduje niezależne działanie obu składników w układzie

pokarmowym gospodarza. Składnik prebiotyczny stymuluje rozwój mikrobioty jelitowej gospodarza, poprawiając tym samym równowagę mikrobiologiczną jelit. Z drugiej strony, mikroorganizmy probiotyczne kolonizują przewód pokarmowy. Taki układ zdefiniowano jako synergizm synbiotyku w stosunku do gospodarza (Dunisławska i wsp., 2023). Mikrobiota jelita ślepego fermentuje prebiotyki stymulując produkcję metabolitów, do których należą SCFA (Snelson i wsp., 2021). Stymulacja prebiotykami sprzyja wzrostowi liczby bakterii *Lactobacillus* i *Bifidobacterium*, które odpowiadają za hamowanie rozwoju bakterii patogennych w organizmie (Mohanty i wsp. 2018). Docierają one do końcowego fragmentu jelit, gdzie wspierają proliferację drobnoustrojów i zwiększają odporność na kolonizację przez patogeny (Khan i wsp., 2020).

Maślan sodu (ang. *sodium butyrate*, SB) jest jedną z substancji bioaktywnych stosowaną jako postbiotyk. Odgrywa rolę w utrzymaniu zdrowego środowiska jelitowego przez obniżanie pH, hamowanie wzrostu szkodliwych mikroorganizmów oraz stymulowanie wzrostu kosmków jelitowych (Bawish i wsp. 2023). Jako jeden z trzech głównych SCFA, kwas masłowy jest niezbędnym źródłem energii dla nabłonka oraz wpływa na prawidłowy, zrównoważony rozwój mikrobioty jelitowej (Deng i wsp., 2023). Oprócz działania jako „paliwo napędowe" w jelicie, maślan może stymulować ekspresję genu *MUC2* oraz wywierać działanie przeciwzapalne i zapobiegać martwiczemu zapaleniu jelit spowodowanemu zakażeniem przez patogeny (Teng i Kim, 2018). SB nie tylko poprawia parametry produkcyjne drobiu, ale także indukuje znaczące zmiany w mikrobiocie jelitowej, poprawiając w ten sposób zdrowie jelit (Wan i wsp., 2022).

Jelita, oprócz pełnienia swoich podstawowych funkcji, współdziałają z innymi organami wewnętrznymi. Do kluczowych organów w tej relacji należą wątroba i mózg. Oś jelitowo-mózgowa to dwukierunkowy układ interakcji między jelitami a ośrodkowym układem nerwowym. Rola mikrobioty jelitowej w tej relacji jest kluczowa, ponieważ tworzy ona złożoną, gęstą sieć z autonomicznym układem nerwowym. W jelicie znajdują się neurony ruchowe, czuciowe i interneurony przekazujące informacje między jelitowym, a ośrodkowym układem nerwowym (Long-Smith i wsp., 2020). Dodatkowo mikrobiota jelitowa produkuje bioaktywne peptydy, neuroprzekaźniki, SCFA, hormony jelitowe oraz aminokwasy, które wpływają na komunikację międzynarządową (Long–Smith i wsp., 2020). Metabolity te biorą udział w przekazywaniu sygnałów w osi jelitowo-mózgowej, jednocześnie stymulując oś przysadka - podwzgórze - nadnercza. Ponadto peptydy bezpośrednio wpływają na funkcje immunologiczne błony śluzowej jelit, z której sygnały docierają do ośrodkowego układu nerwowego (Ding i wsp., 2020).

Jelita i wątroba komunikują się za pośrednictwem żyły wrotnej i krążenia ogólnoustrojowego. Metabolity wytwarzane przez jelita są transportowane przez żyłę wrotną do wątroby. Jednocześnie wątroba transportuje sole żółciowe i cząsteczki przeciwdrobnoustrojowe do światła jelita przez przewody żółciowe. W ten sposób kontroluje nieograniczony wzrost bakterii (Tripathi i wsp., 2018).

2. WYKAZ ARTYKUŁÓW NAUKOWYCH STANOWIĄCYCH CYKL PUBLIKACJI ROZPRAWY DOKTORSKIEJ

1. **Aleksandra Bełdowska**, Marcin Barszcz, Aleksandra Dunisławska, State of the art in research on the gut-liver and gut-brain axis in poultry, *Journal of Animal Science and Biotechnology*, 2023, 14, 37, https://doi.org/10.1186/s40104-023-00853-0
   pkt MNiSW: 200, Impact Factor: 7,00

2. **Aleksandra Bełdowska**, Maria Siwek, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Kamil Gawin, Aleksandra Dunisławska, Impact of *in ovo* administration of xylo- and mannooligosaccharides on broiler chicken gut health, *Poultry Science*, 2024a, 103, 12, 104261, https://doi.org/10.1016/j.psj.2024.104261
   pkt MNiSW: 140, Impact Factor: 3,80

3. Aleksandra Dunisławska, **Aleksandra Bełdowska**, Olha Yatsenko, Maria Siwek, Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens, *Poultry Science*, 2023, 102, 6, 102663, https://doi.org/10.1016/j.psj.2023.102663
   pkt MNiSW: 140, Impact Factor: 4,40

4. **Aleksandra Bełdowska**, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, The effect of sodium butyrate administrated *in ovo* on the health status and intestinal response in broiler chicken, *Poultry Science*, 2024b, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108
   pkt MNiSW: 140, Impact Factor: 3,80

Łącznie: pkt MNiSW 620, Impact Factor: 19.

3. UZASADNIENIE SPÓJNOŚCI TEMATYCZNEJ CYKLU PUBLIKACJI ROZPRAWY DOKTORSKIEJ

Cykl publikacji, stanowiący podstawę do ubiegania się o stopień doktora, opiera się na badaniach nad wpływem substancji bioaktywnych podawanych *in ovo* w 12. dobie inkubacji jaj na zdrowie jelit kurcząt brojlerów, z uwzględnieniem ich oddziaływania na mikrobiotę jelitową. Podjęty kierunek badań uwzględnia rosnące zainteresowanie zdrowiem kurcząt oraz potrzebę opracowania efektywnych sposobów poprawy wydajności produkcji drobiu. Stymulacja *in ovo* wpływa na rozwój mikrobioty jelitowej kurcząt i może mieć długofalowe konsekwencje dla ich zdrowia i wydajności. W publikacjach opisano zastosowanie prebiotyków (ksylooligosacharydów i mannooligosacharydów) oraz postbiotyku (maślan sodu), które po raz pierwszy podane zostały z wykorzystaniem technologii *in ovo* w 12. dobie inkubacji jaj. Stosowane substancje wykazują różnorodne właściwości prozdrowotne, przyczyniając się do poprawy funkcjonowania jelit, stabilizacji mikrobioty oraz wspomagając procesy metaboliczne.

Publikacja przeglądowa nr 1 (Bełdowska i wsp., 2023) zatytułowana „*State of the art in research on the gut-liver and gut-brain axis in poultry*", stanowi przegląd aktualnego stanu wiedzy w zakresie wzajemnych oddziaływań przewodu pokarmowego, mikrobioty jelitowej, wątroby i mózgu u drobiu. Artykuł ten jest istotny dla zrozumienia roli jelit i mikrobioty jelitowej oraz wpływu substancji bioaktywnych i metabolitów bakteryjnych na wątrobę oraz ośrodkowy układ nerwowy kurcząt brojlerów. W **publikacji naukowej nr 2** (Bełdowska i wsp., 2024a) pt. „*Impact of in ovo administration of xylo- and mannooligosaccharides on broiler chicken gut health*", opisano badania and wpływem czterech oligosacharydów (XOS i MOS) podanych *in ovo* na zdrowie jelit u kurcząt, z uwzględnieniem aktywności mikrobioty jelitowej oraz ekspresji genów związanych z odpornością i metabolizmem składników pokarmowych. **Publikacja naukowa nr 3** (Dunisławska i wsp., 2023) pt. „*Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens*" dotyczy badań nad wpływem wyżej wspomnianych prebiotyków podawanych w 12. dobie inkubacji jaj na mitochondria komórek błony śluzowej jelita ślepego i migdałków jelitowych. W **publikacji naukowej nr 4** (Bełdowska i wsp., 2024b) pt. „*The effect of sodium butyrate administered in ovo on the health status and intestinal response in broiler chicken*" opisano badania nad wpływem różnych dawek maślanu sodu podanego *in ovo* na skład i aktywność mikrobioty jelitowej, parametry histologiczne jelit oraz ekspresję genów związanych z odpowiedzią immunologiczną kurcząt brojlerów.

Badania zostały przeprowadzone w ramach projektów OPUS UMO-2021/41/B/NZ9/02562 pt. "Mechanizmy molekularne osi jelitowo-wątrobowej kurcząt" oraz SONATA UMO-2021/43/D/NZ9/01548 pt. „ActEpi: Aktywacja mechanizmów epigenetycznych u drobiu przez programowanie mikrobioty jelitowej" finansowanych przez Narodowe Centrum Nauki.

3.1. WYKAZ SKRÓTÓW

ACTB - beta actin - beta aktyna

ATP6 - ATP synthase 6 - syntaza ATP 6

AVBD1 - avian beta defensin 1 - ptasia beta-defensyna 1

CATHL2 - cathelicidin 2 - katelicydyna 2

CLDN1 - claudin 1 - klaudyna 1

CS - citrate synthase - syntaza cytrynianowa

CYCS - cytochrome C - cytochrom C

D-loop - displacement loop - pętla przesunięcia

EPX (MPO) - eosinophil peroxidase - peroksydaza eozynofilowa

FFAR2 - free fatty acid receptor 2 - receptor wolnych kwasów tłuszczowych 2

FFAR4 - free fatty acid receptor 4 - receptor wolnych kwasów tłuszczowych 4

G6PDH - glucose 6 phosphate dehydrogenase - dehydrogenaza glukozo-6-fosforanowa

GALT - gut-associated lymphoid tissue - tkanka limfatyczna związana z jelitem

GCG - glucagon - glukagon

GLUT1 - glucose transporter 1 - transporter glukozy 1

GLUT2 - glucose transporter 2 - transporter glukozy 1

GLUT5 - glucose transporter 5 - transporter glukozy 1

GOS - galactooligosaccharide - galaktooligosacharyd

IL-1β - interleukin 1 beta - interleukina 1 beta

IL-2 - interleukin 2 - interleukina 2

IL-4 - interleukin 4 - interleukina 4

IL-6 - interleukin 6 - interleukina 6

IL-8 - interleukin 8 - interleukina 8

IL-10 - interleukin 10 - interleukina 10

IL-12 - interleukin 12 - interleukina 12

IL-17 - interleukin 17 - interleukina 17

IFNβ - beta interferon - interferon beta

IFNγ - gamma interferon - interferon gamma

MnSOD - superoxide dismutase mitochondrial - mitochondrialna dysmutaza ponadtlenkowa

MOS - mannooligosaccharide - mannooligosacharyd

MOS3 - mannotriosis - mannotrioza

MOS4 - mannotetraose - mannotetroza

MUC2 - mucin 2 - mucyna 2

MUC6 - mucin 6 - mucyna 2

ND2 - NADH dehydrogenase subunit 2 - podjednostka 2 dehydrogenazy NADH

ND6 - NADH dehydrogenase subunit 6 - podjednostka 2 dehydrogenazy NADH

NRF1 - nuclear respiratory factor 1 - jądrowy czynnik oddechowy 1

PCR - polymerase chain reaction - reakcja łańcuchowa polimerazy

qPCR - quantitative polymerase chain reaction - ilościowa reakcja łańcuchowa polimerazy

RT-qPCR - quantitative reverse transcription polymerase chain reaction – ilościowa reakcja łańcuchowa polimerazy z początkową odwrotną transkrypcją

SB - sodium butyrate - maślan sodu

SCFA - short-chain fatty acids - krótkołańcuchowe kwasy tłuszczowe

TFAM - transcription factor A, mitochondrial - mitochondrialny czynnik transkrypcyjny A

TJAP - tight junction associated protein - białko połączeń ścisłych

XOS - xylooligosaccharide - ksylooligosacharyd

XOS3 – xylotriose - ksylotrioza

XOS4 - xylotetraose - ksylotetroza

3.2. HIPOTEZA BADAWCZA, CEL I ZAKRES BADAŃ

Hipoteza badawcza zakłada, że podanie *in ovo* prebiotyku i/lub postbiotyku w trakcie rozwoju embrionalnego w 12. dobie inkubacji jaj wpływa bezpośrednio na profil mikrobioty jelitowej, co pośrednio skutkuje zmianami fenotypowymi i molekularnymi w jelitach kurcząt brojlerów w 42. dniu po wylęgu.

Poddając weryfikacji powyższą hipotezę podjęto badania, których głównym celem było określenie wpływu stymulacji *in ovo* w 12. dobie inkubacji jaj substancjami bioaktywnymi na fizjologię jelit i zdrowie kurcząt brojlerów.

Celem doświadczenia 1. było określenie wpływu prebiotyków XOS i MOS na fizjologię jelit i zdrowie kurcząt brojlerów, z uwzględnieniem aktywności mikrobioty jelitowej oraz ekspresji genów w błonie śluzowej jelit i migdałkach jelitowych. **Celem doświadczenia 2.** było natomiast określenie wpływu maślanu sodu podanego w różnych stężeniach na stan zdrowia kurcząt, skład i aktywność mikrobioty jelitowej, ekspresję genów i morfologię jelit**.**

Wyznaczono również **cele szczegółowe**, takie jak określenie wpływu na:

- profil bakteryjny w treści pokarmowej jelita biodrowego i ślepego (doświadczenie 1 i 2);
- ekspresję genów w błonie śluzowej jelita biodrowego i ślepego (doświadczenie 1 i 2) oraz migdałkach jelitowych (doświadczenie 1);
- parametry biochemiczne krwi (doświadczenie 2);
- aktywność mikrobioty jelita biodrowego i ślepego (doświadczenie 1 i 2);
- parametry histologiczne jelita biodrowego i ślepego (doświadczenie 2).

3.3. MATERIAŁY I METODY BADAŃ

Jako kryteria oceny wpływu czynników doświadczalnych na mikrobiotę jelitową oznaczono:

- względną liczebność bakterii z wykorzystaniem metody reakcji łańcuchowej polimerazy (ang. polymerase chain reaction, PCR) w czasie rzeczywistym (ang. quantitative polymerase chain reaction, qPCR),
- pH treści jelitowej
- stężenie SCFA za pomocą chromatografu gazowego,
- stężenie amoniaku i aktywność β-glukuronidazy metodami spektrofotometrycznymi.

Jako kryteria oceny funkcjonowania jelita biodrowego i ślepego oznaczono:

- ekspresję genów w błonie śluzowej i migdałkach jelitowych metodą ilościowej odwrotnej transkrypcji reakcji łańcuchowej polimerazy w czasie rzeczywistym (ang. quantitative reverse transcription polymerase chain reaction, RT-qPCR),
- parametry histologiczne za pomocą mikroskopu świetlnego po uprzednim zabarwieniu preparatów histologicznych alcjanem błękitu, kwasem nadjodowym i odczynnikiem Schiff'a oraz diaminami i alcjanem błękitu.

3.3.1. Eksperyment 1 – podanie prebiotyków XOS i MOS *in ovo*

Do doświadczenia wykorzystano 700 sztuk zapłodnionych jaj wylęgowych pochodzących od stada rodzicielskiego kur brojlerów Ross 308. W 7. dobie inkubacji wykonano prześwietlanie jaj, aby wyeliminować niezapłodnione jaja lub jaja z wcześnie obumarłymi zarodkami. W 12. dobie inkubacji jaja zostały losowo podzielone na 5 grup. Do komory powietrznej każdego jaja wstrzyknięto 0,2 ml soli fizjologicznej (kontrola) lub po 0,2 ml roztworu prebiotyku: ksylotriozy (XOS3), ksylotetrozy (XOS4), mannotriozy (MOS3) lub mannotetrozy (MOS4). Oligosacharydy podawano w zawiesinie z 0,5 mg/0,2 ml roztworu NaCl. Jaja inkubowano przez 21 dni. Od 1. do 18. dnia jaja utrzymywano w inkubatorze w temperaturze 37,7°C, przy wilgotności ok. 55%, a od 19 do 21 dnia w klujniku, w temperaturze 37,5°C i wilgotności 70%. Po wykluciu pisklęta umieszczono w kojcach na ściółce z posiekanej słomy pszennej z zachowaniem podziału na grupy doświadczalne. Przez cały okres odchowu ptaki miały swobodny dostęp do świeżej wody i paszy. Pasze starter, grower i finisher, zakupiono w wytwórni pasz, a ich skład był zgodny z normami żywienia kurcząt brojlerów opisanymi przez Smulikowską i Rutkowskiego (2018). Zawartość białka wynosiła odpowiednio 22% dla paszy starter, 20,5% dla paszy grower oraz 19% dla paszy finisher. Koncentracja energii metabolicznej wynosiła średnio 12,50 MJ/kg. Pasza zawierała wszystkie niezbędne dodatki dla kurcząt brojlerów, w tym witaminy i składniki mineralne. W dniach 1, 14, 35 i 42 odchowu kontrolowano spożycie paszy. Na podstawie uzyskanych danych obliczono przyrost masy ciała i współczynnik konwersji paszy. W 42. dniu odchowu, 10 losowo wybranych osobników z każdej grupy zostało uśmierconych w celu pobrania do badań fragmentów jelit, zeskrobin błony śluzowej, treści jelit i migdałków jelit ślepych.

3.3.2. Pomiar pH oraz analiza krótkołańcuchowych kwasów tłuszczowych

Pomiary pH treści jelita biodrowego i ślepego wykonano za pomocą pH-metru, natomiast stężenie SCFA analizowano przy użyciu chromatografu gazowego z kwasem

izokapronowym jako wzorcem wewnętrznym. Stężenie amoniaku i aktywność β-glukuronidazy w obu jelitach oznaczono metodami kolorymetrycznymi.

3.3.3. Względna obfitość bakterii wskaźnikowych

Względna obfitość wybranych bakterii w treści jelit została obliczona według opublikowanego przez Christensen i wsp., (2014).

Analizie poddano względną obfitość następujących bakterii: *Akkermansia muciniphilla, Bifidobacterium* spp., *Clostridium difficile, Escherichia coli, Faecalibacterium prausnitzii* oraz *Lactobacillus* spp.

3.3.4. Względna analiza ekspresji genów

Analizy ekspresji genów dokonano z wykorzystaniem metody RT-qPCR. Względną analizę ekspresji genów przeprowadzono na podstawie metody opisanej przez Livak i Schmittgen (2001), w której względną ekspresję genów obliczono przy pomocy wzoru $2^{-\Delta\Delta CT}$. Analizę ekspresji genów przeprowadzono dla panelu, który obejmował geny związane z wrodzoną odpowiedzią immunologiczną (*IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-17, IL1-β*, *IFNγ*, *IFNβ*), peptydy obronne gospodarza (*AvBD1*, *CATHL2*), geny związane z metabolizmem (*FFAR2*, *FFAR4*, *GLUT1*, *GLUT2*, *GLUT5*) oraz związane z funkcją barierową (*MUC6*, *CLDN1*, *TJAP*). *ACTB* i *G6PDH* zostały użyte jako geny referencyjne.

3.3.5. Analiza kopii mitochondrialnego DNA oraz ekspresji genów mitochondrialnych

Analiza ekspresji genów dla migdałków oraz błony śluzowej jelita ślepego została przeprowadzona dla panelu genów związanych z mitochondriami: *CS*, *MPO*, *CYCS*, *TFAM*, *NRF1*, *ND2*, *MnSOD*. Analizę ekspresji genów przeprowadzono metodą RT-qPCR. Względnej analizy ekspresji genów dokonano na podstawie metody opisanej przez Livak i Schmittgen (2001) i obliczono według wzoru $2^{-\Delta\Delta CT}$. *ACTB* został użyty jako gen referencyjny.

Analizę względnej liczby kopii mitochondrialnego DNA w migdałkach i błonie śluzowej jelita ślepego przeprowadzono przy użyciu dwóch metod obliczeniowych. Weryfikację molekularną dla analizowanych genów *D-loop*, *ATP6*, *ND6* oraz *GCG* przeprowadzono metodą qPCR. Jedna z metod obliczeniowych dotyczyła liczby kopii mitochondrialnego DNA, którą określono według wzoru opisanego przez Zhang i wsp. (2020).

Natomiast druga metoda dotyczyła analizy względnej liczby kopii mitochondrialnego DNA, która została obliczona według wzoru opisanego przez Zhang i wsp. (2020).

3.3.6. Eksperyment 2 – podanie postbiotyku (maślan sodu) *in ovo* w 12 dobie inkubacji jaj

Do doświadczenia wykorzystano 1000 jaj podzielonych na 4 grupy po 250 szt. w każdej. W 12. dobie inkubacji jaja zostały poddane iniekcji 0,2 ml soli fizjologicznej (grupa kontrolna) lub 0,1%, 0,3% lub 0,5% roztworu SB. Inkubację jaj prowadzono w takich samych warunkach jak w doświadczeniu 1. Po wykluciu z każdej grupy wybrano 60 piskląt. Każdą grupę podzielono na 5 powtórzeń po 12 ptaków w każdym. Doświadczenie fermowe przeprowadzono analogicznie jak opisano w punkcie 3.3.1. W 42. dniu odchowu 10 losowo

wybranych osobników z każdej grupy zostało uśmierconych w celu pobrania do badań krwi, fragmentów jelit, zeskrobin błony śluzowej i treści pokarmowej.

3.3.7. Analiza biochemiczna krwi

Parametry biochemiczne osocza krwi analizowano spektrofotometrycznie przy użyciu analizatora MAXMAT PL. Stężenia sodu i potasu mierzono na analizatorze Na/K EasyLyte.

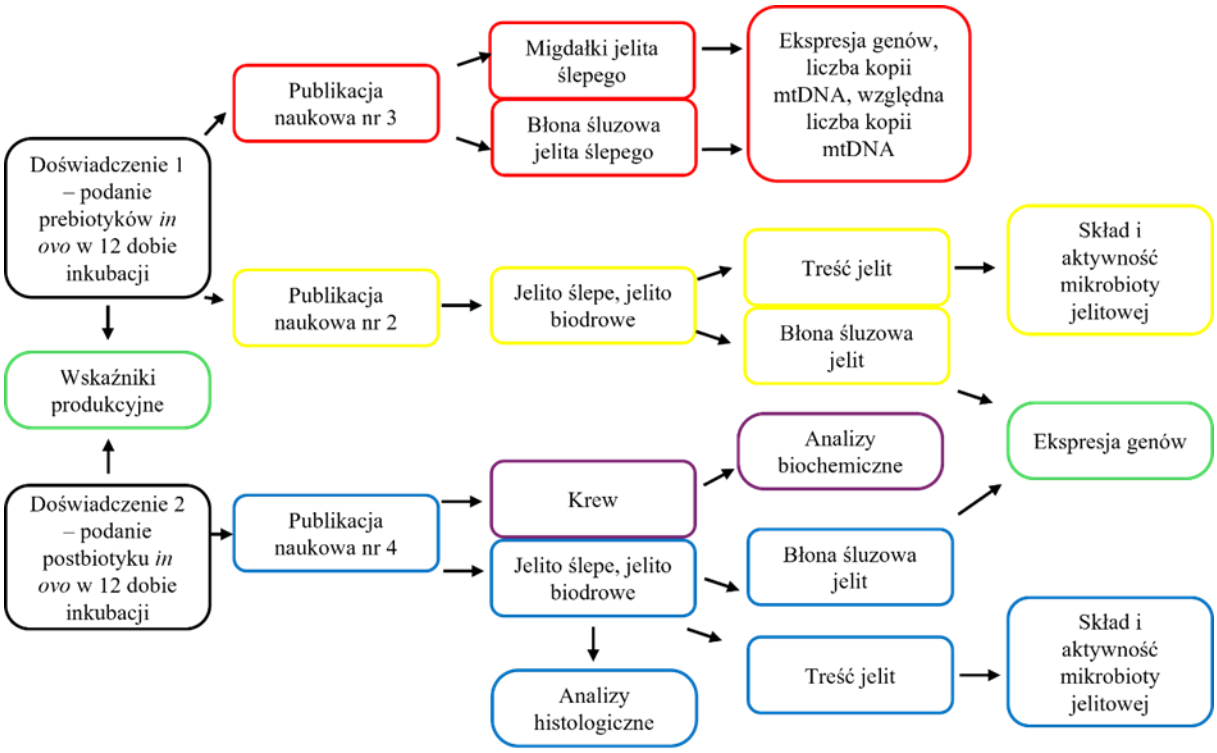
3.3.8. Względna obfitość bakterii wskaźnikowych oraz względna analiza ekspresji genów

Metoda analizy względnej obfitości bakteryjnej po stymulacji maślanem sodu była taki sama jak opisano w punkcie 3.3.3. Analizie poddano względną obfitość następujących bakterii *Akkermansia muciniphilla, Bifidobacterium* spp., *Clostridium difficile*, *Escherichia coli*, *Faecalibacterium prausnitzii* oraz *Lactobacillus* spp.

Analiza ekspresji genów została przeprowadzona dla następującego panelu: geny wrodzonej odpowiedzi immunologicznej (*IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL1-β*, *IFNγ*), peptydy obronne gospodarza (*AvBD1*, *CATHL2*) oraz związane z funkcją barierową (*MUC2*, *MUC6*, *CLDN1*, *TJAP*). *ACTB* oraz *G6PDH* zostały użyte jako geny referencyjne (zgodnie z metodą opisaną w punkcie 3.3.4).

3.3.9. Stężenie SCFA oraz morfologia jelit

Pomiary pH treści jelita biodrowego i ślepego przeprowadzono za pomocą pH-metru, natomiast stężenie SCFA analizowano przy użyciu chromatografu gazowego z kwasem izokapronowym jako wzorcem wewnętrznym. Do pomiarów histologicznych wykorzystano barwione przekroje jelit. W jelicie biodrowym określono wysokość kosmków, szerokość kosmków, głębokość krypt, stosunek wysokości kosmków do głębokości krypt, grubość błony mięśniowej oraz rodzaj mucyn w komórkach kubkowych. Ponadto obliczono powierzchnię kosmków, korzystając ze wzoru Law i wsp. (2007). Na preparatach jelita ślepego wykonano pomiary głębokości krypt i grubości błony mięśniowej oraz oznaczono rodzaj mucyn w komórkach kubkowych.



Rys 1. Schemat przeprowadzonych doświadczeń, cyklu publikacyjnego oraz przeprowadzonych analiz.

3.4. WYNIKI

3.4.1. Eksperyment 1 – podanie prebiotyków XOS i MOS *in ovo* w 12 dobie inkubacji

Stymulacja prebiotykami XOS3, XOS4, MOS3 i MOS4 in *ovo* nie wpłynęła istotnie na parametry produkcyjne kurcząt, pH treści pokarmowej jelita biodrowego i ślepego ani na stężenie SCFA. Stymulacja *in ovo* prebiotykami nie wpłynęła również na stężenie amoniaku w jelicie ślepym oraz aktywność bakteryjnej β-glukuronidazy w jelicie biodrowym i ślepym. W jelicie biodrowym nie zaobserwowano istotnych różnic w poziomie ekspresji genów po stymulacji *in ovo* testowanymi prebiotykami.

Podanie prebiotyków XOS i MOS *in ovo* znacząco wpłynęło na ekspresję genów w jelicie ślepym. Stymulacja spowodowała istotne zmiany w ekspresji genów wrodzonej odpowiedzi immunologicznej (*IL-2*, *IL-12*, *IL-17*), peptydów obronnych gospodarza (*AvBD1*, *CATHL2*) oraz genów związanych z metabolizmem składników pokarmowych (*FFAR4*, *GLUT1*). Podanie MOS3 spowodowało wzrost ekspresji *IL-2* (1.46), *IL-12* (2.13), *IL-17* (2.62), *AvBD1* (1.94), *CATHL2* (1.51), *FFAR4* (2.5) oraz *GLUT1* (2.14) względem kontroli. Natomiast podanie XOS4 spowodowało wzrost ekspresji genów *IL-2* (2.08), *IL-12* (2.23), *IL-17* (3.18) i genów związanych z metabolizmem kwasów tłuszczowych i glukozy *FFAR4* (3.11) oraz *GLUT1* (2.13) względem kontroli. Wyżej opisane wyniki przedstawiono w publikacji nr 2.

Podanie MOS3 wpłynęło istotnie na zwiększenie populacji bakterii *Bifidobacterium* spp. (0.00014) w jelicie biodrowym względem kontroli (0.00004). Obfitość bakterii *Bifidobacterium* spp. w jelicie ślepym wzrosła w grupach eksperymentalnych XOS3 (0.08),

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MOS3 (0.01) oraz MOS4 (0.001) względem kontroli (0.0008). Liczebność bakterii *Escherichia coli* wzrosła ponad 10-krotnie względem kontroli w grupie MOS3 (0.004). W przypadku bakterii *Faecalibacterium prausnitzii* zanotowano istotny wzrost w grupie XOS3 (0.01) oraz wzrost *Lactobacillus* spp. w grupach MOS3 (0.018) oraz MOS4 (0.018) w jelicie ślepym.

Istotną zmianę ekspresji genów mitochondrialnych wywołało podanie XOS4 i MOS3. Oba prebiotyki spowodowały wzrost poziomu ekspresji genów w jelicie ślepym. Ponad dwukrotnemu wzrostowi w obu grupach doświadczalnych względem kontroli uległy geny *EPX*, *CYCS*, *NRF1*, *ND2* oraz *MnSOD*. W przypadku migdałków jelita ślepego zaobserwowano istotny spadek ekspresji we wszystkich 4 grupach doświadczalnych dla poszczególnych genów: *CS*, *EPX*, *CYCS*, *TFAM*, *NRF1*, *ND2* oraz *MnSOD*.

Najwyższą wartość względnej liczby kopii mitochondrialnego DNA dla błony śluzowej jelita ślepego wykazano w grupie MOS4. Najniższą wartość względnej wartości dla liczby kopii mitochondrialnego DNA w błonie śluzowej jelita ślepego wykazano w grupie XOS4. Wartość dla wszystkich grup eksperymentalnych w tej tkance była bliska 1. Ponadto całkowita liczba kopii mitochondrialnego DNA była stabilna w obu tkankach (błona śluzowa jelita ślepego, migdałki jelita ślepego). Wyżej opisane wyniki zaprezentowano w publikacji nr 3.

3.4. 2. Eksperyment 2 – podanie postbiotyku (maślanu sodu) *in ovo* w 12 dobie inkubacji

Stężenie maślanu sodu nie wpłynęło istotnie na parametry wzrostu kurcząt brojlerów. Stymulacja postbiotykiem wpłynęła na zwiększone stężenie chlorków we krwi w grupach stymulowanych 0.1% SB (215 mM/l), 0.3% SB (184 mM/l) oraz w grupie 0.5% SB (209 mM/l) względem kontroli (178 mM/l). Stężenie kwasu moczowego zależne było od dawki; w kontroli wynosiło (205 μM/l), dla grupy z najniższą dawką maślanu stężenie kwasu moczowego spadło (183 μM/l), natomiast dla średniej i najwyższej dawki stężenie wynosiło odpowiednio (226 i 308 μM/l).

Stymulacja postbiotykiem spowodowała wzrost populacji bakterii *Bifidobacterium* spp. dla każdej z grup w jelicie biodrowym, natomiast w grupie stymulowanej 0,5% SB wzrost ten był istotny, ponad 10-krotny względem kontroli. Nie wykazano istotnych statystycznie różnic w populacji bakterii *Lactobacillus* spp., *Escherichia coli* oraz *Faecalibacterium prausnitzii* w jelicie biodrowym. W jelicie ślepym wykazano kilka istotnych zmian w populacji bakterii po stymulacji *in ovo* postbiotykiem. Populacja *Bifidobacterium* spp. znacząco wzrosła w grupie stymulowanej 0.3% SB (0.0006) względem kontroli (0.0002). Dodatkowo wystąpiła istotna różnica między grupą, która otrzymała 0.3% SB a grupą stymulowaną 0.5% SB (0.0002). Obfitość bakterii *Lactobacillus* spp. była istotnie mniejsza w każdej z grup poddanych iniekcji niż w grupie kontrolnej (0.038). Populacja *Feacalibacterium* wzrosła istotnie w grupach stymulowanych 0.1% SB (0.006) oraz 0.3% SB (0.007) względem grupy kontrolnej (0.003).

Poziom ekspresji genów *IL-1β*, *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *CATHL2*, *CLDN* oraz *MUC2* nie zmienił się istotnie w jelicie biodrowym w skutek stymulacji *in ovo* postbiotykiem. Wykazane zostały różnice dla dwóch genów związanych z odpowiedzią immunologiczną. W grupie stymulowanej 0.1% SB (0.4) zaobserwowano spadek poziomu ekspresji genu *IL-4* względem kontroli, natomiast dla genu *IFNγ* nastąpił znaczny wzrost w grupie, której podano 0.3% SB (1.87) względem grupy stymulowanej 0.1% SB (0.59). Istotne były również różnice w poziomie ekspresji *AvBD1* w grupie, która otrzymała 0.1% SB (2.92) względem kontroli

oraz pomiędzy grupą stymulowaną 0.1% SB a grupą z 0.3% SB (1.55). Różnice względem kontroli wystąpiły również dla genu *TJAP* w grupach stymulowanych 0.3% SB (0.6) i 0.5% SB (0.24) oraz dla genu *MUC6* w grupach, które otrzymały 0.1% SB (0.4) oraz 0.5% SB (0.48).

Panel badanych genów w jelicie ślepym był taki sam jak w przypadku jelita biodrowego. Istotne różnie nastąpiły dla genu *IL-1β* w grupie z najwyższą dawką SB (2.27) oraz dla genu *IL-8* po stymulacji najniższą dawką (4.21). Zmiany wykazano również dla genów mucyn. Poziom ekspresji *MUC2* wzrósł w grupach z 0.1% SB (3.97) oraz 0.3% SB (3.85) względem kontroli. W przypadku *MUC6* ekspresja uległa obniżeniu w grupie stymulowanej 0.1% SB (0.28) oraz wzrostowi w grupie, która otrzymała iniekcję 0.3% SB (0.43) względem grupy z 0.1% SB.

W jelicie biodrowym kurcząt brojlerów nie zaobserwowano wpływu stymulacji *in ovo* SB na pH treści pokarmowej i poziom SCFA. W jelicie ślepym stymulacja 0,1% i 0,3% SB znacząco zwiększyła stężenie kwasu propionowego w porównaniu z grupą kontrolną i grupą, która otrzymała 0,5% SB. Zaobserwowano również wpływ na stężenie kwasu masłowego, które było większe u ptaków stymulowanych 0,1% SB (12.51 μM/g) niż u ptaków stymulowanych 0,3% SB (8.92 μM/g) i 0,5% SB (7.51 μM/g). Stymulacja SB nie wpłynęła na stężenia pozostałych SCFA i pH treści pokarmowej jelita ślepego.

Stymulacja SB nie wpłynęła na parametry morfologiczne, takie jak wysokość, powierzchnia kosmka, głębokość krypty i ilość komórek kubkowych w jelicie biodrowym. W jelicie ślepym, głębokość krypt była większa w grupach stymulowanych 0,1% i 0,5% SB, niż w grupie kontrolnej. Uzyskane wyniki zaprezentowano i opisano w publikacji nr 4.

3.5. DYSKUSJA

Dotychczasowe badania przeprowadzone przez zespół Katedry Biotechnologii i Genetyki Zwierząt Politechniki Bydgoskiej im. Jana i Jędrzeja Śniadeckich dowodzą, że podanie *in ovo* w 12. dobie inkubacji pojedynczej dawki synbiotyku składającego się z *Lactobacillus salivarius* oraz GOS wpływa na wzrost populacji pożytecznych bakterii *Lactobacillus* spp. w jelicie ślepym brojlerów kurzych (Dunisławska i wsp., 2017). Podanie wyłącznie prebiotyku GOS wpłynęło na wzrost populacji korzystnych bakterii *Lactobacillus* spp. i *Bifidobacterium* spp., a także na korzystny profil ekspresji genów związanych z odpowiedzią immunologiczną oraz integralnością błony śluzowej jelita czczego, krętego, ślepego oraz dwunastnicy (Sławińska i wsp., 2019).

Wyniki doświadczenia 1., w którym podawano prebiotyki XOS i MOS *in ovo* wykazały ponad dwukrotny wzrost populacji bakterii *Lactobacillus* spp. i *Bifidobacterium* spp. w jelicie ślepym oraz kilkukrotny wzrost *Bifidobacterium* spp. w jelicie biodrowym kurcząt po stymulacji MOS3. Wyniki te wskazują na modulujące działanie mannooligosacharydu w przewodzie pokarmowym kurcząt brojlerów prowadzące do stymulacji wzrostu pożytecznych bakterii (Di Pede i wsp., 2024). O działaniu bifidogennym można wnioskować na podstawie wzrostu bakterii *Bifidobacterium* spp. w jelicie biodrowym oraz ślepym. Z kolei XOS4 spowodował wzrost poziomu ekspresji genów interleukin prozapalnych: *IL-2, IL-12* oraz *IL-17*, które wzmacniają odpowiedź immunologiczną oraz stymulują aktywację komórek odpornościowych. Wzrost ekspresji *GLUT1* można uznać za korzystny ze względu na jego funkcje. Gen koduje białko odpowiedzialne za ułatwianie podstawowego wychwytu glukozy, niezbędnego do wzrostu i rozwoju większości komórek w organizmie (Kono i wsp., 2005). Receptory *FFAR* odgrywają rolę w procesie wykrywania kwasów tłuszczowych i ich regulacji, szczególnie w mózgu i jelitach. Zazwyczaj wzrost ekspresji *FFAR4* następuje bezpośrednio po posiłku, co może regulować poposiłkowe mechanizmy zachowań związanych ze spożywaniem pokarmów wysokotłuszczowych (Roy i wsp., 2023). Dodatek XOS nie wpłynął na parametry wzrostu kurcząt, co może być spowodowane faktem, iż w doświadczeniu własnym XOS był podany w jednorazowej dawce do jaja. Dawka użyta w doświadczeniu mogła być niewystarczająca do oddziaływania na wskaźniki produkcyjne. Skuteczność dodatku paszowego w postaci XOS została potwierdzona przez Yadav i wsp. (2024), którzy wykazali, że dodatek tego prebiotyku na poziomie 0,5% znacząco zmniejszył pobranie paszy i poprawił wykorzystanie paszy u brojlerów. Maesschalck i wsp. (2015) również zaobserwowali poprawę współczynnika konwersji paszy, gdy do diety na bazie pszenicy i żyta dodano XOS. Jako prebiotyk, XOS promuje wzrost pożytecznych bakterii jelitowych i zwiększa produkcję SCFA w jelitach brojlerów (Wang i wsp., 2021). W doświadczeniu 1. stymulacja XOS nie wpłynęła na stężenia SCFA ani na stężenie amoniaku. Wszystkie ptaki w badaniach własnych były żywione taką samą paszą, co mogło mieć wpływ na otrzymane wyniki. Singh i wsp. (2022) zaobserwowali zwiększenie stężenie SCFA po stymulacji XOS3 w ilości 3 mg/0,5 ml.

Bakteryjna β-glukuronidaza jest enzymem, który hydrolizuje glukuronidy syntetyzowane w wątrobie w czasie detoksykacji ksenobiotyków (Pellock i Redinbo, 2017). Bierze zatem udział w krążeniu jelitowo-wątrobowym substancji uprzednio zneutralizowanych w wątrobie. Aktywność tego enzymu była znacznie większa w treści pokarmowej jelita ślepego niż biodrowego, co jest zgodne z wynikami analizy względnej liczebności bakterii. Aktywność β-glukuronidazy stwierdzono u bakterii *Bacteroides*, *Bifidobacterium* i *Eubacterium* (Beaud i wsp., 2005). W badaniu własnym wykazano, że liczebność *E. coli* i *Bifidobacterium* spp.

była znacznie większa w jelicie ślepym niż w jelicie w biodrowym, co może częściowo tłumaczyć różnicę w aktywności β-glukuronidazy między tymi fragmentami jelit. Wykazano, że podanie prebiotyków *in ovo* nie wpłynęło na aktywność tego enzymu między poszczególnymi grupami badawczymi. Brak efektu prebiotyków podawanych *in ovo* może świadczyć o tym, że wydzielanie żółci z wątroby nie zostało zaburzone u kurcząt brojlerów. Istotny był również fakt, że wszystkie ptaki były karmione tą samą dietą. Każda dieta dla kurcząt opierała się na zbożach i śrucie sojowej, która jest źródłem izoflawonów będących również substratami dla β-glukuronidazy (Tuśnio i wsp., 2014).

Interakcja pomiędzy mitochondriami komórek błony śluzowej a mikrobiotą jelitową odgrywa istotną rolę w utrzymaniu homeostazy jelit. Sygnały mikrobiologiczne docierające do mitochondriów znajdujących się w komórkach nabłonka jelitowego mogą zmieniać ich funkcję metaboliczną. Czynniki, które wpływają na mitochondria, mogą prowadzić do stanu zapalnego nabłonka jelitowego (Zhang i wsp., 2022). Wzajemne oddziaływanie między mikrobiotą jelitową a mitochondriami jest możliwe przez SCFA. Kwas masłowy jest źródłem energii dla komórek nabłonka i może mieć wpływ na metabolizm mitochondriów (Zhang i wsp., 2022). W badaniu własnym w ramach doświadczenia 1. wykazano, iż podane *in ovo* XOS4 i MOS3 zwiększają ekspresję genów *EPX*, *CYCS*, *NRF1*, *ND2* i *MnSOD* w błonie śluzowej jelita ślepego, natomiast w migdałkach jelita ślepego nastąpił spadek ekspresji dla wyżej wymienionych genów. Peroksydaza eozynofilowa (EPX) koduje białko występujące w ziarnistościach cytoplazmatycznych eozynofilów. Eozynofile promują homeostazę mikrobiomu jelitowego i ochronę przed zakażeniami żołądkowo-jelitowymi. *EPX* wykazuje aktywność przeciwbakteryjną wobec bakterii Gram-ujemnych (Percopo i wsp., 2018). Gen somatyczny cytochromu C (*CYCS*) koduje enzym cytochromu C, który odgrywa rolę w kompleksie łańcucha transportu elektronów w mitochondriach i podczas apoptozy. W normalnych warunkach białko to znajduje się wewnątrz mitochondriów. Uwalnianie go do cytoplazmy następuje podczas niezapalnej apoptozy komórek (Khan i wsp., 2019). W badaniach przeprowadzonych przez Tao i wsp. (2021) wykazano, że wzrost ekspresji *CYCS* w nerkach brojlerów związany był z występowaniem rakotwórczej aflatoksyny, która indukowała apoptozę komórek nerkowych przez szlak mitochondrialny. *NRF1* jako efektor oddziaływań jądrowo-mitochondrialnych jest niezwykle istotny dla biogenezy mitochondriów. Z kolei *TFAM*, który jest głównym regulatorem genu mitochondrialnego, jest niezbędny do utrzymania mitochondrialnego DNA wraz z dynamiką mitochondriów, przyczyniając się do przeżycia komórek. Na podstawie badań własnych można przypuszczać, że XOS4 i MOS3 podawane *in ovo* modulują mikrobiotę jelitową kurcząt, co ma wpływ na indukowaną biogenezę mitochondriów.

W przeprowadzonym badaniu własnym w ramach doświadczenia 2. wykazano, że podanie SB nie miało wpływu na przyrost masy ciała ani współczynnik konwersji paszy. Brak różnic wskazuje, że stymulacja *in ovo* SB jest bezpieczna dla ptaków i nie upośledza ich wzrostu ani wykorzystania paszy. Kurczęta brojlery były zdrowe przez cały okres odchowu, co zostało potwierdzone wysoką przeżywalnością i parametrami biochemicznymi krwi. Brak wpływu na aktywność aminotransferaz, a także na stężenie białka całkowitego, albuminy, bilirubiny i cholesterolu świadczy o braku szkodliwego wpływu na wątrobę ptaków. Stymulacja SB nie wpłynęła na stężenie SCFA w jelicie biodrowym, natomiast miała wpływ na stężenia SCFA w jelicie ślepym kurcząt. Stymulacja 0,1% i 0,3% SB zwiększyła stężenie kwasu propionowego, co sugeruje wzrost bakterii *Bacteroidetes, Megasphaera* spp. lub innych producentów propionianu, którzy mogą wykorzystywać 3 różne ścieżki do jego produkcji. Podanie 0,1% SB zwiększyło również stężenie kwasu masłowego w jelicie ślepym.

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Wynik ten można przypisać większej populacji bakterii *F. prausnitzii,* która należy do gatunków produkujących ten kwas. Porównanie efektów, jakie SB wywierał na SCFA oraz błonę śluzową jelita biodrowego i ślepego, wskazuje na większą specyficzność SB w stosunku do jelita ślepego. Można to wyjaśnić wyższą wrażliwością na maślan sodu komórek nabłonkowych i bakteryjnych w jelicie ślepym.

Wykazano również wzrost ekspresji genów związanych z odpowiedzią immunologiczną. W jelicie ślepym zanotowano istotny wzrost ekspresji genów dwóch cytokin prozapalnych: *IL-1β* w grupie stymulowanej 0.5% SB oraz *IL-8* w grupie stymulowanej 0.1% SB. Wzrost ekspresji tych interleukin następuje podczas infekcji, uszkodzenia tkanek lub innych bodźców zapalnych, takich jak narażenie na alergeny, toksyny lub stres (Al-Qahtani i wsp., 2024). Poziom ekspresji *MUC2* wzrósł w grupie z 0.1% i 0.3% SB, natomiast dla tych samych grup poziom ekspresji *MUC6* zmniejszył się względem kontroli. Melhem i wsp. (2021) odkryli, że TNF-α zwiększa ekspresję *MUC2*, ale nie wpływa na ekspresję *MUC6*. Różnica ta może wynikać z faktu, że TNF-α aktywuje specyficzne ścieżki sygnalizacyjne, takie jak NF-κB, które selektywnie regulują geny odpowiedzialne za produkcję określonych mucyn, takich jak MUC2. W jelicie biodrowym nastąpił wzrost ekspresji *AvBD1* w grupach stymulowanych SB. Alsafy i wsp. (2024) wykazali, że brojlery, które otrzymywały wodę zawierającą 0,98 mg SB cechowały się zwiększoną ekspresją genu *AvBD1* oraz zwiększoną powierzchnią chłonną (długością) kosmków jelitowych w jelicie biodrowym. Poziom ekspresji *TJAP* oraz *MUC6* w jelicie biodrowym zmniejszył się po stymulacji SB. *TJAP* należy do genów związanych z połączeniami ścisłymi. Li i wsp. (2023) wykazali, że wysokie stężenia kwasu moczowego w surowicy krwi (707,2 mM/l) mogą zmniejszać produkcję białek połączeń ścisłych i mucyny. Jest to zatem zgodne ze wzrostem stężenia kwasu moczowego w grupach stymulowanych SB sodu w badaniach własnych. Dodatkowo Li i wsp. (2023) wykazali, że niskie stężenia SB (2 mM) zwiększają, a wysokie stężenia zmniejszają produkcję białek połączeń ścisłych i okludyn. Może to wynikać z faktu, że wysokie stężenia SB mogą hamować proliferację komórek.

Przeprowadzone analizy wykazały, że stymulacja 0,5% SB zwiększyła populację bakterii *Bifidobacterium* spp. w jelicie biodrowym, natomiast w jelicie ślepym efekt bifidogenny zaobserwowano po iniekcji 0.1% oraz 0.3% SB. Może to być spowodowane specyfiką tych segmentów układu pokarmowego. Funkcją jelita biodrowego jest wchłanianie składników odżywczych, a skład mikrobioty wpływa na dostępność składników odżywczych i szybkość wchłaniania a tym samym na wskaźniki produkcyjne ptaków. Mikrobiota jelita ślepego ma zdolność trawienia pokarmów bogatych w celulozę, skrobię i oporne polisacharydy. Zdolność wielu szczepów bakteryjnych w jelicie ślepym do wzrostu na arabinoksylanie, polisacharydzie ze ściany komórkowej roślin, wskazuje na ważną rolę mikrobioty jelita ślepego w trawieniu zbóż. Dodatkowo jelito ślepe jest głównym miejscem absorpcji wody oraz transportu i absorpcji składników odżywczych (Stanley i wsp., 2014). W związku z różnicami w fizjologii jelita biodrowego i ślepego, wzrost populacji *Bifidobacterium* spp. mógł następować pod wpływem indywidualnej dawki maślanu.

Wu i wsp. (2018) wykazali, że stosowanie SB jako dodatku paszowego zwiększyło zagęszczenie liczebności komórek kubkowych i wydzielanie śluzu w jelicie czczym i biodrowym u kur. Liczba komórek kubkowych produkujących kwaśne mucyny została również zwiększona w jelicie cienkim przez suplementację diety SB (Sikandar i wsp., 2017). Zasadniczo, zwiększona wysokość kosmków i zmniejszona głębokość krypt w jelicie cienkim były uznawane za oznaki zdrowia jelit, ponieważ są wskaźnikami prawidłowego rozwoju nabłonka (Chiang i wsp., 2010). Elnesr i wsp. (2020) opisali, że długość i szerokość kosmków

były zależne od dodatku SB do paszy dla brojlerów. W 21. dniu życia długość kosmków wzrosła o około 55% i 27%, a w 42. dniu długość kosmków wzrosła o około 39% i 18% w porównaniu z grupą kontrolną. Guilloteau i wsp. (2010) wykazali, że kwas masłowy stymuluje wzrost kosmków jelitowych u drobiu i świń. W badaniu własnym nie zaobserwowano wpływu SB na wysokość kosmków jelitowych ani na ich powierzchnię w jelicie biodrowym kurcząt. Stymulacje dawkami 0,1% i 0,5% SB zwiększyły głębokość krypt w jelicie ślepym względem kontroli. Zwiększenie głębokości krypt wiąże się z większą szybkością proliferacji komórek nabłonkowych (Xue i wsp., 2018).

Przeprowadzone badania wskazują na indywidualny wpływ poszczególnych substancji bioaktywnych na odpowiedź ze strony jelit. Wpływ ten zależny jest od rodzaju substancji, zastosowanej dawki oraz odcinka przewodu pokarmowego.

3.6. PODSUMOWANIE

Przeprowadzone doświadczenia, polegające na określeniu odpowiedzi jelitowej kurcząt brojlerów na stymulację *in ovo* prebiotykiem i postbiotykiem, pozwoliły na potwierdzenie postawionej hipotezy. Zaproponowane substancje bioaktywne wpływają na zmiany zachodzące w jelitach (biodrowym i ślepym) oraz w migdałkach jelita ślepego. Przyczyniają się one do zmiany liczebności bakterii pożytecznych oraz chorobotwórczych wpływając pośrednio na zmiany fenotypowe i molekularne w jelitach kurcząt. Publikacja przeglądowa nr 1 pozwoliła na szczegółowy przegląd aktualnej wiedzy i podsumowanie najważniejszych informacji dotyczących substancji bioaktywnych podawanych *in ovo*, ich wpływu na mikrobiotę jelitową, funkcjonowanie jelit oraz osi jelito-wątroba-mózg.

Na podstawie doświadczeń opisanych w cyklu publikacji stwierdzono, że:

- stymulacja *in ovo* za pomocą prebiotyków XOS4 i MOS3 wywołała istotne zmiany w ekspresji genów, w tym tych związanych z odpowiedzią immunologiczną, peptydami obronnymi oraz kodującymi białka odpowiedzialnymi za wykrywanie składników odżywczych. Zmiany te sugerują potencjalną poprawę ogólnej zdolności immunologicznej oraz regulacji metabolicznej rozwijającego się zarodka kurzego,
- podanie *in ovo* prebiotyków XOS3, MOS3 i MOS4 stymuluje rozwój korzystnych bakterii (*Bifidobacterium* spp., *Lactobacillus* spp., *F. prausnitzii*) w jelitach kurcząt brojlerów. Tak wczesna stymulacja mikrobioty może przyczynić się do poprawy trawienia, wchłaniania składników odżywczych oraz bardziej efektywnego funkcjonowania układu immunologicznego u kur,
- prebiotyki XOS3, XOS4, MOS3 i MOS4 wpływają na mitochondria komórkowe w migdałkach jelita ślepego oraz błonie śluzowej jelita ślepego,
- dawka maślanu sodu 0.3% wpływa na odpowiedź jelitową na poziomie zmian mikrobioty jelitowej i zmian ekspresji genów związanych z odpowiedzią immunologiczną i barierą jelitową,
- stymulacja *in ovo* maślanem sodu powoduje zmiany w wybranych parametrach biochemicznych krwi, aktywności mikrobioty oraz parametrach morfologicznych błony śluzowej jelita ślepego. Stężenia SCFA są zależne od dawki maślanu sodu,
- maślan sodu podawany w stężeniu 0,3% ma najwyższy potencjał w kierunku poprawy i/lub utrzymania zdrowotności jelit.

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4. STRESZCZENIE

Odpowiedź jelitowa na stymulację prebiotykiem i postbiotykiem podanym *in ovo* w trakcie rozwoju embrionalnego

mgr inż. Aleksandra Bełdowska

Słowa kluczowe: *in ovo*, maślan sodu, mikrobiota jelitowa, oligosacharydy, zdrowie jelit

Zdrowie jelit jest kluczowym czynnikiem wpływającym na wzrost, dobrostan oraz wydajność brojlerów, ponieważ prawidłowe funkcjonowanie błony śluzowej i mikrobioty jelitowej warunkuje efektywne trawienie, wchłanianie składników odżywczych oraz ochronę przed patogenami. Przeprowadzone badania miały na celu ocenę wpływu substancji bioaktywnych, takich jak prebiotyki i postbiotyk, podawanych metodą *in ovo* w 12. dobie inkubacji jaj na zdrowie jelit, poprzez analizy molekularne oraz histologiczne. W tym kontekście wykorzystano ksylo- i mannooligosacharydy (XOS i MOS) jako prebiotyki oraz maślan sodu (SB) jako postbiotyk. Stymulacja *in ovo* prebiotykami wykazała, że substancje te modulują skład mikrobioty jelitowej, zwiększając liczebność pożytecznych bakterii, takich jak *Bifidobacterium spp.* i *Lactobacillus spp.* Podanie MOS3 znacząco zwiększyło liczbę bakterii *Bifidobacterium spp*. w jelicie biodrowym i ślepym, a także wpłynęło na zwiększenie liczby bakterii *Escherichia coli*. Działanie to wpłynęło na zmniejszenie liczebności potencjalnych patogenów. Ponadto, prebiotyki znacząco wpłynęły na ekspresję genów związanych z odpowiedzią immunologiczną, w tym interleukin *IL-2, IL-12* i *IL-17* oraz genów związanych z metabolizmem, takich jak *GLUT1* i *FFAR4*. Zaobserwowano także zmiany w funkcjonowaniu mitochondriów komórek jelitowych, wskazujące na ich związek z mikrobiotą i metabolizmem składników odżywczych. Zmiany w ekspresji genów mitochondrialnych, takie jak wzrost ekspresji genów *EPX, CYCS, NRF1, ND2* oraz *MnSOD*, zostały zauważone w grupach stymulowanych *XOS4* i *MOS3*, wskazując na potencjalny wpływ prebiotyków na funkcjonowanie mitochondriów. Podanie *in ovo* maślanu sodu wpłynęło na wzrost stężenia krótkołańcuchowych kwasów tłuszczowych, takich jak kwas masłowy i propionowy, w jelicie ślepym. SB zwiększył liczebność bakterii *Bifidobacterium spp.*, jednocześnie zmieniając ekspresję genów związanych z integralnością błony śluzowej, takich jak *MUC2* i *IL-1β*. Efekty działania maślanu sodu różniły się w zależności od dawki co podkreśla znaczenie specyficznego oddziaływania poszczególnych substancji na mikrobiotę i funkcje jelit. Wyniki badań wskazują, że stymulacja *in ovo* prebiotykami (XOS, MOS) i postbiotykiem (SB) stanowi skuteczne narzędzie do poprawy zdrowia jelit, wspierając odpowiedź immunologiczną. Wnioski na podstawie otrzymanych wyników wskazują, że stymulacja *in ovo* prebiotykami i postbiotykiem jest skuteczną metodą poprawy zdrowia jelit, wspierając odpowiedź immunologiczną i optymalizując procesy trawienne, co może przekładać się na lepszą wydajność produkcyjną brojlerów.

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5. ABSTRACT

Intestinal response to prebiotic and postbiotic *in ovo* stimulation during embryonic development

MSc Aleksandra Bełdowska

Key words: *in ovo*, sodium butyrate, gut microbiota, oligosaccharides, gut health

Gut health is a key factor influencing the growth, well-being and performance of broilers, as the proper functioning of the intestinal mucosa and microbiota determines effective digestion, absorption of nutrients and protection against pathogens. The aim of the study was to assess the effect of bioactive substances, such as prebiotics and postbiotics, administered *in ovo* on the day 12 of egg incubation on gut health, through molecular and histological analyses. In this context, xylo- and mannanoligosaccharides (XOS and MOS) were used as prebiotics and sodium butyrate (SB) as a postbiotic. *In ovo* stimulation with prebiotics showed that these substances modulate the composition of the gut microbiota, increasing the number of beneficial bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp. MOS3 administration significantly increased the number of Bifidobacterium spp. in the ileum and cecum, and also increased the number of *Escherichia coli*. This effect reduced the number of potential pathogens. In addition, prebiotics significantly affected the expression of genes related to the immune response, including interleukins *IL-2*, *IL-12* and *IL-17*, and genes related to metabolism, such as *GLUT1* and *FFAR4*. Changes in the functioning of intestinal cell mitochondria were also observed, indicating their relationship with the microbiota and nutrient metabolism. Changes in mitochondrial gene expression, such as increased *EPX*, *CYCS*, *NRF1*, *ND2*, and *MnSOD* gene expression, were observed in the XOS4 and MOS3 stimulated groups, indicating a potential effect of prebiotics on mitochondrial function. *In ovo* administration of sodium butyrate increased the concentration of short-chain fatty acid, such as butyric and propionic acids, in the cecum. SB increased the abundance of *Bifidobacterium* spp., while changing the expression of genes related to mucosal integrity, such as *MUC2* and *IL-1β*. The effects of sodium butyrate varied depending on the dose, emphasizing the importance of specific effects of individual substances on the gut microbiota and function. The results of the study indicate that *in ovo* stimulation with prebiotics (XOS, MOS) and postbiotics (SB) is an effective tool to improve gut health by supporting the immune response. Conclusions based on the obtained results indicate that *in ovo* stimulation with prebiotics and postbiotics is an effective method of improving intestinal health, supporting the immune response and optimizing digestive processes, which may translate into better production performance of broiler.

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6. ZAŁĄCZNIKI

6.1. KOPIE ARTYKUŁÓW NAUKOWYCH STANOWIĄCYCH CYKL PUBLIKACJI ROZPRAWY DOKTORSKI

6.2. OŚWIADCZENIE AUTORA ROZPRAWY DOKTORSKIEJ

6.3. OŚWIADCZENIA WSPÓŁAUTORÓW ARTYKUŁÓW NAUKOWYCH

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REVIEW

Open Access

State of the art in research on the gut-liver and gut-brain axis in poultry

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Abstract

The relationship between the intestines and their microbiota, the liver, and the neuronal system is called the gut-liver-brain axis. This relationship has been studied and observed for a relatively short time but is considered in the development of research focused on, e.g., liver diseases and intestinal dysbiosis. The role of the gut microbiota in this relationship is crucial, as it acts on poultry's performance and feed utilization, affecting meat and egg quality. The correct composition of the intestinal microbiota makes it possible to determine the essential metabolic pathways and biological processes of the individual components of the microbiota, allowing further speculation of the role of microbial populations on internal organs such as the liver and brain in the organism. The gut microbiota forms a complex, dense axis with the autonomic and enteric nervous systems. The symbiotic relationship between the liver and gut microbiota is based on immune, metabolic and neuroendocrine regulation, and stabilization. On the other hand, the gut-brain axis is a bidirectional interaction and information transfer system between the gastrointestinal tract and the central nervous system. The following paper will discuss the current state of knowledge of the gut-liver-brain axis of poultry, including factors that may affect this complex relationship.

Keywords Chicken, Crosstalk, Interaction, Intestines, Microbiota

Background

The gut microbiota has an essential function in the organism, including modulation of the immune response, digestion and further metabolism of nutrients, obtaining energy for the host, and shaping the feed intake and utilization level. Animal studies have shown that the intestinal microbiota is active not only in the intestines but also interacts with other organs in the digestive system, such as the liver, and organs outside, such as the brain [1]. The gut-brain axis is a bidirectional system of interactions between the gastrointestinal tract and the central nervous system (CNS). The role of the gut microbiota in this relationship is crucial because it forms a complex, dense network with the autonomic nervous system and the enteric nervous system. The intestine comprises motor neurons, sensory neurons, interneurons, and mucosa that transmit information between the CNS and the enteric nervous system (ENS). Intestinal neurons affect the microbiota physiology, absorption, secretion, blood flow, and communication. Intestinal neurons are connected to the gastrointestinal tract using primary messengers [2]. The vagus nerve is the main intermediary in communication between the CNS and the ENS. It is made of 80% afferent fibers and 20% of drainage fibers. The afferent neurons of the vagus nerve produce cholecystokinin (CCK) and serotonin (5-hydroxytryptamine, 5-HT) peptide receptors. The vagus nerve is the main component of the parasympathetic nervous system. The termination of the vagus nerve is located in the layer of

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the gastric and small intestine mucosa. In the mucosa of the gastrointestinal tract, there are enteroendocrine cells (EEC). They are responsible for the secretion of more than 90% serotonin in the body. In addition, EEC express receptors that react to metabolites of bacterial origin. The gut microbiota communicates with the CNS via several possible pathways [3]. The microbiota uses one of five possible pathways: the neuroanatomical pathway, the neuroendocrine pathway, i.e., the hypothalamic-pituitary-adrenal (HPA) axis, the gut microbiome metabolism pathway, and the intestinal mucosal barrier or immune system to communicate with the nervous system. Communication through the neuroendocrine and neuroimmune systems is called remote communication. The HPA axis represents the neuroendocrine system responsible for the body's response to the stressor. The factors that cause stress in animals include stress related to the external environment, such as heat stress and stress associated with, e.g., increasing the level of pro-inflammatory cytokines. These cytokines activate corticotropin-releasing factor (CRF) in the hypothalamus, stimulating adrenocorticotropin hormone (ACTH) secretion in the pituitary gland. At the same time, ACTH releases cortisol from the adrenal glands [4].

Numerous studies have shown that the intestinal microbiota plays a crucial role in the gut-brain axis because it produces bioactive peptides, neurotransmitters, short-chain fatty acid (SCFA), intestinal hormones, and branched-chain amino acids that affect inter-organ communication [2]. These bioactive compounds are involved in transmitting signals within the gut-brain axis, simultaneously stimulating the HPA axis. In addition, peptides directly affect the immune system of the intestinal mucosa, from which signals reach the CNS [5]. The gut-brain axis as a bidirectional system includes the endocrine, nervous and immune systems, allowing the host brain to influence the gastrointestinal tract and the organism's homeostasis [6].

Due to increased animal mortality caused by liver disease and its complications, researchers became interested in the relationship between the liver and other internal organs. In addition, the liver synthesizes and transports bile salts and antimicrobial molecules to the intestinal lumen through the bile ducts. In this way, it controls the unlimited growth of intestinal bacteria in the intestines [7]. On the other hand, intestinal microbiota produces numerous compounds that affect the liver, e.g., SCFA. Gut-liver-brain axis is a term that has been used and described recently. The increase in its popularity and the number of publications about it have been noticeable since 2016. This review aims to collect and define the primary information on the structure and function of the gut-liver-brain axis of poultry.

Intestinal microbiota

The intestinal microbiota consists of commensal, pathogenic, and concomitant microorganisms [1]. These microorganisms include bacteria, yeast, viruses, and protozoa [8]. It has been proven that the intestinal microbiota affects the organism's immune response, both adaptive and innate immunity [9]. The amount and composition of the microbiota vary depending on the place of colonization. *Lactobacillus* bacteria dominate the upper gastrointestinal tract in chickens while *Clostridium*, *Enterococcus*, and *Lactobacillus* are most abundant in the small intestine and ceca [10]. Such a diversity of bacteria is associated with the function of the digestive organs since gastric juices reduce the pH, which promotes colonization by lactic acid bacteria, e.g., *Lactobacillus* spp. [11]. In the ceca, the food content stays longer during the digestive process. There is also the highest concentration of SCFAs synthesized by intestinal bacteria [12]. The butyrate belonging to the SCFA is produced by the gut bacteria such as *Faecalibacterium prausnitzii*, *Clostridium* spp., and *Fusobacterium*. Moreover, *Bifidobacterium* and *Lactobacillus* bacteria have an anti-inflammatory effect and stimulate lipid metabolism in the liver, mainly by increasing the production of SCFA. These bacteria can decarboxylate essential amino acids, thereby producing amine by-products. Excess SCFA, which is not metabolized by intestinal epithelial cells, is transported through the hepatic vein to the liver, which can be incorporated as precursors to gluconeogenesis, lipogenesis, and cholesterologenesis. Acetate and propionate inhibit endogenous lipolysis [13]. SCFAs can also act as signaling molecules. They are also associated with synthesizing neuroactive molecules, including leptin, which is transported to the brain through circulation. SCFA, by regulating the appetite, reduces the adipocyte tissue of the liver. They affect the brain causing a feeling of satiety, which reduces food intake, mainly by stimulating the secretion of GLP-1 from endocrine cells. GLP-1 suppresses the appetite by stimulating the hepatic fibers of the vagus nerve. SCFAs stimulate adipocytes to synthesize and secrete leptin. Leptin is a satiety hormone that targets the neurons of the hypothalamus to increase satiety and reduce the storage of lipids in the liver [14]. The intestinal microbiota is a source of neuromediators and hormones like serotonin, catecholamine, melatonin, and histamine that directly regulate the functioning of the intestines and indirectly modulate the functions of extraintestinal organs such as the brain, kidneys, and liver [15]. The interaction between the intestinal microbiota and the host organism is bidirectional. Microorganisms shape the functioning

and development of the immune system, while the host organism's immune system shapes the composition and diversity of the microbiota in the intestines. Communication between the microbiome and parenteral organs occurs directly with the help of toll-like receptors (TLRs) and indirectly with the help of bacterial metabolites and signaling molecules [16]. A properly balanced diet rich in fiber and unsaturated fats contributes to the increase in the abundance of anti-inflammatory bacteria, which include *Bifidobacterium* and *Akkermansia*. These microorganisms are responsible for strengthening the intestinal barrier, preventing the translocation of microorganisms through the intestinal wall and the resulting endotoxemia [17]. Calefi et al. [18] showed that *Clostridium perfringens*, along with the heat stress, induced a negative behavioral response in broiler chickens and increased the expression of c-fos, the cellular proto-oncogene of the early cellular response, in the medial nucleus of the hypothalamus and the amygdala nucleus. The composition of the intestinal microbiota in broiler chickens is presented in Table 1.

Intestinal mucosa

The mucous membrane comprises lamina propria, epithelium, and smooth muscles. The outer layer of the small intestine is lined with absorbent cylindrical cells (enterocytes) alternately with goblet cells and enteroendocrine cells [26]. Goblet cells (GCs) are polarized epithelial cells that secrete mucins, the main constituents of mucus. Intestinal mucus is the host's first line of defense. It protects the surface of the epithelium from pathogens, enzymes, and mechanical damage occurring during the digestion process [27]. Bacteria and components produced by goblet cells are recognized by the sensory system of the immune and intestinal cells. The intestinal mucosa is densely colonized by microorganisms capable of metabolic activity [28]. Intestinal mucus should be a barrier, catching and immobilizing pathogens while allowing nutrients to penetrate the surface of the epithelium. The compact inner layer prevents the penetration of pathogenic bacteria such as *Clostridium perfringens* and *Escherichia coli* into intestinal epithelial cells [29]. More than 90% of nutrient absorption occurs in the small intestine, with a thinner layer of mucus. In contrast, a thicker layer of mucus is found in the large intestine, preventing excessive bacterial colonization [27, 30]. Mucin is a major component of cytoplasmic granules produced by goblet cells [31]. Movements of the cytoskeleton are regulated by constitutive secretion, moving secretory granules toward the cell surface. This constant release results in the maintenance of the mucus layer. It has been observed that bacteria destroying the mucosal surface stimulate the more rapid release of stored mucin granules. The physiological dynamics of mucins are influenced by bacterial metabolites such as SCFA, lactic acid, secondary bile acids, and ammonia, which regulate immunity and intestinal mucosa physiology [32–35]. The absence of these gut bacteria in uninfected chickens reduces the number of cup cells and decreases *MUC2* gene expression [36]. The rapid production of mucins in the intestinal villi facilitates digestion and protects the villi surface from microbial invasion [32]. The sulfate group protects mucins from degradation by bacterial enzymes in the gut. Sulfated mucins reduce the ability of the pathogenic bacterium *Campylobacter jejuni* to penetrate the intestinal mucus in poultry [37]. The inner layer of the mucosa contains transmembrane mucins, while the outer layer contains secretory mucins [27]. Prebiotic galactooligosaccharides (GOS) delivered *in ovo* increase *MUC* gene expression [32] and also stimulates the growth of intestinal villi and, thus goblet cells [38]. Alemka et al. [39] demonstrated the cytotoxic effects of mucins against *Salmonella* bacteria [39]. Intestinal dysbiosis disrupts the expression of the *MUC2* gene. Oxidative stress caused by a high-fat diet increased cytokines IL-1β and IL-17 and simultaneously decreased MUC expression. The intestinal bacterium *F. prausnitzii* is an important butyrate producer with anti-inflammatory properties, while *Akkermansia muciniphila* degrades mucin in the intestinal lining, causing syntrophic interactions and stimulation of intestinal metabolites. Co-cultures of *A. muciniphila* with butyrate-producing bacteria result in syntrophic growth. Butyrate affects glucose and energy homeostasis by activating intestinal gluconeogenesis [40]. It also stimulates mucus secretion [36, 41], thus its impaired metabolism in the colon epithelium may result in a thinner adherent mucus layer [42]. One factor that may inhibit butyrate metabolism is hydrogen sulfide produced by the intestinal microbiota during the catabolism of sulfur amino acids. Hydrogen sulfide may also damage disulfide bridges of mucins, contributing to the intestinal barrier's impairment [43].

Gut-brain axis

The brain and intestines participate in bidirectional communication with the help of the endocrine and nervous systems. This connection has been called the gut-brain axis. Bacterial metabolites and host hormones such as leptin and glucagon-like peptide 1 and 2 regulate host metabolic homeostasis, development, health, and behavior. Changes in gut microbiota composition can affect gut health and brain changes, such as altering monoamine concentrations in crucial brain areas, i.e., decreases in norepinephrine (NE), epinephrine (E), and 5-HT in the hypothalamus and dopamine in the midbrain [44]. It has been observed that any intestinal infection activates

Table 1 The composition of the microbiota in broiler chickens, considering the sections of the digestive tract and the compounds produced by the bacteria

Bacteria	Place of occurrence in the digestive system	Compounds produced by bacteria	Compounds description	Reference
Bifidobacterium	Caeca, crop, gizzard, esophagus, proventriculus	Vitamins K, B_1, and B_2, lactic acid	Lactic acid reduces pH, facilitates digestion and absorption of metabolic products, improves intestinal peristalsis, reduces cholesterol levels, and prevents diarrhea and indigestion.	[19]
Clostridium	Caeca, ileum	Propionate, butyrate	The ability to strongly bind propionate to Olfr78 receptors and G-protein-coupled receptor 41 (GPR41) is associated with the hypotensive effect of propionate, while Olfr78, in combination with propionate, increases blood pressure. Propionate, by interacting with the GPR43 protein present on adipose tissue cells and intestinal endocrine cells, stimulates the secretion of the intestinal hormones PYY (peptide YY) and GLP-1 (glucagon-like peptide-1), which consequently reduces the appetite and slows down the absorption of glucose.	[20, 21]
Enterobacter	Caeca, crop, gizzard, ileum	Histamine	The action of histamine is related to H receptors (H1-H4). It acts as a neurotransmitter and local hormone, modulates the work of the stomach, and heart work, smooths muscle contractions and circadian rhythm, and maintains body temperature.	[22]
Escherichia coli	Ileum	Hydrogen sulfide	It is a toxic gas secreted by the *E. coli* in the ileum. Excess H_2S is a problem in commercial poultry houses; exposure of the hen's internal organs to it causes immune dysregulation in hens.	[23]
Faecalibacterium	Caeca, large intestine	Butyrate	Its reduction contributes to a decrease in the integrity of the gastrointestinal barrier. The increase in the abundance of butyrate restores bone density and osteocyte activity and reduces inflammation in the skeletal system.	[24]
Lactobacillus	Caeca, crop, gizzard, esophagus, ileum, proventriculus	Acetylcholine, serotonin, dopamine, gamma-aminobutyric acid (GABA)	Vagus nerve GABA receptor expression and hyperpolarization of enteric nervous system neurons affect pain sensation by the nervous system. Serotonin, as a neurotransmitter and tissue hormone, participates in the transmission of impulses between cells of the nervous system; it affects emotional states, concentration, and memory.	[25]

the midbrain serotonergic system by increasing levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the hypothalamus. Accordingly, reduced levels of monoamines increase the abundance of pathogenic bacteria such as *Escherichia coli*, *Clostridium perfringens*, and *Salmonella* spp. in hens [44]. Intestinal commensal bacteria participate in the metabolism of undigested food residues, from which the body draws additional energy. The degradation of protein and carbohydrates also produces neuroactive components [11]. Neuroactive molecules include, for example, serotonin, which exerts a local influence on the regulation of physiological processes. Adequate serotonin levels positively affect gastrointestinal motility by increasing small intestinal peristalsis and decreasing gastric hydrochloric acid secretion. Decreased brain serotonin levels and increased catecholamine levels are found in animals fed only diets low in tryptophan and rich in tyrosine. The deficiency of serotonin causes a lack of appetite and aggression seizures. On the other hand, excess serotonin increases body temperature [45].

One neuronal pathway transmits information to the CNS via the vagus nerve afferent fibers, which can shape host behavior. The structural components of bacterial cell walls and products of bacterial metabolism are responsible for the host's immune response by activating enteroendocrine cells that affect the nervous system locally and systemically [46]. The intestinal nervous system is the main connection between the intestinal microbiota and the host organism. It is the second most complex nervous circuit in the organism. Through neural networks and neurotransmitters, it closely connects to the CNS. Because of the number of neurons in the enteric nervous system, it is called the second brain [47]. The brain affects gut physiology, microbiota composition, and the immune system. Neurotransmitters, including NE and E, stimulate the growth of beneficial microbial bacteria such as *Lactobacillus* spp. or *Bifidobacterium* spp. [48]. Through communication with the brain, the intestinal nervous system affects, among others, the cerebral cortex, amygdaloid body, or hypocampus. These structures are responsible for the organism's memory processes [49]. An effect of the microbiome-gut-brain axis on social behavior among animals was observed. Disturbance of the composition of microorganisms and the state of intestinal dysbiosis increases the sense of danger and can cause animals to separate from other individuals [50]. Hill et al. [51] observed the influence of the gut-brain relationship and the state of satiety in animals. Several compounds with antagonistic functions mediate endocrine regulation of appetite, i.e., those that increase food intake: neuropeptide Y (NPY) ghrelin and orexins A and B, and those associated with reduced hunger such as leptin, cholecystokinin, glucagon-like peptide-1 (GLP-1), and pancreatic peptides PP (pancreatic polypeptide) and peptide YY (PYY). Ghrelin and leptin are two compounds with opposing activities concerning energy balance and appetite regulation. The integration of signals that regulate appetite occurs in the arcuate nucleus of the hypothalamus. Groups of neurons are responsible for processing sent impulses into neural and behavioral responses. These consist of initiating a new meal when hunger appears or stopping further eating when satiety appears. The arcuate nucleus contains two antagonistically acting systems. The orexigenic system first includes orexin A and B and NPY neurons, which express appetite-stimulating substances. These substances reduce energy expenditure under starvation conditions and stimulate food intake. The second system is the anorexigenic system consisting of proopiomelanocortin (POMC) and α-melanotropic hormone (MSH). These substances decrease appetite and energy expenditure [51]. Bacterial components and metabolites stimulate the satiety pathway by stimulating endocrine cells after feed intake. Bacterial peptides like glucagon-like peptide-1 (GLP-1) and glucagon-like peptide 2 (GLP-2) act directly in the hypothalamus. They are responsible for the long-term regulation of appetite [52]. A study conducted on germ-free Japanese quails found that the state of intestinal dysbiosis reduces the emotional response to fear and social difficulties without much impact on animal growth [53]. Gentle pecking of feathers in chickens is considered normal social behavior, while strong pecking is already considered a manifestation of aggression [54]. This harmful behavior was linked to the gut-brain axis because the lines of aggressive and non-aggressive chickens exhibited a variable gut microbiome. Pecking feathers can lead to increased stimulation of the intestinal wall, thereby impairing serotonin signaling [55]. Intestinal pathogens usually cause disease states in the host organism. Such symptoms are generally accompanied by a decrease in feed intake, as a consequence of which the growth of animals is slowed down. To reduce the losses associated with the reduction of feed intake, Bacillus spores may be given to animals because they increase the frequency and duration of feeding [56]. Giving chickens tryptophan modifies the intestinal microbiota, reducing serum serotonin and heat shock protein levels. The metabolism of tryptophan to serotonin has been linked to feather pecking in birds, and its supplementation reduces this behavior [54]. Giving pro or prebiotics as a feed additive is the most practical way to shape the intestinal microbiota. In the case of poultry, these additives can also be given *in ovo* on d 12 of incubation [54]. Since many breeding situations can cause stress during rearing, and this condition affects the microbiome, it is important to take care of the proper microbiome. Bacteria synthesize neurotransmitters,

including 5-HT, which acts locally. In the small intestine, 5-HT is released into the mucous membrane, affecting intestinal peristalsis, stimulating bicarbonate secretion during digestion, and dilating blood vessels [57]. In controlled conditions, microorganisms of the gastrointestinal tract play beneficial roles. They participate in competing with pathogenic bacteria and keeping the structure of intestinal mucus intact [27]. In addition, they ferment undigested polysaccharides into SCFA and provide vitamins, especially from group B [58]. The gut microbiota is a source of peripheral hormones and neurotransmitters, such as 5-HT and dopamine. These molecules directly communicate the state of intestinal health through the vagus nerve fibers, up to the brainstem and other areas of the brain. Stressful signals through peripheral and central pathways activate the hypothalamus-pituitary-adrenal axis (HPA). This can alter the composition and function of the gut microbiota, as well as the function of the intestinal epithelium [44]. The release of corticotropin-secreting factor from the hypothalamus stimulates the release of adrenocorticotropic hormone from the anterior pituitary lobe into the circulatory system. This causes the secretion of corticosteroids from the adrenal glands, such as corticosterone, in birds. Corticosteroids affect the gastrointestinal tract through direct interactions with gut cells and bacteria, thus leading to the release of cytokines (interleukin-6), which affect the brain and regulate appetite, mood, and cognitive function [59]. Regulation of appetite occurs in the hypothalamus and brainstem. Intestinal nutrients stimulate the secretion of cholecystokinin, the hormone responsible for satiety [60]. Synapses have been discovered in enteroendocrine cells, which at the same time are cells of the sensory epithelium of the intestine. They transmit sensory signals from the intestinal environment to the brain [61]. A simplified diagram of the mechanism of interaction between the microbiota and the brain is presented in Fig. 1.

Gut-liver axis

The term gut-liver axis describes the close functional-anatomical relationship between the liver and the gut [62]. Owing to the numerous occurrences of liver diseases, interest began to be taken in the relationship between the intestinal microbiota and the liver. Intestines and the liver communicate via the portal vein and systemic circulation. Metabolites produced by the intestines are transported through the portal vein to the liver. At the same time, the liver transports bile salts and antimicrobial molecules to the intestinal lumen through the bile ducts. In this way, it controls the unlimited growth of bacteria. Antimicrobial peptides and molecules (AMPs) are a diverse class of naturally occurring molecules. They are produced as the first line of defense by all multicellular organisms. These proteins can exhibit broad activity to neutralize fungi, bacteria, yeast, viruses, and cancer cells. Antimicrobial proteins include interferon: alpha (α), beta (β), and gamma (γ) [7]. A diseased liver cannot properly inhibit bacterial overgrowth, eliminate harmful by-products, and accelerate regeneration. The liver is also one of the immune organs, activating adaptive and innate immunity mechanisms after exposure to intestinal bacteria in the circulatory system [63]. The liver is an organ with immune properties. One of the liver functions is phagocytosis, the engulfment of particles originating in tissues or entering the body from outside, primarily with blood from the portal vein. These particles are degraded cell fragments, denatured proteins, lipoproteins, viruses, bacteria, and fungi. These particles are degraded in the liver macrophages and the Kupffer cells. Kupffer cells are sedimented macrophages found between endothelial cells in the wall of sinusoidal vessels in the liver. Their main functions include participation in the body's immune mechanisms through phagocytosis of bacteria and phagocytosis of cancer cells [63]. In addition, the liver is the main site of plasma protein

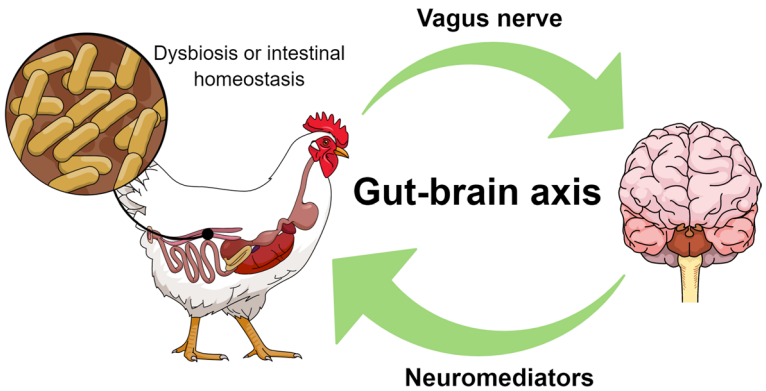

Fig. 1 The mechanism of communication of the gut-brain axis

synthesis. Hepatocytes, the liver cells, are responsible for the secretion of immunoglobulins, albumin, and fibrinogen. Hence, liver dysfunction usually decreases plasma protein production [64]. The enterohepatic axis represents a close bidirectional relationship between the intestine and the liver. The liver is an organ exposed to the products of digestion and absorption, in addition to all factors coming from the intestines, which include bacteria and components of bacterial origin, e.g., lipopolysaccharides (LPS), SCFAs, ammonia, phenols, toxins, and carcinogens previously neutralized in the liver, which are re-released by the bacteria and end up in the gut-liver circulation. The liver produces bile which is later stored in the gallbladder. Bile is composed of emulsifying fats, bile salts, and bile pigments. These include bilirubin, which is a breakdown product of hemoglobin. The small intestine uses liver bile to break down fats. In addition, the liver stores glycogen, buffers blood glucose levels and thus participates in carbohydrate metabolism. Excess sugars in the liver are converted into fatty acids. The liver is also involved in the breakdown of amino acids, a process in which liver cells convert a toxic by-product (ammonia) into urea [65].

The venous system of the portal circulation determines the enterohepatic axis and emphasizes the importance of anatomical and functional interaction of the gastrointestinal tract and liver [66]. The portal vein is a direct venous outflow from the intestine. Increased intestinal barrier permeability automatically exposes the liver to numerous toxic components of intestinal origin, including intestinal bacteria such as *Escherichia coli* [67]. Intestinal dysbiosis is associated with increased intestinal permeability and, consequently, with exposure of the liver to bacterial components. These are called molecular patterns and are divided into two groups, pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). Both patterns can cause liver damage [68]. Pathogen-specific patterns (PSPs) act directly on hepatocytes and/or cells of the liver's innate immune system, including Kupffer cells and starlit cells. The activated immune system of the liver stimulates pro-inflammatory, antiviral, and antiapoptotic pathways in hepatocytes. Such a reaction has both positive and negative effects. The harmful effect is the activation of the immune response and the release of pro-inflammatory cytokines, while the positive effects include hepatocyte reconstruction and cytoprotection. The liver, through bile acids and secretory immunoglobulin A (sIgA) affects the intestinal microbiota, regulating the microbial population. Immunoglobulin synthesis in the gut is one of the body's initial protective responses to pathogenic bacteria in the intestinal contents. Bile produced in the liver is the main source of sIgA in the intestinal lumen. sIga's primary function is to prevent pathogenic bacteria and viruses from attaching to and attacking erythrocytes. The concentration of immunoglobulins in the gut depends on the amount of sIgA in the gastrointestinal tract and can vary from day to day [69, 70]. In the future, therapies are predicted in which it will be possible to use an artificial microbiota to reduce the permeability of the intestinal barrier and reduce the release of pro-inflammatory cytokines in the intestine. Disturbed integrity of the intestinal barrier caused by dysbiosis leads to an increase in bacterial translocation and metabolic endotoxemia, which activates the hepatic TLR system, thus the local inflammatory response of the liver [71]. Bacterial metabolites, which include SCFAs and bile acids, are heavily involved in normal liver function and reduced lipogenesis and liver inflammation. Aberrations occurring in the composition, diversity, and function of the commensal microbiome lead to increased intestinal permeability, LPS production, ethanol production, and bile production. All these metabolites and factors combined with lipids from food can cause liver disorders. These include steatosis, inflammation, and liver damage. Liver disorders such as primary cholangitis or spotted liver disease in poultry farming can be caused by both qualitative and quantitative changes in the gut microbiota [72, 73]. A healthy liver is a barrier between systemic circulation and the intestines. In the case of disturbances in the functioning of the liver, this barrier is dysfunctional. Links were found between liver disease and the composition of the microbiome.

The diagram of the relationship between the microbiota and the gut is presented in the Fig. 2.

Factors affecting the gut-liver-brain axis in poultry

Nutrients

Nutrients significantly affect the brain, liver, and most internal organs by affecting their development and functioning during health and disease. Diet is the most important modulator of the intestinal microbiota, both in terms of its development and biosynthetic abilities. Amino acids are one of the essential nutrients supplied to the host by the intestinal microbiota. They act as neurotransmitters (*L*-glutamate). They can also be precursors for the synthesis of neurochemicals, including serotonin, gamma-aminobutyric acid (GABA), dopamine, and norepinephrine. Strains of *Lactobacillus* and *Bifidobacterium* bacteria can metabolize amino acids [74]. This is beneficial in the context of animals, where intestinal microorganisms provide amino acids that are unavailable in their daily diet. Amino acids, the end product of protein digestion, are absorbed into the blood vessels of the intestinal villi and transported to the liver through the portal vein. Amino acids act as precursors and signal the animal's

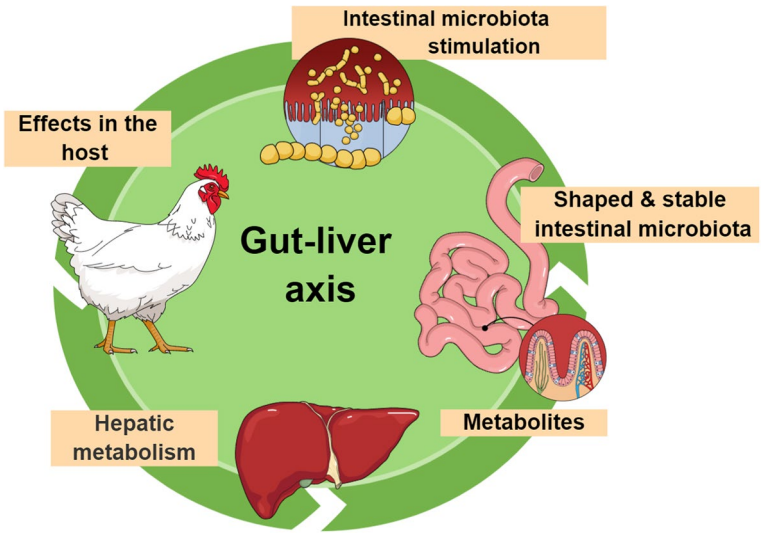

Fig. 2 Simplified diagram of the gut-liver axis mechanism in chicken

nutritional status to the brain [74]. Excessive consumption of sugars affects changes in the brain's functioning and its impairment. It has been mainly observed that sugars reduce animal learning ability and memory [75].

Excessive fiber consumption stimulates the abundance of *Bacteroides thetaiotaomicron*, which increases the amount of free sialic acid. Such a reaction may contribute to the growth of the pathogenic bacterium *Clostridium difficile* [76]. Dysregulation of SCFA interferes with metabolism and sleep [77]. In turn, omega-3 deficiency increases aggression and arousal [78]. Dietary fiber increases the ratio of Firmicutes to Bacteroidetes, while the ketogenic diet causes the growth of *Akkermansia*, which modulates host amino acids' metabolism [79]. The diet-microbiome interaction is based on the action of metabolites and nutritional components derived from the diet on the host organ systems. Salah et al. [80] experimented on chicken broilers exposed to heat stress (8 h, 34 °C) and fed a diet supplemented with curcumin (100 mg/kg diet). These studies showed that the addition of curcumin to a diet of heat stressed-chickens doubled the level of coenzyme Q10 in the liver [80].

Additionally, curcumin reduced the influence of thermal stress on the level of the enzyme Na/K ATPase in the liver. The addition of curcumin reduced the percentage of unwanted fat deposits in the abdominal cavity in heat-stressed broilers, which may be due to its soothing effect on energy metabolism. Curcumin increased serotonin levels in the brain of broilers to the level of the control group. This study shows that nutrients are crucial in the brain's functioning, and undisturbed animal behavior [80].

Nutrition and compounds produced by bacteria can also affect up and down-regulation of gene and protein expression. Downward regulation is when a cell reduces the amount of a cellular component, such as RNA or protein, in response to external stimuli. An example is a cellular decrease in receptor expression in response to its increased activation by hormones or neurotransmitters. This results in a decreased sensitivity to the molecule. During downward regulation, intestinal cells produce signaling molecules that circulate in the blood and pass through the blood-brain barrier to the CNS. Salt in the diet stimulates the response of Th17 cells in the intestine, which induces an increased amount of interleukin-17 in the plasma. IL-17 affects the endothelial cells of the brain and inhibits the production of nitric oxide, thereby reducing brain perfusion [81]. Upward regulation involves the response of liver cells exposed to xenobiotic molecules. This increases the degradation of such molecules. Up-down adjustment is carried out thanks to operating three systems: autonomic nervous system (ANS), enteric nervous system (ENS), and central nervous system (CNS). ANS refers to the sympathetic and parasympathetic nerves, which controls the motility of the gastrointestinal tract and regulates blood flow in the digestive tract and the secretion of digestive juices. ANS also includes mast cells (mastocytes), found in the most outstanding amounts in blood vessels and around the endings of nerve fibers. Mastocyte granules are rich in histamine and heparin, stimulating the secretion of prostaglandins and cytokines. Their function is to receive and transmit signals to the nervous system. Their role in the mechanisms of the acquired immune response is based on the ability to present the antigen and direct the action

of the released cytokines and other humoral factors [82]. ENS reacts to gastrointestinal microorganisms and converts chemical signals from the environment into nerve impulses, which are then spread to the intestines and other organs [83]. The CNS regulates the sympathetic and parasympathetic nerves, affecting the digestive system [84].

Probiotics and prebiotics

Probiotics are cultures of living microorganisms used as functional components to shape and maintain the proper state of health of the body. They act on the intestinal microbiota to increase the activity of digestive enzymes, reduce pathogen development, and stimulate the immune system [85]. Probiotics work properly only when they survive in the gastrointestinal tract. The main probiotic bacteria are *Lactobacillus* and *Bifidobacterium* [86]. Probiotics produce lactic acid or SCFA. Studies conducted on poultry provided information on the regulating effect of *Bifidobacterium infantis* on excessive stress response through the hypothalamic-pituitary-adrenal axis [87]. In addition, numerous studies have shown that probiotics reduce the negative effects of stress. The gut microbiota can influence the central nervous system via the gut nervous system and the immune system under stress. This is due to the fact that stress increases intestinal permeability. This allows commensal microorganisms to translocate through the intestinal mucosa and interlocate with immune cells and neurons of the enteric nervous system [6]. Probiotics help in bacterial colonization of the intestines, which is crucial for the proper development and growth of the immune and endocrine systems. A probiotic consisting of *Bacillus subtilis* given to chickens prevents complications after exposure to heat stress. In turn, the addition of *Lactobacillus* reduces the population of *Escherichia coli* in the cecum. These are the positive effects of probiotics on heat stress [88]. The cecal microbiota ferments prebiotics in the form of undigested carbohydrates and, as a result, stimulates the production of metabolites, including SCFAs. SCFAs, particularly butyrate, enhance the integrity of the intestinal mucosa by binding to endocrine L-cells [89]. In turn, prebiotics are substances that stimulate the growth of beneficial microorganisms [90].

The most popular are oligosaccharides GOS, mannanoligosaccharides (MOS), fructooligosaccharides (FOS), xylooligosaccharides (XOS), and inulin are fermented by intestinal bacteria, which produce SCFA and lactic acid. Stimulation with prebiotics promotes the growth of the abundance of *Lactobacillus* and *Bifidobacterium* bacteria, which are responsible for inhibiting the growth of pathogenic bacteria in the body [91]. Donalson et al. [92] showed that a diet enriched with 0.75% fructooligosaccharides reduced the occurrence of *Salmonella* spp. in the liver and ovaries. This is due to an increase in the abundance of lactic acid bacteria in the intestines and an increase in intestinal peristalsis [92]. Fowler et al. [93] studied the effect of MOS (250 ppm) on ROSS 308 broilers. They showed that oral administration of this prebiotic from the cell wall of *Saccharomyces cerevisiae* alleviates the effects of heat stress in chickens by increasing the abundance of butyrate-producing bacteria in the intestines. MOS prevents adherence and colonization of the intestine and liver by pathogenic bacteria *E. coli* and *Salmonella* spp. Additionally, MOS increases the height of the intestinal villi. Prebiotics selectively stimulate anti-inflammatory taxa growth and inhibit proinflammatory taxa growth [93]. *Lactobacillus* and *Bifidobacterium* bacteria reduce fat accumulation in the liver and minimize serum lipid concentration [94].

Intestinal dysbiosis

Following infection with bacteria that cause intestinal dysbiosis such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Enterococcus faecalis*, liver disease has been noted in hens. These pathogens were shown to cause extracellular amyloid deposition, and such a phenomenon is avian liver amyloid degeneration. A common disease occurring due to dysbiosis is fatty liver in chickens. Liposaccharides produced by *Escherichia coli* are endotoxins derived from the outer membrane of Gram-negative bacteria. They are detected in blood from the portal vein, indicating that the intestinal epithelium absorbs LPS and, if overdosed, can induce liver disease. The inflammatory response induced by LPS causes fatty liver. Dysbiosis is defined as an imbalance between the amount of harmful and defensive intestinal bacteria. It can affect the degree of hepatitis or liver fibrosis [13, 62].

Intestinal dysbiosis is involved in the pathogenesis of autoimmune liver diseases. These include primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Both conditions are chronic liver diseases, which development is mediated by the immune system. These dysfunctions are characterized by portal inflammation and slow progression. The causes of these diseases are intestinal dysbiosis, a change in the composition of the intestinal microbiota, a change in the composition of bile acids, unfavorable bacterial products (PAMP), and their metabolites [68]. Cirrhosis of the liver is characterized by the loss of liver cells and irreversible cicatrization. Intestinal dysbiosis in liver cirrhosis is accompanied by impaired intestinal barrier function and pathological distribution of bacteria. Bacterial components and toxins reach the liver through the damaged intestinal barrier, simultaneously accelerating the already present liver damage and

increasing the systemic inflammatory response. Prebiotics and probiotics prevent cirrhosis [95]. The use of probiotics reduces bacterial translocations, reduces anti-inflammatory effects, and reduces the release of pro-inflammatory cytokines, including TNF-α [96]. Intestinal dysbiosis has been classified as one of the main factors provoking pathogenesis in the liver, which affects the entire gut-liver-brain axis.

Heat stress

Stress is the host organism's physiological and psychological response to the disturbance of homeostasis. The digestive tract responds to various stressors, including heat stress [97]. The combination of too high ambient temperature with high relative humidity results in heat stress. It impairs the growth rate and development of the microbiota [98]. It is one of the main environmental challenges when the balance between the energy produced and the energy released from the body is impaired [99]. In poultry production, heat stress is considered one of the main factors negatively affecting egg and meat production and the general welfare of the flock through changes in intestinal microbiota [97]. Digestive tract organs exposed to stress are more susceptible to diseases. Birds exposed to stress factors, e.g., too high temperature and humidity of the environment, poor ventilation, or too long exposure to sunlight, have disturbed energy homeostasis [100].

Heat stress in chickens increases exposure to pathogenic intestinal bacteria such as *Salmonella* spp. caused by increased intestinal membrane permeability. This microorganism can also be detected in the liver, spleen, and muscles [101]. Exposure to high temperatures limits food intake in broilers. This is associated with changes in the activity of appetite-regulating peptides: anorexigenic peptides of the corticotropin-releasing factor family and orexigenic neuropeptide Y. These peptides act peripherally with the HPA axis [102]. Heat stress reduces food intake in laying hens, reduces laying capacity, and increases animal losses [103]. Under the influence of too high temperature, the intestinal mucosa of chickens is damaged, resulting in limited transport of nutrients [104]. In chickens subjected to heat stress, an increased abundance of *Escherichia coli* and *Clostridium difficile*, which produce alpha toxins that cause necrotizing enterocolitis, was found [105]. Two mechanisms mediate the impact of heat stress on the intestinal epithelium. The first mechanism is the production of reactive oxygen and nitrogen species in response to too high temperatures [106]. The second mechanism involves the production of pro-inflammatory cytokines, which is facilitated by thermal stress [107]. These cytokines include interleukin-2, produced by T cells [108].

The HPA axis is a system that mediates the body's response to stressors. Its activation releases ACTH and stimulates corticosterone production in birds [109], increasing its level in the blood. Consequently, lower food absorption, reduced immune response, and inflammation development are observed [110]. Corticosterone, cytokines, and selected hormones are factors common to the CNS, the immune system, and the endocrine system. Two catecholamines, epinephrine and norepinephrine, regulate the synthesis of cytokines reducing the expression of pro-inflammatory interleukin-12 and interferon-gamma, and increasing that of regulatory interleukin-10 [14]. The parasympathetic system receives signals from visceral organs and tissues or sends them back to the HPA axis [18]. In studies on chickens, it was shown that heat stress and irritation with *Clostridium* led to a decrease in the concentration of serotonin, epinephrine and norepinephrine in the hypothalamus and dopamine in the midbrain [111]. Dietary supplements (probiotics, prebiotics, and synbiotics) were used to alleviate the effects of heat stress [112]. The intestinal microbiota is sensitive to changing temperatures. A differentiated microbiome is necessary to maintain optimal regulation of signaling pathways in the host organism [113].

Heat stress threatens both humans and animals. Animals have an organism-specific thermal comfort zone necessary for proper functioning physiological functions. When the temperature exceeds the comfort zone's upper limit, heat stress begins in the animals [114]. The organisms of most animals developed phenotypic reactions (e.g., reduced daily feed intake) in response to heat stress. Unfortunately, a higher temperature is a beneficial factor for the growth and development of pathogens in the host [115]. In defense against the harmful effects of heat stress, animal organisms, including their microbiome, have developed molecular responses to repair damages and protect against their deterioration. Chronic exposure to heat stress reduces the number of type 1 T cells in the body while increasing the number of type 2 T lymphocytes. The imbalance causes changes in cytokine production [116]. Chronic heat stress causes an increase in the abundance of A-8 thermal shock proteins in chickens. These are proteins involved in the immune response [117]. Rapid and extensive transcriptional changes follow the heat shock. Transcription factors activating protein-1 (AP-1), a regulator of transcription and immunity, are activated in the gut to increase the immune response [118]. Heat stress stimulates the sudden and rapid release of the anti-inflammatory cytokines IL-1α, IL-8, and IL-10 into the bloodstream.

Heat stress decreases the expression of zonula occludens 1 in the jejunum and occludin in the ileum of broiler chickens, whereas it increases the expression

of IL-1β, IL-6, IFN-γ and TLR4 in both segments of the small intestine. The exposure to heat also contributes to a reduced abundance of *IL-10* mRNA in jejunum and ileum, showing that the impaired balance between pro- and anti-inflammatory cytokines contributes to the disruption of intestinal barrier function [119]. Heat stress lowers the expression of cholecystokinin mRNA in the duodenum and jejunum [120]. Under the influence of heat stress, the intestinal mucosa increases the ability to absorb sugars by up-regulation of GLUT expression. A decrease in GLUT2 expression was observed in the gut in broiler chickens after prolonged exposure to light [121]. The hypothalamic-pituitary axis affects the intestinal microbiota through ASN and the brain [122]. During *Salmonella typhimurium* infection, intestinal neurons produce IL-18, essential for the production of an antimicrobial protein in goblet cells [123]. Prolonged heat stress activates the HPA axis, increasing TNF-β and corticosterone concentration. Corticosterone disrupts the composition of the intestinal microbiota, causing an increase in the permeability of the gastrointestinal tract to pathogenic bacteria [124].

Summary

Although the concept of the gut-liver-brain axis is relatively new, the number of articles on the subject is small. It can be assumed that with an increase in understanding of the functioning of this relationship in the future, many poultry diseases will be reduced or eliminated. In addition, deepening knowledge and further targeted research will contribute to eliminating losses in animal production, including poultry production worldwide. For animal breeders, the proper growth and development of livestock are crucial. Therefore, it is essential to understand the inter-organ impact to control and eliminate unwanted economic losses through relevant factors.

Abbreviations

ACTH	Adrenocorticotropin hormone
ANS	Autonomic nervous system
AP-1	Transcription factors activating protein-1
CNS	Central nervous system
CRF	Cytokines activate corticotropin-releasing factor
DAMP	Damage-associated molecular patterns
E	Epinephrine
ENS	Enteric nervous system
FOS	Fructooligosaccharides
GABA	Gamma-aminobutyric acid
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide 2
GOS	Galactooligosaccharide
HPA	Hypothalamic-pituitary-adrenal
LPS	Lipopolysaccharides
MOS	Mannanoligosaccharides
MSH	α-melanotropic hormone
NE	Norepinephrine
PAMP	Pathogen-associated molecular patterns
PBC	Primary biliary cirrhosis
POMC	Proopiomelanocortin
PP	Pancreatic polypeptide
PSC	Primary sclerosing cholangitis
PSP	Pathogen-specific patterns
SCFA	Short-chain fatty acid
sIgA	Secretory immunoglobulin A
TLR	Toll-like receptor
XOS	Xylooligosaccharides
5-HIAA	5-Hydroxyindoleacetic acid

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Poultry Science

Impact of in ovo administration of xylo- and mannooligosaccharides on broiler chicken gut health

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ABSTRACT The intestinal mucosa creates a connection between the gut microbiota and the host. This study aimed to modify the gut microbiota of broiler chickens by in ovo stimulation with xylo-oligosaccharide (**XOS**) and manno-oligosaccharide (**MOS**) prebiotics and to determine the changes occurring in specific gut segments. Three hundred incubated eggs of Ross 308 broiler chickens on the 12th d of incubation were injected with: saline (control), xylotriose (**XOS3**), xylotetrose (**XOS4**), mannotriose (**MOS3**) or mannotetrose (**MOS4**). Tissue and digesta samples were collected post-mortem from 8 randomly selected individuals from each group, on d 42 after hatching. Gene expression analysis in the cecum and ileum was performed by RT-qPCR for a panel of genes: innate immune response genes (*IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-1β, IFNγ, IFNβ*), nutrient sensing and nutrient transport genes (*FFAR2, FFAR4, GLUT1, GLUT2, GLUT5*), host defence peptides (*AvBD1, CATHL2*), and barrier function genes (*MUC6, CLDN1, TJAP*). The relative abundance of bacteria was determined by qPCR for individual bacteria (*Akkermansia muciniphilla, Bifidobacterium* spp., *Clostridium difficile, Escherichia coli, Faecalibacterium prausnitzii,* and *Lactobacill*us spp.). Stimulation with prebiotics caused changes in the abundance of bacteria especially *Lactobacillus* spp. and *Bifidobacterium* spp. in the cecum. The abundance of both genera increased in each study group compared to the control group. The highest abundance of *Bifidobacterium* spp. in the ileum was found in the MOS3 group compared to the control group. There were changes in the XOS4 and MOS3 groups in the expression of: *FFAR4, GLUT1, AvBD1, CATHL2, IL-2, IL-12*, and *IL-17* in the caecum. In conclusion, in ovo administration of prebiotics increased intestinal colonization by bacteria. The prebiotics influenced gene expression levels via changes in the gut microbiota.

Key words: microbiota, prebiotic

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INTRODUCTION

The intestinal mucosa is the first line of defense, protecting the epithelial surface from pathogens and mechanical damage during digestion (Duangnumsawang et al., 2021). Mucus is responsible for stimulating colonization by commensal bacteria and providing an optimal environment for digestion and simplifying nutrient transport. The intestinal mucosa is densely colonized by microorganisms capable of metabolic activity (Forder et al., 2007). The intestinal mucosa should act as a barrier, trapping and immobilizing pathogens while at the same time allowing nutrients to penetrate the epithelial surface (Duangnumsawang et al., 2021). The intestinal mucosa includes the lamina propria, epithelium, and smooth muscle. The epithelium of the small intestine is composed mainly of cylindrical epithelial cells (enterocytes) alternating with goblet cells (Slawinska et al., 2019). The sensory system of immune and intestinal cells recognizes bacteria and their metabolites. This leads to the activation of the host's innate immune response, which involves secretion of cytokines: interleukin (***IL****)-1β, IL-4, IL-13*, and *IL-20*. Lymphoid tissue, in turn, forms the intestinal immune barrier. Innate gut barrier mechanisms include cytokines, mucins, and host defence

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peptides (**HDPs**) (Slawinska et al., 2019; Duangnumsawang et al., 2021). The intestinal microbiota plays an important role in maintaining intestinal health and influencing the overall performance of chickens. Under undisturbed homeostasis of the host body, intestinal bacteria mainly colonize the outer layer of mucus. They break down mucin proteins and glycans, using them as a potential energy source. Under undisturbed conditions, the inner layer is an impenetrable barrier to bacteria (Khan et al., 2020; Josenhans et al., 2020).

Environmental factors largely influence the composition of the microbiome. Metabolites produced by the microbiome, which include short-chain fatty acids (**SCFA**), are involved in host-microbiome communication and are responsible for maintaining barrier function and immune homeostasis. To optimize the gut microbiota, stimulation with bioactive substances, including prebiotics, are being used. Prebiotics are mostly oligosaccharides that are not digested by host enzymes. They make their way to the downstream (lower) parts of the intestines, where they promote the growth and proliferation of microorganisms. Feeding prebiotics to poultry strengthens the intestinal microbiota by improving host performance and activating resistance to colonization of intestinal pathogens such as *Salmonella* and *Campylobacter*. The most common prebiotics include galacto-oligosaccharides, manno-oligosaccharides, fructo-oligosaccharides, and xylo-oligosaccharides (De Maesschalck et al., 2015; Khan et al., 2020). The aim of this study was to modify the host gut microbiota by in ovo stimulation on d 12 of egg incubation with xylo-oligosaccharide and manno-oligosaccharide prebiotics, and to determine the changes occurring in intestines (ileum and caecum) in broiler chickens after stimulation.

MATERIALS AND METHODS

Experimental Setup

Hatching eggs from the parent flock of Ross 308 broiler hens were purchased from a commercial hatchery, the total number of eggs was 700. On d 7, the eggs were candled (ovoscope, Fermo, Piotrów, Poland), and 300 fertilized eggs were chosen for the experiment. On d 12 of incubation, the eggs were randomly divided into 5 groups (60 eggs in each group) and injected into the air chamber with 0.2 mL of saline (0.2 mmol/L) (control) or with one of the prebiotic solutions: xylotriose (**XOS3**), xylotetrose (**XOS4**), mannotriose (**MOS3**), or mannotetrose (**MOS4**). Oligosaccharides were administered at 0.5 mg/0.2 mL NaCl solution. Washable, nontoxic white glue "slime elmers" was applied to the resulting holes. Incubation was performed in a single-stage incubator and hatcher (Jarson, Gostyń, Poland). The eggs were incubated for 21 d. From 1 to 18 d, the eggs were kept in the incubator, while from 19 to 21 d in the hatcher. The incubator was set to 37.7°C, 55/60% humidity, and 50/60% ventilation (inlet open). The temperature in the hatcher was 37.5°C, humidity 70%, and ventilation 80%. The incubator was opened daily for control activities, verification of the microclimate, and dynamic short-term cooling due to the technical solution in the used laboratory incubator. In the incubator, the eggs were placed on trays (6 trays − repetitions with 10 eggs per each group) with automatic rotation by 45° every hour. On d 7 of embryonic development, the candling of eggs was performed to eliminate unfertilized eggs or eggs with early dead embryos. Similarly, the eggs were checked on the day of the transfer to the hatcher (d 18). The hatching data are shown in Table 1. After hatching, divided groups of birds were placed in pens on chopped wheat-straw bedding. Animals received feed and water ad libitum. Starter, grower, and finisher feeds were purchased from a feed factory. Their composition complied with the feeding standards for broiler chickens according to the nutritional recommendations. The protein content was 22, 20.5, and 19% in starter, grower, and finisher diets, respectively. The metabolizable energy was, on average, 12.50 MJ/kg. The feed contained all the necessary additives for broiler chickens, including vitamins and mineral ingredients. The feed composition was declared by the manufacturer.

Growth Performance

The rearing lasted 42 d. In each group, 48 broiler chickens were divided into 4 replications (pens) and kept at a stocking density not exceeding 33 kg of

Table 1. Hatching parameters of broiler chickens stimulated in ovo by different prebiotics.

Item	Group[1]					SEM	*P*-value
	Control	XOS3	XOS4	MOS3	MOS4		
Eggs in total (sum) on d 12	60.00	60.00	60.00	60.00	60.00	0.000	-
Eggs on d 18 (sum)	60.00[a]	60.00[a]	60.00[a]	58.00[b]	60.00[a]	0.091	0.034
Hatched chicks (sum)	57.00	54.00	56.00	54.00	53.00	0.316	0.724
Crippled and weak chicks (sum)	1.00	0.00	0.00	0.00	0.00	0.067	0.452
Unhatched eggs (sum)	3.00	6.00	4.00	4.00	7.00	0.321	0.737
% of chicks hatched from eggs in total	95.00	90.00	93.33	90.00	88.33	1.579	0.724
% of chicks hatched from eggs placed in the hatcher (on d 18)	95.00	90.00	93.33	93.16	88.33	1.607	0.744
% of chicks crippled and weak	1.67	0.00	0.00	0.00	0.00	0.333	0.452
% of eggs not hatched from eggs in total	5.00	10.00	6.67	6.67	11.67	1.604	0.737
% of eggs not hatched from eggs placed in the hatcher (on d 18)	5.00	10.00	6.67	6.84	11.67	1.607	0.744

[a,b]the mean values marked with different letters in the row differ statistically significantly at $P < 0.05$, SEM, standard error of the mean.
[1]XOS3 − xylotriose, XOS4 − xylotetrose, MOS3 − mannotriose, MOS4 − mannotetrose.

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livestock per 1 m^2 of the surface. Environmental conditions were provided for broiler chickens as described by Biesek et al. (2022). A starter diet was used from d 1 to d 14, a grower from d 15 to d 35, and a finisher from d 36 to d 42. The chickens were weighed (**BW**) on d 1, 14, 35, and 42, and feed intake (**FI**) was recorded. Body weight gain (**BWG**) and feed conversion ratio (**FCR**) were calculated based on the data obtained. Viability was also calculated for each group. On d 42, eight randomly selected individuals from each group were sacrificed to collect intestinal mucosa and digesta samples from ileum and cecum.

The experiment was conducted following the applicable regulations in Poland. The slaughter of the birds was carried out under the applicable regulations on the handling of animals during slaughter, including humane treatment. According to directive no. 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes, the consent of the Ethics Committee was not required. According to Act of January 15, 2015 on the protection of animals used for scientific or educational purposes (item 266, Journal of Laws of the Republic of Poland) slaughtering to collect tissues and organs from animals, is not a procedure. The chickens were stunned using percussive blows to the head (firm and accurate blows to the head provoking severe damage to the brain). It was done following applicable acts: Council Regulation (**EC**) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing (mechanical methods) and Directive no. 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes (methods of animal killing). Decapitation was performed by cutting off the head between the occipital condyle and the first cervical vertebra. There was rapid bleeding of the carcass.

Sample Collection

Intestinal mucosa scraped off the epithelium from cecum and ileum for gene expression was collected in a stabilizing buffer (fixRNA, EURx, Gdansk, Poland). Digesta samples for analyses of microbiota activity indices were taken from the distal part of the ileum and ceca and immediately frozen in dry ice. Mucosa and intestinal contents for isolation of bacterial DNA were collected and stored at -20°C until further processing.

Measurement of Digesta pH and Short-Chain Fatty Acid Analysis

Ileal and cecal digesta pH was measured using a SevenMulti pH-meter (Mettler-Toledo, Warsaw, Poland) after mixing with ultra-pure water in a 1:2 ratio (w/v). Then, pH of the sample was adjusted to 8.2 by adding 1 M NaOH to convert SCFA to their sodium salts. After centrifugation (10 min, 1,800 *g*, room temperature), the supernatants were stored at -20°C until further analysis. The concentration of SCFA was analyzed as described earlier by Barszcz et al. (2011) using the HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with isocaproic acid as the internal standard.

Ammonia Analysis

Ammonia concentration in the cecal content was measured spectrophotometrically according to Taciak et al. (2015). The absorbance of the color complex formed during the reaction of ammonium ion with Nessler's reagent was measured at 425 nm using a Maxmat PL biochemical analyzer (Erba Diagnostics France SARL, Montpellier, France). The concentration of ammonia was calculated from a standard curve prepared using NH_4Cl solution.

Analyses of β-Glucuronidase Activity in Intestinal Digesta

Digesta samples (ca. 0.5 g) were homogenized for 30 s at 18,000 rpm with 2.5 mL of ice-cold potassium phosphate buffer (pH 6.8 at 37°C) with 1% bovine serum albumin. The samples were sonicated and centrifuged (10,000 *g*, 20 min, 4°C). Supernatants were stored at -40°C for further analyses. The activity of bacterial β-glucuronidase was determined spectrophotometrically according to the method described previously by Barszcz et al. (2011), using phenolphthalein β-D-glucuronide as a substrate. The absorbance was measured using a Unicam UV 300 spectrophotometer set at 540 nm.

Relative Abundance of Bacteria

Total bacterial/stool DNA was isolated from approximately 120 mg of intestinal content of ileum and cecum, which were lysed and purified using the GeneMATRIX Stool DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions. The storage temperature of the DNA samples was -20°C. The extracted DNA was subjected to quantitative and qualitative evaluation by spectrophotometric method using NanoDrop2000 (Thermo Scientific Nanodrop Products, Wilmington, NC). The relative abundances of *Akkermansia muciniphilla, Bifidobacterium* spp., *Clostridium difficile, Escherichia coli, Faecalibacterium prausnitzii,* and *Lactobacillus* spp. in intestinal content were determined using quantitative PCR (**qPCR**) carried out on a LightCycler 480 II System (Roche-Diagnostics, Basel, Switzerland). The qPCR reactions mixture contained SG onTaq qPCR Master Mix (2x) (EURx, Gdansk, Poland), 1 μM of each primer specific to 16S rRNA (synthesized by Sigma-Aldrich, Schnelldorf, Germany) and 20 ng of bacterial DNA template. The thermal profile of the qPCR reaction was carried out as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of amplification consisting of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The fluorescence was measured at the end of each extension step. PCR efficiency for each pair of bacterial primers was calculated in the LightCycler 480 II

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Table 2. Bacterial primer sequences used in qPCR reaction (F- Forward primer; R-Reverse primer).

Bacteria	Primer sequences (Forward/Revers)	Reference
Universal bacteria	F: ACTCCTACGGGAGGCAGCAGT R: GTATTACCGCGGCTGCTGGCAC	(Christensen et al., 2014)
Akkermansia muciniphila	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	(Candela et al., 2012)
Bifidobacterium spp.	F: GCGTGCTTAACACATGCAAGTC R: CACCCGTTTCCAGGAGCTATT	(Christensen et al., 2014)
Clostridium difficile	F: TTGAGCGATTTACTTCGGTAAAGA R:TGTACTGGCTCACCTTTGATATTCA	(Penders et al., 2005)
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA	(Huijsdens et al., 2002)
Faecalibacterium prausnitzii	F: ACCATGAGAGCCGGGGGG R: GGTTACCTTGTTACGACTT	(Lund et al., 2010)
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	(Christensen et al., 2014)

software from a standard curve prepared for 5 dilutions (1x, 0.5x, 0.25x, 0.125x, and 0.0625x) of pooled bacterial DNA template. The relative abundances of the bacteria were calculated as follows:

Relative Abundances [%] = (E universal)$^{\text{Ct universal}}$/ (E target)$^{\text{Ct target}}$, (Christensen et al., 2014)

where E universal is the efficiency of qPCR with primers for all bacteria, Ct universal is the Ct values for reaction with primers for all bacteria, E target is the efficiency of qPCR with primers specific for target bacteria, Ct target is the Ct values for reaction with primers for target bacteria (*Akkermansia muciniphilla, Bifidobacterium* spp., *Clostridium difficile, Escherichia coli, Faecalibacterium prausnitzii* and *Lactobacillus* spp. (Table 2)).

Gene Expression

Total RNA was isolated from approximately 100 mg of ileal, and cecal mucosa, which were homogenized in 0.2 mL of chloroform and 1 ml RNA Extracol (EURx, Gdansk, Poland) using a TissuesRuptor homogenizer (Qiagen GmbH, Hilden, Germany). RNA was purified from the solution and contaminant using a GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland) following the manufacturer's instructions. Each RNA sample was quantitatively and qualitatively evaluated using the NanoDrop 2000 (Thermo Scientific Products). Gene expression analysis was performed for the gene panel, which included innate immune response genes (*IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL1-β, IFNγ, IFNβ)*, host defense peptides (*AvBD1, CATHL2)*, nutrient sensing genes (*FFAR2, FFAR4, GLUT1, GLUT2, GLUT5)* and barrier function genes (*MUC6, CLDN1, TJAP). ACTB* and *G6PDH* were used as reference genes (Table 3). Gene expression analysis was performed by qPCR with initial reverse transcription. cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania). The qPCR reaction was performed using LightCycler 480 II. The qPCR reactions mixture contained Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 1 μM of each primer specific to the target gene (synthesized by Sigma-Aldrich, Schnelldorf, Germany) and 70 ng of cDNA. The thermal profile of the qPCR reaction was carried out as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 45 s and melting curve. The ΔΔCT algorithm calculated relative gene expression. The amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analysis was performed using SAS statistical software (SAS Enterprise Guide 8.3; SAS Institute Inc., Cary, NC). The significance of gene expression and the effect on bacterial abundance was analyzed by one-way ANOVA. However, the significance of the influence of intestinal section, substance and interaction (intestinal section x substance) was calculated using a 2-way ANOVA followed by Tukey's HSD post hoc test, for which the classifying variable (tissue and group) and the dependent variable (tested gene).

RESULTS

Production Data

Data are presented as hatchability rates in Table 1. The sum of fertilized and non-dead eggs, the number of hatched, crippled, weak chicks, and unhatched eggs was calculated. Values are given as sums and percentages of eggs laid and fertilized in Table 1.In the MOS3 group, a significantly lower number of eggs were placed in the hatcher on the 18th d of incubation ($P = 0.034$). During the egg candling, it was found that the developing chicken embryos were dead in 2 hatching eggs, which were visible through a bloody ring adhering to the eggshell of the eggs. After opening the eggs, it was found that death occurred on d 14 (medium mortality) and d 17 (late mortality) (Table 1).

Table 3. Genes primer sequences used in RT-qPCR reaction (F- Forward primer; R-Reverse primer).

Gene	Name	Primer sequences (Forward/Revers)	Reference
ACTB	Actin, beta	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	(Slawinska et al., 2019)
G6PDH	Glucose 6 phosphate dehydrogenase	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	(Slawinska et al., 2019)
IL1β	Interleukin 1 beta	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	(Slawinska et al., 2019)
IL-2	Interleukin 2	F:GCTTATGGAGCATCTCTATCATCA R: TTGGGCAGGTTGAGGTTGTT	(Slawinska et al., 2019)
IL-4	Interleukin 4	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	(Biesek et al., 2021)
IL-6	Interleukin 6	F:AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	(Sławinska et al., 2014)
IL-8	Interleukin 8	F:AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	(Sławinska et al., 2014)
IL-10	Interleukin 10	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	(Biesek et al., 2021)
IL-12	Interleukin 12	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	(Slawinska et al., 2019)
IL-17	Interleukin 17	F: CCGTCTTCTGCTGAGAGGAGTG R: ACCGTTGTTCCGTCCCATCAC	(Biesek et al., 2021)
IFNβ	Beta interferon	F: ACCAGATCCAGCATTACATCCA R: CGCGTGCCTTGGTTTACG	(Biesek et al., 2021)
IFNγ	Gamma interferon	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	(Biesek et al., 2021)
AvBD1	Avian beta defensin 1	F: AAACCATTGTCAGCCCTGTG R: TTCCTTAGAGCCTGGGAGGAT	(Slawinska et al., 2019)
CATHL2	Cathelicidin 2	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	(Slawinska et al., 2019)
CLDN1	Claudin 1	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	(Slawinska et al., 2019)
TJAP1	Tight junction associated protein	F: AGGAAGCGATGAATCCCTGTT R: TCACTCAGATGCCAGATCCAA	(Slawinska et al., 2019)
MUC6	Mucin 6	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	(Slawinska et al., 2019)
FFAR2	Free fatty acid receptor 2	F: GCTCGACCCCTTCATCTTCT R: ACACATTGTGCCCCGAATTG	(Slawinska et al., 2019)
FFAR4	Free fatty acid receptor 4	F: AGTGTCACTGGTGAGGAGATT R: ACAGCAACAGCATAGGTCAC	(Slawinska et al., 2019)
GLUT1	Glucose transporter 1	F: AGATGACAGCTCGCCTGATG R: GTCTTCAATCACCTTCTGCGG	(Slawinska et al., 2019)
GLUT2	Glucose transporter 2	F: GGAGAAGCACCTCACAGGAA R: CAGGCTGTAACCGTACTGGA	(Slawinska et al., 2019)
GLUT5	Glucose transporter 5	F: ACGGTTCCCAGAGCAAGTTA R: GTCTTGCATGTATGGGGCTG	(Slawinska et al., 2019)

There were no statistically significant differences in growth performance parameters (Table 4).

Intestinal Microbiota Activity

In ovo stimulation with oligosaccharides did not affect ileal and cecal digesta pH. SCFA concentration in the ileum and ceca were similar in all groups and did not differ from the control group (Table 5). Also, there was no effect on ammonia concentration in the ceca of broiler chickens. Bacterial β-glucuronidase activity was considerably higher in ceca than in the ileum but was unaffected by in ovo stimulation with oligosaccharides (Table 6).

Gene Expression

Table 7 shows the significance of the effects of gut section (ileum, cecum) substance (XOS3, XOS4, MOS3, MOS4) and interaction (gut section x substance). In the ileum, no significant differences were noted in the level of gene expression after in ovo stimulation with prebiotics. Administration of prebiotics in ovo significantly affected gene expression in the cecum. Stimulation caused significant changes in the expression of innate immune response genes, host defense peptides, and nutrient-sensing genes. The MOS3 group shows an increase in the expression of all tested genes. The XOS4 group shows an increase in the expression of interleukins and nutrient-sensing genes. Figures 1, 2, and 3 show statistically significant changes in gene expression levels in the cecum.

Relative Abundance of Bacteria

Prebiotic administration in ovo had a significant effect on the relative abundance of bacteria in the ileum and cecum. In the case of the abundance of *Bifidobacterium* spp., their amount in the ileum in the MOS3 group increased more than 4 times compared to the remaining groups ($P < 0.05$). In the cecum, in the MOS3 group, there was a more than 2 times increase in the abundance of *Bifidobacterium* spp., *Lactobacillus* spp., and *Escherichia coli* as compared to the control group ($P < 0.05$).

Table 4. Growth performance of chickens stimulated in ovo by different prebiotics.

Item[1]	Groups[2]					SEM	*P*-value
	Control	XOS3	XOS4	MOS3	MOS4		
BW (g)							
D 1	46.48	46.25	45.75	46.63	46.79	0.204	0.461
D 14	432.30	439.82	431.08	431.71	424.15	4.400	0.577
D 35	1962.72	1924.78	1926.04	1948.13	1877.15	16.550	0.892
D 42	2520.53	2480.86	2484.78	2546.49	2451.32	27.855	0.589
BWG (g)							
D 1 – 14	385.82	393.57	385.33	385.08	377.35	4.400	0.872
D 15 – 35	1530.42	1484.96	1494.96	1516.42	1453.00	14.566	0.880
D 36 – 42	557.80	556.08	558.74	598.35	574.17	24.928	0.539
Total	2474.05	2434.61	2439.03	2499.86	2404.53	27.857	0.986
FI (g)							
D 1 – 14	459.75	470.37	459.90	480.30	458.54	4.536	0.872
D 15 – 35	2498.13	2571.79	2352.10	2459.62	2372.71	36.077	0.525
D 36 – 42	1206.37	1340.89	1267.91	1256.83	1171.36	34.283	0.296
Total	4164.25	4383.04	4079.91	4196.75	4002.61	68.038	0.629
FCR (kg/kg)							
D 1 – 14	1.20	1.20	1.20	1.25	1.22	0.017	0.509
D 15 – 35	1.63	1.73	1.57	1.62	1.63	0.025	0.860
D 36 – 42	2.37	2.54	2.33	2.12	2.04	0.125	0.386
Total	1.69	1.80	1.68	1.68	1.67	0.022	0.771
Viability (%)	87.50	89.58	95.83	95.83	97.92	2.059	0.300

[1]BW – body weight; BWG – body weight gain; FI – feed intake; FCR – feed conversion ratio; SEM, standard error of the mean
[2]XOS3 – xylotriose, XOS4 – xylotetrose, MOS3 – mannotriose, MOS4 – mannotetrose

Table 5. Digesta pH and SCFA concentrations in the ileum and cecum of broiler chickens stimulated in ovo by different prebiotics.

Groups	Short chain fatty acids, μmol/g digesta						pH
	Acetate	propionate	isobutyrate	butyrate	isovalerate	valerate	
Ileum							
Control	12.44	1.58	0.27	1.62	0.07	0.24	6.41
XOS3	9.52	0.44	0.24	0.08	0.00	0.18	6.51
XOS4	9.88	0.55	0.41	0.00	0.03	0.25	6.61
MOS3	9.40	0.59	0.39	0.02	0.00	0.29	7.45
MOS4	7.90	0.56	0.32	0.27	0.00	0.22	6.82
SEM	0.878	0.178	0.031	0.228	0.010	0.022	0.140
P	0.6108	0.2263	0.3753	0.1112	0.1826	0.6092	0.1614
Cecum							
Control	59.41	18.49	1.30	9.09	1.06	1.11	7.41
XOS3	57.97	17.78	1.36	9.23	1.11	1.12	7.41
XOS4	44.87	14.40	1.14	6.93	1.01	0.96	7.72
MOS3	59.91	16.61	1.32	7.11	1.13	1.02	7.51
MOS4	57.33	20.33	1.29	8.35	1.12	1.13	7.33
SEM	1.968	0.852	0.047	0.411	1.086	0.039	0.062
P	0.0757	0.2382	0.6376	0.2474	0.9163	0.5478	0.3285

SEM, standard error of the mean, XOS3 – xylotriose, XOS4 – xylotetrose, MOS3 – mannotriose, MOS4 – mannotetrose.

Table 6. Ammonia concentration (μM/g digesta) and β-glucuronidase (U/g digesta) activity in the intestinal content of broiler chickens stimulated in ovo by prebiotics.

Groups	Ammonia	β-glucuronidase	
	Cecum	Ileum	Cecum
Control	38.24	0.09	141.38
XOS3	30.95	0.41	140.52
XOS4	29.02	0.31	149.62
MOS3	35.75	0.19	194.65
MOS4	43.78	1.37	149.35
SEM	1.801	0.207	14.53
P	0.0757	0.2926	0.7766

SEM, standard error of the mean, XOS3 – xylotriose, XOS4 – xylotetrose, MOS3 – mannotriose, MOS4 – mannotetrose

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The relative abundance of *Lactobacillus* spp. was more significant in the cecum of chickens in the MOS4 group than in other groups ($P < 0.05$). Figure 4 shows the relative abundance of bacteria in the ileum, while Figure 5 shows the relative abundance of bacteria in the cecum.

DISCUSSION

The current study is a continuation of the research on the impact of in ovo stimulation with bioactive substances on chicken intestinal microbiota. Particularly important, in this case, are direct and indirect effects of prebiotics on intestinal health in broiler chickens. Previously, the positive effect of galacto-oligosaccharides on

Table 7. Effects of experimental groups, intestinal segment and their interaction on genes expression in chicken intestinal mucosa.

Gene	Intestine[1]	Substance[2]	Intestine x Substance[3]
Cytokine genes			
IL2	< 0.0001	< 0.05	Ns
IL12	< 0.0001	ns	Ns
IL17	ns	ns	< 0.01
Host defence peptide (HDP) genes			
AVBD1	< 0.01	ns	< 0.05
CATHL2	< 0.0001	ns	Ns
Nutrient sensing genes			
FFAR4	< 0.001	ns	< 0.0001
GLUT1	< 0.001	ns	< 0.05

Effects.
[1]Intestinal segment (ileum, cecum).
[2]In ovo delivery of XOS3, XOS4, MOS3, MOS4.
[3]The interaction between intestinal segment and in ovo delivery substances.

the development of the microbial population and the gene expression in the mucosa in each section of the intestine was described by Slawinska et al., 2019 (Rinttilä and Apajalahti, 2013). Modification of the intestinal microbiota is possible already at the stage of embryo development by administration of bioactive substances directly to the egg. In ovo stimulation is a process involving the injection of specific bioactive substances, including prebiotics, into the egg's air chamber on d 12 of incubation (Siwek et al., 2018). XOS and MOS used in the research belong to oligosaccharides of hemicellulose origin. They are obtained, among others, from guar gum, corn cobs, palm kernel expeller, and locust bean gum. The basis for in ovo stimulation with prebiotic MOS is its interaction with the host organism's cells to induce an immune response, the effect on the activity of enzymes, and the modulation of the microbiota. Mannan-oligosaccharide is produced by beta-mannases derived from bacteria and fungi MOS. It increases animal performance by reducing harmful bacteria while stimulating an increase in the abundance of beneficial bacteria (Rezaei et al., 2015; Ayimbila and Keawsompong, 2022). Manno-oligosaccharides stimulate nutrient absorption and immune response. Additionally, they increase the production of SCFAs, including acetate, butyrate, and propionate. The present study showed an up-regulation of *IL-2, IL-12,* and *IL-17* gene expression in the cecum in of birds stimulated with XOS3 and MOS4. According to the results obtained by Kim et al., 2014, an increase in the expression of *IL-12* and *IL-17* may indicate the presence of infection in chickens or the formation of inflammation. This is an innovative finding because, according to the literature, both prebiotics are used to activate the intestinal microbiota by increasing the abundance of beneficial microbes (Peng et al., 2020). In the studies described by Ayimbila and Keawsompong, 2022, it was proven that MOS stimulates the growth of *Lactobacillus* spp. and *Bifidobacteria* spp. in the intestines of broilers. These results are consistent with the obtained values, which show more than a 2-fold increase in *Lactobacillus* spp. and *Bifidobacterium* spp. in the cecum and a several-fold increase in *Bifidobacterium* spp. in the ileum. The results proved the prebiotic effect of MOS, which modulates the environmental conditions in the digestive tract of chickens and ensures the appropriate

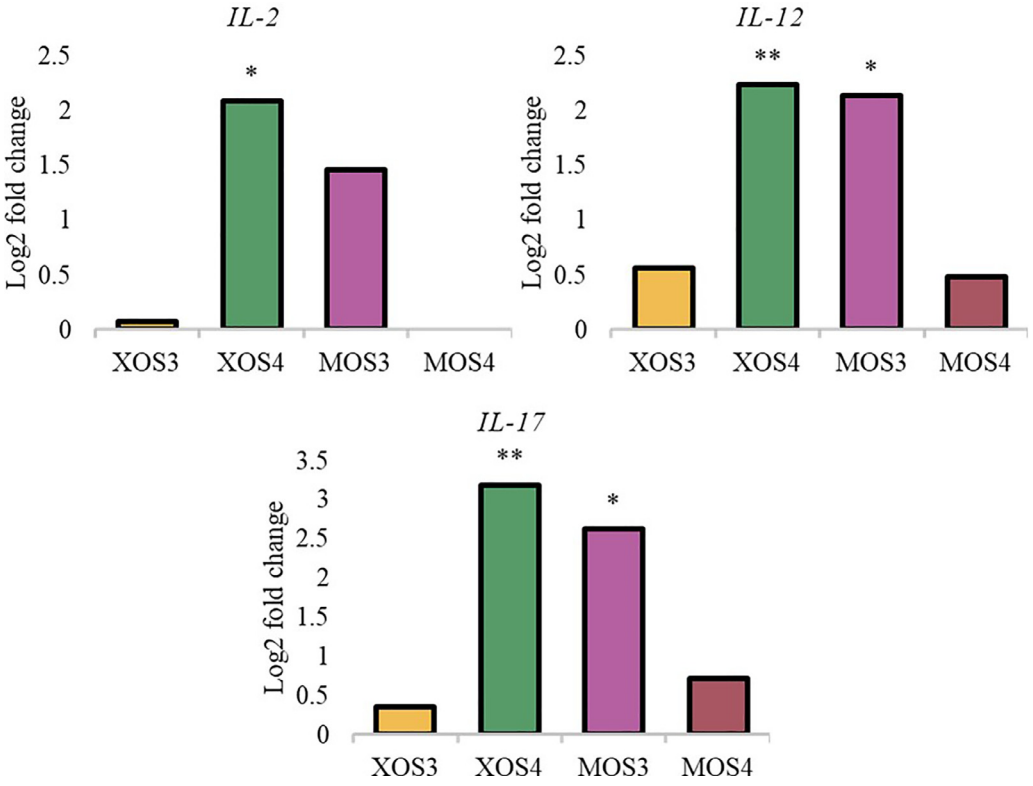

Figure 1. Expression of innate immune response genes in the caecum. The relative gene expression was calculated by the ΔΔCT algorithm and the amount of the target gene was calculated by the 2-ΔΔCT formula. Asterisks indicate means that differ significantly from the control group at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). XOS3 – xylotriose, XOS4 – xylotetrose, MOS3 – mannotriose, MOS4 – mannotetrose.

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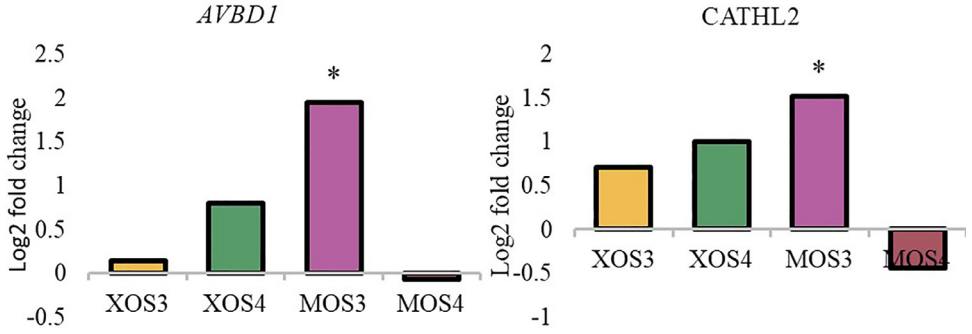

Figure 2. Expression of host defense peptides genes in the caecum. The relative gene expression was calculated by the $^{\Delta\Delta}C_T$ algorithm and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula. Asterisks indicate means that differ significantly from the control group at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). XOS3 − xylotriose, XOS4 − xylotetrose, MOS3 − mannotriose, MOS4 − mannotetrose.

microbiocenosis by stimulating the growth of beneficial bacteria. In groups subjected to in ovo stimulation with MOS, an increase in the expression of host defense peptides *AvBD1, CATHL2* was observed in the cecum. These genes correspond to the innate immunity and the mucosal defense. Intestinal HDP genes are associated with mucins, creating an immunological and mechanical barrier of the host. Their main functions include antimicrobial activity. They also participate in the process of wound healing as well as immunomodulation and chemotaxis. (Akbari et al., 2008; Slawinska et al., 2019) HDP gene expression depends on microbial modulation. Defensins, which include *AvBD1*, are defence peptides expressed mainly in epithelial cells. Their main function is to protect the host against bacterial, viral, and fungal infections. They have the ability to inhibit the growth of pathogenic bacteria. In turn, cathelicidins, including that encoded by *CATHL2* gene, are host-protective proteins that play a significant role in the innate and adaptive immunity. Similarly to defensins, they can eliminate pathogens and modulate the immune response (Dunislawska et al., 2022). Higher expression of *AvBD1* and *CATHL2* genes in the current study may indicate an inflammatory response. This might suggest a disease state of the host organism, and necessity to defend the body against pathogens. Increased expression of both genes in the MOS3 group might be related to a significant increase in the abundance of *Escherichia coli*.

XOS has a bifidogenic effect, improves the absorption of nutrients, and stimulates the immune response. Dietary supplementation with XOS can improve growth performance of chickens by positively enhancing immune function and improving gut health. As a prebiotic, XOS promotes the growth of beneficial intestinal bacteria and increases the production of SCFA in the intestines of broilers (Wang et al., 2021). In the XOS-stimulated groups, the abundance of *Lactobacillus* bacteria increased. SCFA are the main energy source for the intestinal microbiota and intestinal epithelial cells. Ding et al., 2018 observed that XOS improved gut health and immune function by increasing SCFA, including butyric acid and *Bifidobacteria* counts in the cecum of chickens. The results obtained from the current research confirm the stimulatory effect of XOS3 and XOS4 on *Bifidobacterium* spp. population in the cecum, and of MOS3 and MOS4 in both intestinal sections. The current research demonstrated an increased expression of *FFAR4* and *GLUT1* genes in the cecum after stimulation with XOS4 and MOS3. Both genes are nutrient-sensing genes. Groups that showed a significant increase in *GLUT1* expression appear advantageous due to its functions. *GLUT1* is responsible for facilitating basal glucose uptake, essential for most cells' growth and development (Kono et al., 2005).

The intestinal microbiota produces many metabolites which may affect the host. SCFA are the end-products

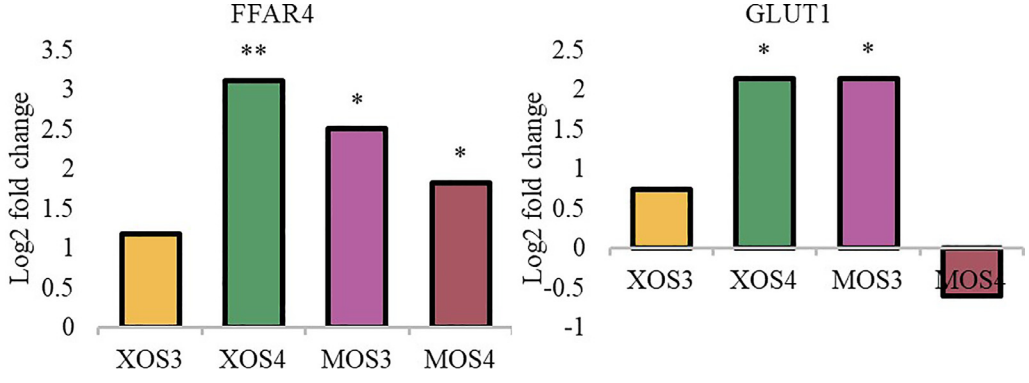

Figure 3. Expression of nutrient sensing genes in the caecum. The relative gene expression was calculated by the ΔΔCT algorithm and the amount of the target gene was calculated by the 2-ΔΔCT formula. Asterisks indicate means that differ significantly from the control group at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). XOS3 − xylotriose, XOS4 − xylotetrose, MOS3 − mannotriose, MOS4 − mannotetrose.

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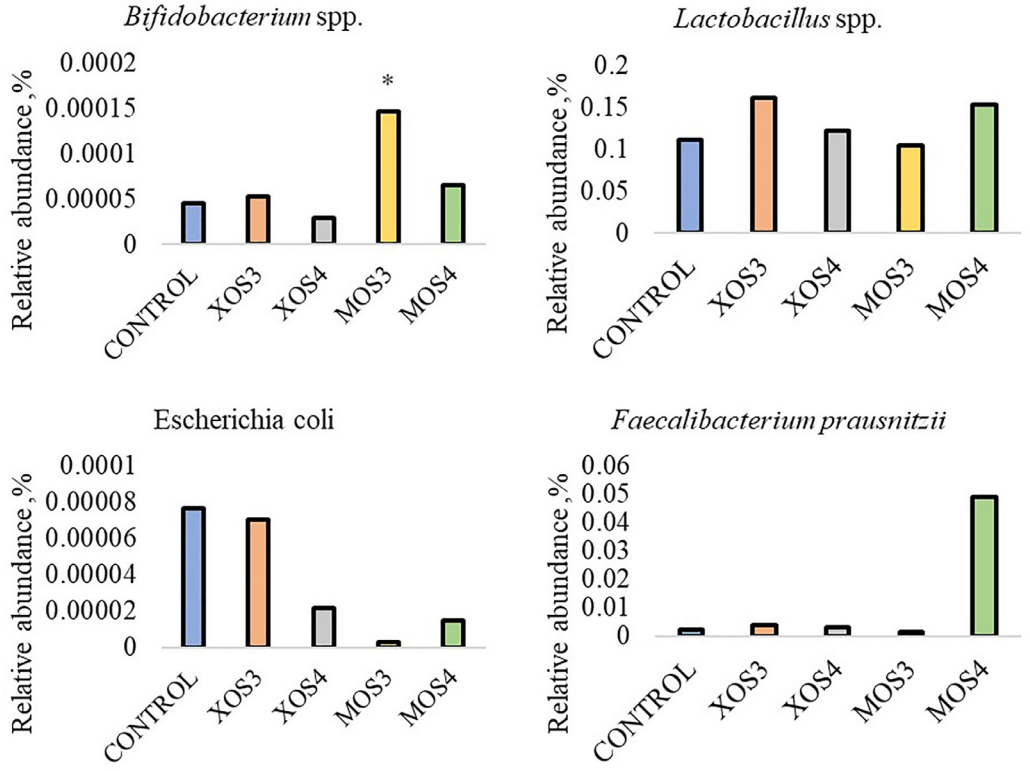

Figure 4. Relative abundance of bacteria in the ileum contents after in ovo stimulation with prebiotics. Asterisks indicate means that differ significantly from the control group at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). XOS3 − xylotriose, XOS4 − xylotetrose, MOS3 − mannotriose, MOS4 − mannotetrose.

of carbohydrate and protein fermentation, while branched-chain fatty acids (iso-butyrate, iso-valerate), ammonia, amines, as well as phenolic and indolic compounds are formed during proteolysis (Taciak et al., 2017). In ovo stimulation of broiler chickens with oligosaccharides did not affect the indices of microbial activity in the ileum and cecum. Only a tendency toward a reduction of acetic acid concentration was found in the cecum of birds stimulated with XOS4. This trend may suggest that the population of bacteria being its producers, for example, *Bacteroides-Prevotella* group (Louis et al., 2007) was reduced. However, in the current study, their abundance was not studied. Therefore, more detailed analysis of microbiota composition should be done in the future research to determine the effect of in ovo stimulation with prebiotics on microbial ecology of broiler chickens. Since acetic acid is a bacterial metabolite, which can be utilized in lipogenesis in the liver and as a fuel for skeletal muscles (2002) the effect of prebiotic administration during embryonic development on peripheral tissues of chickens should be also analysed.

The current research showed also a tendency toward higher ammonia concentration in the cecum of birds stimulated in ovo with MOS4. The results may suggest that this oligosaccharide has a potential to intensify deamination of amino acids or degradation of blood urea as these reactions lead to ammonia release (Windey et al., 2012). The concentration of ammonia in the intestinal content also depends on the absorption by the epithelium (Lupton and Blanchant, 1989; Windey et al., 2012). Thus, the tendency to its higher level found in the MOS4 group might result from the intensified proteolysis or impaired absorption. It may be also speculated that MOS4 inhibited assimilation of ammonia by the cecal bacteria, which use it for the synthesis of their own protein (Blachier et al., 2007).

Beside SCFA and ammonia, bacterial β-glucuronidase activity was also measured in the current study. This enzyme hydrolyses glucuronides synthesized in the liver and secreted with the bile (Pellock and Redinbo, 2017). Thus, it participates in entero-hepatic circulation of substances formerly detoxified in the liver. The activity of this enzyme was considerably greater in the cecal than ileal digesta, which is line with the results of analysis of the relative abundance of bacteria. The β-glucuronidase activity was found in *Bacteroides, Bifidobacterium, Eubacterium*, and *Ruminococcus*. Its gene was described for *E. coli, Lactobacillus gasseri*, and *Staphylococcus* spp., and identified also in *Clostridium perfringens* (AKAO, 1999, 2000; Russell and Klaenhammer, 2001; Beaud et al., 2005). In the current study, the abundance of *E. coli* and *Bifidobacterium* spp. was much greater in the cecum than in the ileum, which may partially explain the difference in β-glucuronidase activity between these segments. In the current study, it was demonstrated that in ovo administration of oligosaccharides did not affect the activity of this enzyme despite the abundance of *E.coli* and *Bifidobacterium* spp. differed between groups. However, these bacteria are not predominating in the intestinal content of broiler chickens and changes in their population size seems to have no impact on β-glucuronidase activity. The lack of

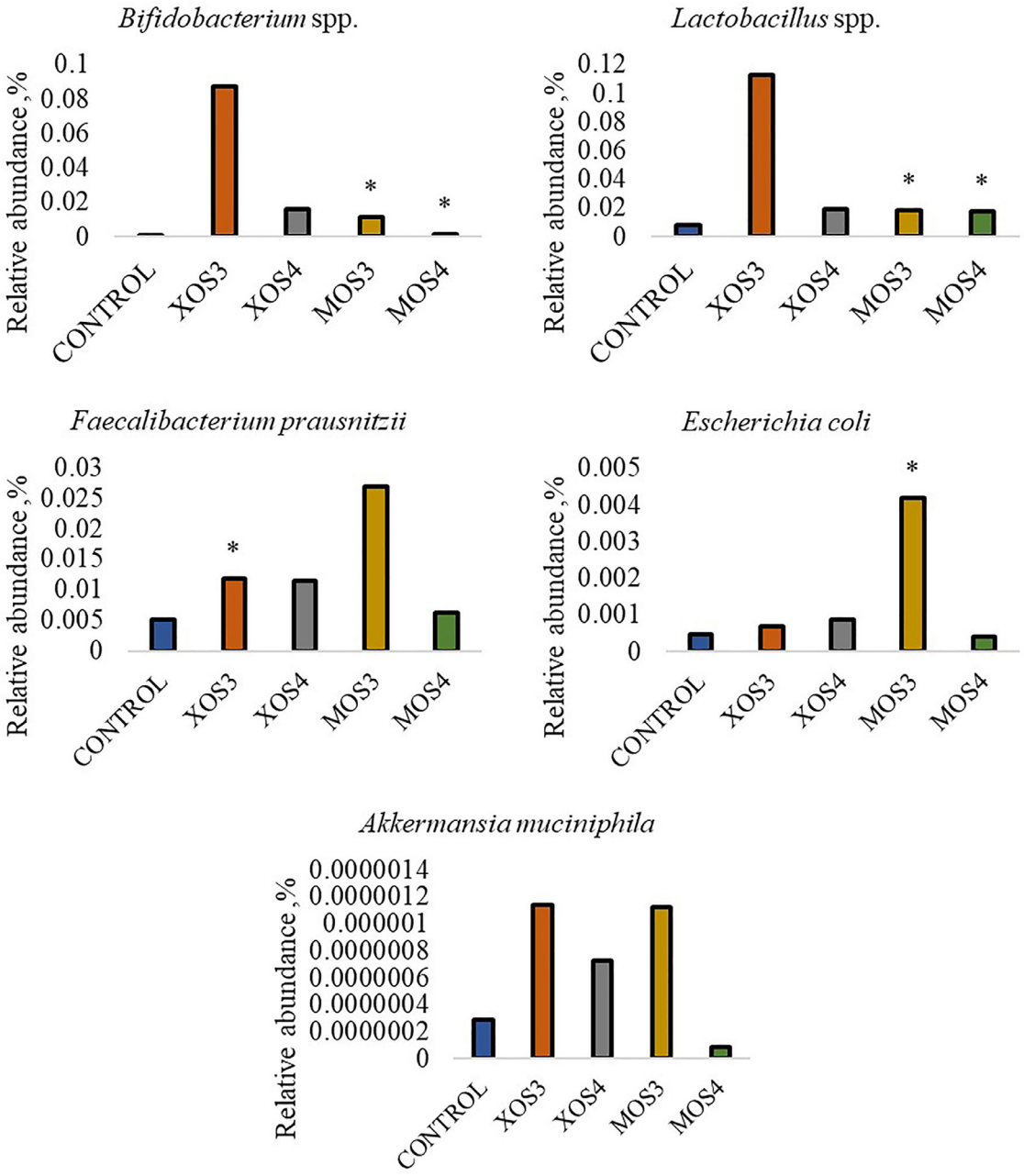

Figure 5. Relative abundance of bacteria in the cecal contents after in ovo stimulation with prebiotics. Asterisks indicate means that differ significantly from the control group at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***). XOS3 − xylotriose, XOS4 − xylotetrose, MOS3 − mannotriose, MOS4 − mannotetrose.

effect of prebiotics administered in ovo indicate that the bile secretion from the liver was not affected in chickens. The fact that all birds were fed the same diets was also of importance. Each diet for chickens (starter, grower, and finisher) was based on cereals and soybean meal, the latter being a source of isoflavones (Tuśnio et al., 2014). These compounds are metabolized in the liver by binding to glucuronic acid and then secreted as glucuronides with bile (Dabek et al., 2008). Tuśnio et al. 2020 demonstrated that feeding diets without soybean meal reduced the activity of β-glucuronidase in the colon of pigs. In the current research, the soybean meal content in a diet was the same for each group, which ensured similar availability of substrates (isoflavone glucuronides) for the intestinal microbiota.

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CONCLUSIONS

1. In ovo stimulation with prebiotics resulted in significant changes in the genes expression, such as those involved in the innate immune response, host defense peptides and nutrient sensing genes. These changes imply a potential improvement in the overall immune capacity and metabolic regulation of the developing chicken embryo. Potentially leading to improved broiler health and growth performance after hatching.
2. In ovo prebiotic injection stimulates the growth of beneficial bacteria in chickens intestines. Such early

stimulation of the microbiota can lead to better digestion, nutrient absorption and a more efficient immune system in chickens.

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Data Availability Statement: All data are available from the corresponding author (Bełdowska Aleksandra) of the manuscript.

Ethics Declarations: All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments. The research was done under the consent of the Departmental Animal Welfare Team of the Bydgoszcz University of Science and Technology (No. 2/2022) acting for the Ethics Committee (Bydgoszcz, Poland). All methods followed the ARRIVE guidelines.

DISCLOSURES

The authors declare no conflicts of interest.

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Poultry Science

Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens

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ABSTRACT Mitochondria are cellular organelles that are the place of many metabolic processes and thus have a significant impact on the proper functioning of the organism. These organelles respond easily to environmental stimuli and cellular energy demands. To ensure the proper functioning of mitochondria, a high supply of specific nutrients is needed. Literature reports suggest that a favorable profile of the intestinal microbiota may improve the functioning of the mitochondria. The gut microbiota transmits a signal to the mitochondria of the mucosa cells. This signaling alters mitochondrial metabolism, activates cells of the immune system, and alters intestinal epithelial barrier functions. The aim of the study is to determine the relative number of mtDNA copies and to analyze the mitochondrial expression of genes related to respiratory chain proteins and energy metabolism in the intestinal mucosa and cecal tonsils of broiler chickens injected on the d 12 of egg incubation with various prebiotics. 300 incubated eggs of Ross 308 broiler chicken on d 12 of incubation were injected with: control group with physiological saline, prebiotics: XOS3, XOS4, MOS3, and MOS4. On d 42 after hatching, 8 individuals from each group were sacrificed. Cecal mucosa and cecal tonsils were collected postmortem for DNA and RNA isolation. Relative mitochondrial DNA copy number analysis was performed by qPCR method using 2 calculation methods. Gene expression analysis of the cecal tonsils and cecal mucosa was performed by RT-qPCR for the gene panel selected based on literature data and gene functions related to mitochondria: *CS, EPX (MPO), CYCS, TFAM, NRF1, ND2, MnSOD (SOD2)*. As the results showed the overall mt DNA copy number is stable in both tissues. The significant change in gene expression in cecal mucosa was induced by XOS4 and MOS3. Both prebiotics caused upregulation of gene expression. In cecal tonsils all prebiotics caused downregulation of entire set of genes under the analysis. Statistically significant results of gene expression were detected for *CYCS, ND2, NRF, TFAM* for all experimental groups.

Key words: cecal tonsils, intestines, microbiome, mitochondrial stimulation, mucosa

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INTRODUCTION

Mitochondria are cellular organelles that are the site of many metabolic processes and thus significantly impact the organism's proper functioning. These organelles respond quickly to environmental stimuli and cellular energy needs (Javadov et al., 2020). To ensure the proper functioning of mitochondria, a high supply of the right nutrients is needed. The "fuel" for the mitochondria to produce ATP is glucose, ketone bodies, and oxygen. The production of ATP, in combination with electron transport and translation of mitochondrial proteins, is an essential function of mitochondria (Osellame et al., 2012). Bottje et al. (2002) showed a relationship between the feed's energy content and mitochondria's functioning. They proved that the mitochondrial functions and the activity of the respiratory chain are closely related to the feed composition in male broiler breeders. It can be assumed that differences in the activity of the respiratory chain complex in the muscles of birds may be caused, among other things, by the expression of proteins related to mitochondria. Literature reports suggest that a favorable profile of the intestinal microbiota may improve the functioning of the mitochondria. The gut microbiota transmits a signal to the mitochondria of the mucosa cells. This signaling alters mitochondrial metabolism, activates immune system cells, and alters the intestinal epithelium's barrier function. Scientific evidence suggests that altered mitochondrial function in intestinal mucosa cells may be linked to gut disease (Jackson and Theiss, 2020). In

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poultry production, the health of the intestines and the maintenance of homeostasis are key factors in maintaining profitable production. The gut microbiota plays a key role in keeping poultry healthy. It affects the host's organism by regulating the immune response, metabolic and digestive processes, as well as absorption of nutrients (Wickramasuriya et al., 2022). The interaction between the gut microbiome and the host is extremely important in the early stages of host organism development. Intestinal bacteria provide, among other things, the necessary stimuli to stimulate the immune response of chicks, making adults less susceptible to infections and inflammatory diseases. In addition, pathogenic bacteria can attack the mitochondria in the host cells, leading to the weakening of the organism (Tiku et al., 2020). The interaction between the gut microbiota and the host mitochondria is becoming an important research area in poultry production. In order to understand these mechanisms, it is necessary to study molecular signaling between the gut microbiota and the mitochondria to determine the mechanisms of the host microbiota-mitochondria linkage. Many years of our own research prove that in poultry, effective stimulation of the intestinal microbiota is possible by administering bioactive substances during embryonic development (on d 12 of egg incubation) (Villaluenga et al., 2004; Bednarczyk et al., 2016). These bioactives have direct impact on the host gut microbiota and indirect on the host organisms. This interaction is initiated already during embryo development and the effects are lifelong and observed at many levels in phenotypic parameters and molecular indicators. The rationale behind the in ovo administration of bioactives on d 12 of egg incubation and lifelong effects on microflora, growth traits, feed efficiency, intestinal morphology meat microstructure and quality, immune system development, physiological characteristic, and host transcriptome is presented in our review article (Siwek et al., 2018). Our research in proteomic analysis (Dunislawska et al., 2021a) showed that stimulation of the intestinal microbiota during embryonic development with a synbiotic affects changes in the production of mitochondrial proteins. Based on the facts mentioned above, a hypothesis has been made that the administration of prebiotics during the embryonic development of broiler chickens affects the condition of mitochondria in the intestinal tissue and the immune tissue associated with the intestines.

The aim of the study is to determine the relative number of mtDNA copies and to analyze the mitochondrial expression of genes related to respiratory chain proteins and energy metabolism in the intestinal mucosa and cecal tonsils of broiler chickens injected on the d 12 of egg incubation with various prebiotics.

MATERIALS AND METHODS

Experimental Setup

Three hundred eggs of Ross 308 broiler chicken were incubated using an automated incubator at 37.8°C and relative humidity of 61 to 63%. On d 12 of incubation, the eggs were randomly divided into 5 experimental groups (60 eggs per group). The groups were injected with: 1) control group mock-injected with 0.2 mmol/L physiological saline (0.9%), 2) prebiotic 1—xylotriose (**XOS3**) 0.5 mg, 3) prebiotic 2—xylotetraose (**XOS4**) 0.5 mg, 4) prebiotic 3—mannotriose (**MOS3**) 0.5 mg, 5) prebiotic 4—mannotetraose (**MOS4**) 0.5 mg. Eggs were injected into an air chamber with 0.2 mL of the aqueous solution of each substance. Washable, nontoxic glue was applied to the resulting holes. After hatching, divided groups of birds were placed in pens on bedding (12 birds/pen). Feed and water were delivered manually and were available ad libitum. The feeding regime was applied according to the requirements of Ross broiler chicken. On d 42 after hatching, 8 randomly selected individuals from each group were sacrificed. Cecal mucosa and cecal tonsils were collected postmortem. Collected tissues were stored in stabilizing buffer (fixRNA, EURx, Gdansk, Poland) for future isolation of nucleic acids.

The experiment was conducted following the applicable regulations. The slaughter of the birds was carried out under the applicable regulations on the handling of animals during slaughter, including humane treatment. According to directive no. 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes, the consent of the Ethics Committee was not required. According to Act of January 15, 2015 on the protection of animals used for scientific or educational purposes (item 266, Journal of Laws of the Republic of Poland) slaughtering to collect tissues and organs from animals, is not a procedure.

Analysis of Mitochondrial Gene Expression

Total RNA was isolated from approximately 50 mg of tissues which was homogenized in 1 mL RNA Extracol (EURx, Gdansk, Poland) using a TissueRuptor homogenizer (Qiagen GmbH, Hilden, Germany) ($n = 8$/group). RNA was purified from the solution and contaminant using a GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland) following the manufacturer's instructions. Each RNA samples were quantitatively and qualitatively evaluated using the NanoDrop 2000 (Thermo Scientific Products, Wilmington, NC) and electrophoresis on an agarose gel. The storage temperature for isolated RNA was −20°C.

Gene expression analysis of the cecal tonsils and cecal mucosa was performed for the gene panel selected based on literature data and gene functions related to mitochondria: *CS, EPX (MPO), CYCS, TFAM, NRF1, ND2, MnSOD (SOD2)*. Gene expression analysis was performed by quantitative PCR with initial reverse transcription (**RT-qPCR**). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania). The qPCR reaction was performed using LightCycler 480 II (RocheDiagnostics, Basel, Switzerland) as

Table 1. Primer sequences used in the RT-qPCR reaction.

Gene	Full name	Gene ID (NCBI)	Primer sequence (5′→3′)
CS	Citrate synthase	100858903	F: GCATTTTCCAAGGGTGAGCC R: CTGTAGGGCTGCAGGAGTG
EPX (MPO)	Eosinophil peroxidase	417467	F: AAGCAACTTCTGCAGGACTGA R: AGGTTGAGATGACCGCTCTG
CYCS	Cytochrome C	420624	F: CCATGAAGGTTGGGTCCAGT R: CTCTGTGTGTGTTCCTGGTCT
TFAM	transcription factor A, mitochondrial	373888	F: CCTACGAGAGGGGAGGGG R: TCGATCCCTGTGTGAACTGC
NRF1	Nuclear respiratory factor 1	416677	F: AAAAGCCCAGAGCTGAATGGT R: GGCACCGTGCAAAGAGAGAA
ND2*	NADH dehydrogenase subunit 2	63549482	F: ATCAGCCCTAATCCTCTTCTC R: GTGGCTATTGGGGTTATTTCT
MnSOD (SOD2)	superoxide dismutase 2, mitochondrial	374042	F: GCAGCCTGTGCAAATCAAGA R: ACATCTCATCCATTTGCCTCTGA

*Yang et al. (2020)

described in Dunislawska et al., 2021a. The qPCR reactions mixture contained Maxima SYBR Green qPCR Master Mix intercalating dye (Thermo Fisher Scientific, Waltham, MA), 1 μM of each primer (synthesized by Merck, Darmstadt, Germany), and 140 ng of cDNA. Sequences of primers were based on literature or were designed by NCBI Primer BLAST tool (Ye et al., 2012). The optimal melting temperature for qPCR was 58°C. Primer sequences are shown in Table 1. Relative gene expression analysis was conducted by using the ΔΔCt method using *ACTB* (Sevane et al., 2014) as a reference gene (Livak and Schmittgen, 2001). Statistical analysis was performed by using a Student *t* test (* indicates statistical significance $P \leq 0.05$).

Relative Mitochondrial DNA Copy Number

Total DNA was isolated from approximately 25 mg of tissues, which were lysed and purified using the GeneMATRIX Tissues DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions (n = 8/group). The extracted DNA was subjected to quantitative and qualitative evaluation by a spectrophotometric method using NanoDrop2000 (Thermo Scientific Nanodrop Products, Wilmington, NC) and electrophoresis. The DNA was prepared for the next analysis step and stored at −20°C.

Relative mitochondrial DNA copy number analysis in cecal tonsils and cecal mucosa was performed using 2 calculation methods. The molecular verification was provided by using LightCycler 480 II (RocheDiagnostics, Basel, Switzerland). The qPCR reactions mixture contained SG onTaq qPCR Master Mix intercalating dye (EURx, Gdansk, Poland), 1 μM of each primer (synthesized by Merck, Germany), and 2 uL of the sample (50 ng of DNA). The primers for the analyzed genes *D-loop, ATP6, ND6, GCG* were from Zhang et al., 2020. The thermal profile of the qPCR reaction was carried out according to the dye manufacturer's protocol as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of amplification: denaturation at 94°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. Thermal cycling consisting of fluorescence was measured at the end of each extension step. The generated Ct values were analyzed by 2 calculation methods.

MtDNA copy number was performed according to (Zhang et al., 2020) by using the formula:

$$\text{mtDNA copy number} = 2 \times \text{the copy number of D} - \text{loop (and separately respectively : ATP6 or ND6)/GCG copy number}$$

From the generated values of mtDNA copy number for individual genes, the average was calculated. A Student *t* test (*) was also performed between the control group and the individual study groups.

Relative mtDNA copy number analysis was performed according to Venegas and Halberg (2012) by using formula:

$$(\text{gDNA average Ct}) - (\text{mtDNA average Ct}) = \text{delta Ct}$$

$$2 \times 2(\Delta\text{Ct}) = \text{mtDNA content}$$

$$\text{Relative mtDNA content} = \text{mtDNA target/mtDNA control}$$

GCG gene was used as gDNA reference, *D-loop* gene was used as mtDNA reference.

A Student *t* test (*) was also performed between the control group and the individual study groups. Standard errors of the mean (**SEM**) were applied as a variation parameter within the groups.

RESULTS

Mitochondrial DNA Copy Number

The mitochondrial DNA copy number (mt DNA-CN) for cecal mucosa and cecal tonsils are presented in Figure 1. The average mt DNA-CN for cecal mucosa equals to 1.30 for XOS3, MOS3, MOS4 and is slightly higher for the control group (1.31) and XOS4 group (1.32). The mt DNA-CN for cecal tonsils is the lowest for XOS3 (1.32) and the highest for MOS4 (1.40). The

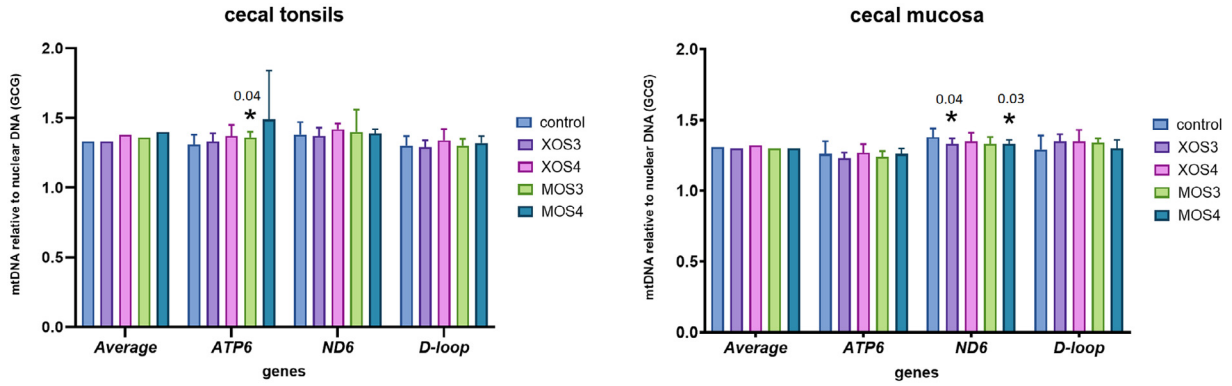

Figure 1. Mitochondrial DNA copy number relative to nuclear DNA in cecal tonsils and cecal mucosa after in ovo stimulation with prebiotics.

mt DNA-CN for 3 other groups have intermediate values: 1.37 for XOS4; 1.36 for MOS3, and 1.33 for the control group. The relative mt DNA-CN for cecal mucosa and cecal tonsils are presented in Figure 2. The highest value for relative mt DNA-CN for cecal mucosa was detected in group MOS4. Overall the relative mt DNA-CN in this tissue is close to 1. The lowest value for relative mt DNA-CN in cecal mucosa was detected in group XOS4. The values calculated for all experimental groups are close to 1. The overall mt DNA copy number is stable in both tissues.

Gene Expression

The results of gene expression in cecal mucosa are presented in Figure 3. Statistically significant results were detected for 5 (*CYCS, EPX, MnSOD, ND2, NRF*) out of 7 analyzed genes. The significant change of gene expression was induced by XOS4 and MOS3. Both prebiotics caused upregulation of gene expression. The results of gene expression in cecal tonsils are presented in Figure 4. All 4 prebiotics (XOS3, XOS4, MOS3, MOS4) caused downregulation of entire set of genes under the analysis. Statistically significant results of gene expression were detected for *CYCS, ND2, NRF, TFAM* for all experimental groups. *MnSOD* gene was significantly downregulated in groups that received: XOS3, XOS4 and MOS3. The expression was *CS* gene was significantly decreased after administration: XOS3, XOS4, and MOS4. The *EPX* gene was significantly downregulated in the group that obtained XOS3 during embryo development.

DISCUSSION

Current study is a follow-up analysis of an impact of bioactives administered in ovo directly on host microbiota and indirectly on host organisms in particular on cecal tonsils and intestinal mucosa. Previous reports focused on these 2 tissues concerned transcriptomic changes of selected genes related to immune responses (*IL4, IL6, IL12, IL8, IL1β, IFN-β, IFN-γ*) and energy metabolism (*ACOX2, BRSK2, APOA1*) upon administration of prebiotics or synbiotics in ovo (Sławinska et al., 2014; Dunislawska et al., 2017; Dunislawska et al., 2021b). It is hypothesized that the interaction between the host microbiota and host organism might be realized through mitochondria. Mitochondria respond to the gut microbiota through several ways: regulating energy production, altering redox balance and regulating immune reactions by attenuating TNFα induced and inflammation induced oxidation (Clark and Mach, 2017). Therefore the current report focused on impact of in ovo administered prebiotics on mtDNA copies and to analyze the mitochondrial expression of genes related to

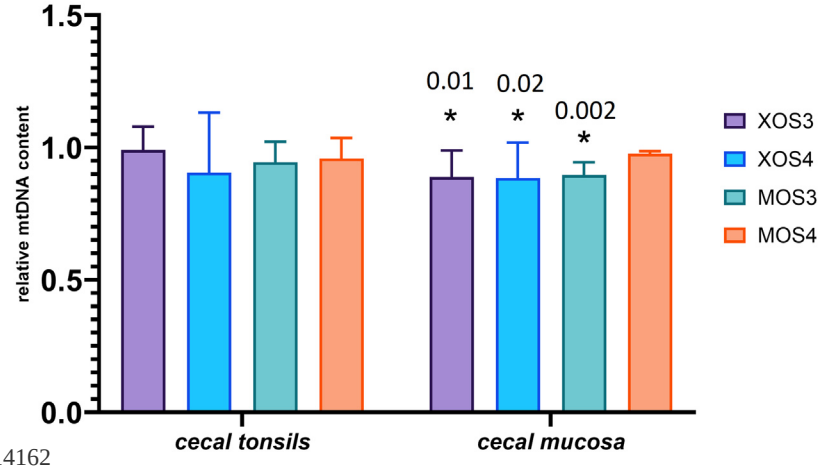

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Figure 2. Relative mtDNA content in cecal tonsils and cecal mucosa after in ovo stimulation with prebiotics.

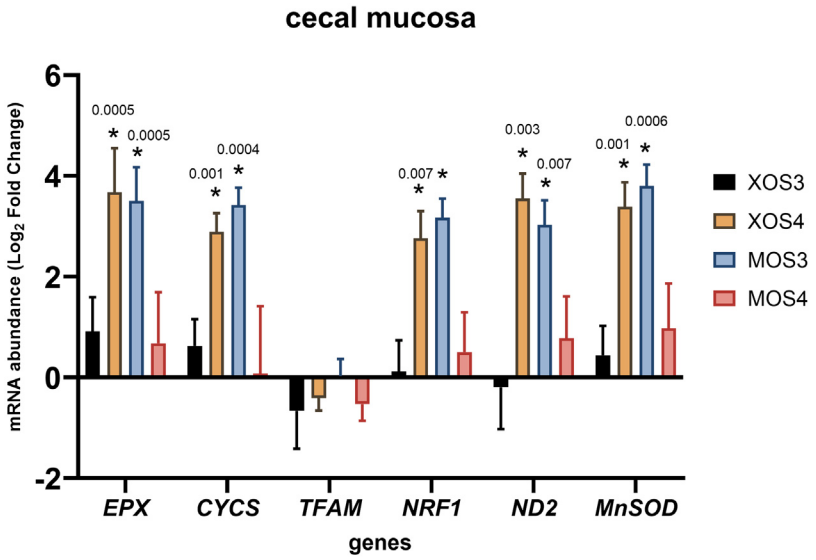

Figure 3. Mitochondrial gene expression analysis in cecal mucosa after in ovo stimulation with prebiotics; * $P \leq 0.05$, P value is numerically represented.

respiratory chain proteins and energy metabolism in cecal mucosa and cecal tonsils of adult chicken.

Microbiota inhabiting gut intestines play multiple roles for the host organism. The primary function is participations in food digestion and providing the nutrients for the growth of host organisms. Besides that, it plays other important roles such as the development of the functional intestines, maintaining a well-balanced immune system and interaction with the nervous system.

Mitochondria are the primary energy centers, which upon oxidization of fats and sugars produce adenosine triphosphate (**ATP**). Mitochondrial oxidative phosphorylation (**OXPHOS**) is based on electron transport, cell respiration, ATP synthesis and fatty acid β-oxidation. Reactive oxygen species (**ROS**) and reactive oxygen nitrogen species (**RONS**) are a side product of the respiration process. These reactive molecules play an essential role in cell signaling and homeostasis but might have a damaging impact on cell structure if released in excessive amount. Mitochondria also play a significant role in the initiation of inflammation by inflammasomes activating caspase-1 and further secretion of inflammatory cytokines (Clark and Mach, 2017). Mitochondria have further significant impact on intestinal epithelial cell metabolism, immunity, stress, and apoptosis (Zhang et al., 2022).

The number of the mitochondrial genome is cell-dependent and present in multiple copies. A higher number of mt DNA copies are present in cells with high ATP demands, such as the heart and skeletal muscle. Hence in spleen or liver, cells with low energy requirements, the mt DNA-CN is around 100. A reduced mt DNA-CN might be a suitable biomarker of mitochondria dysfunction (Castellani et al., 2020). In the current study, administration of the XOS and MOS prebiotics did not

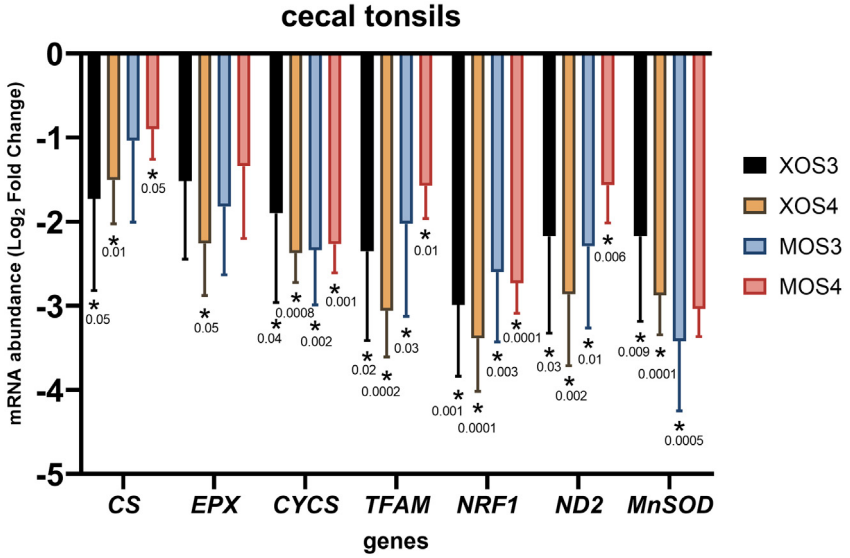

Figure 4. Mitochondrial gene expression analysis in cecal tonsils after in ovo stimulation with prebiotics; * $P \leq 0.05$, P value is numerically represented.

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have a significant impact on the number of mtDNA copy number. There were also no differences between analyzed tissues, since both tissues fall in the category of low energy requirements.

The mechanisms responsible for the interaction between the host and its microbiota are still under study. One hypothesis suggests the important role of mitochondria in the cross-talk between the host and microbiota (Saint-Georges-Chaumet et al., 2015). The main reason for mitochondria's central role might be their prokaryotic origin. Both microbiota and mitochondria have a circular genome, ribosome prokaryotic signature sensitive to antibiotics, and maternal inheritance. Both microbiota and mitochondria are able to activate formyl peptide receptor (**FPR**) in response to autophagy. Autophagy is a process responsible of the elimination of senescent cellular macromolecules or organelles. The bioenergetic status of the cell is responsible for the activation of the process of autophagy (Onnis et al., 2018). The gut microbiota regulates the transcriptional coactivators, transcription factors, and enzymes responsible for mitochondrial biogenesis (*PGC-1α, SIRT1, AMPK*) (Clark and Mach, 2017). Mitochondrial ROS production, on the other hand, regulates gut microbiota by modulating intestinal barrier function and mucosal immune responses (Clark and Mach, 2017).

The content and the quality of microbiota species are related to diet, presence of pathogens, and overall health conditions. So far, studies in humans indicate an association between mitochondrial mt DNA haplogroups and microbial composition (Ma et al., 2014). In the host organisms, the gut microbiota faces the physical and chemical barrier created by intestinal epithelial tissue. Every 3 to 5 d, intestinal stem cells in crypts differentiate and formulate new epithelial cells. In parallel, functions in mitochondria are modified (Zhang et al., 2022).

The cross talk between microbiota and mitochondria is realized by short-chain fatty acid (**SCFA**). The SCFA, microbial metabolites, are a signal to intestinal epithelial cells. Butyrate, which is a source of energy for epithelial cells might have an impact on mitochondrial metabolism. Butyrate might upregulate peroxisome proliferator-activated receptor-gamma coactivator (**PCG**)-1 alpha to enhance the electron transport chain (**ETC**) activity of OXPHOS. Butyrate catalyzed by the mitochondria to NADH might upregulate the expression of mitochondrial uncoupling protein 2 (**UCP2**). The metabolism of colonic epithelial cells is biased toward oxidative phosphorylation. This direction of the metabolism maintains the consumption of oxygen at a high level, which creates a hypoxic environment that supports the presence of obligating anaerobes (Zhang et al., 2022). Prebiotics XOS and MOS used in the current study belong to the hemicellulose-derived oligosaccharides (**HDO**). Their biological properties are similar to fructooligosaccharides. HDO is produced from low-value substrates such as locust bean gum, guar gum, konjac gum, and agro-waste products: corn cob, copra meal, and palm kernel cake. These substrates are subjected to enzymatic hydrolysis using hemicellulases: endo-β-(1→4) xylanase and endo-β-(1→4) mannanase (Jana et al., 2021). In the study performed by Singh et al. (2022) XOS and MOS with different degrees of polymerization were used to modulate chicken microbiota during embryo development (Singh et al., 2022). The administration of these oligosaccharides in ovo had an impact on SCFA. In particular the level of butyrate was significantly upregulated after XOS injection.

Bile acids produced by the gut microbiota are the second way to regulate mitochondria energy metabolism. Secondary bile acids interact with mitochondria by modulating transcription factors related to lipid and carbohydrate metabolism (Clark and Mach, 2017). XOS supplemented in the diet of young piglets increased the number of Lactobacillus bacteria and increased the amount of microbiota metabolites: SCFA and bile acids (Tang et al., 2022). Similar results were obtained in the study on XOS supplementation in the diet of hamsters (Abdo et al., 2021). Hence, we might speculated that similar mechanism of cross talk between microbiota and mitochondria took place in the current experiment on broiler chickens.

In the current study, the impact of the XOS and MOS on the microbiota itself and, subsequently on the mitochondria was estimated based on the activity of genes belonging to markers of mitochondrial biogenesis. The nuclear respiratory factor (**NRF1**) is coding a protein, transcription factor activating genes, which are responsible for mitochondrial DNA transcription and replication, and nuclear genes required for respiration. Mitochondrial transcription factor A (***TFAM***) encodes a mitochondrial transcription factor, and the coded protein plays a role in mitochondrial DNA replication and repair (Stelzer et al., 2016). A study performed on a chicken DT40 cell line showed that the chicken TFAM gene plays a role in maintaining the mtDNA copy number (Matsushima et al., 2003). In the current study, the administration of XOS and MOS in ovo, either did not change the expression of the TFAM gene (cecal mucosa) or the gene was significantly downregulated (cecal tonsils). That is in agreement with the general overall stable value for mt DNA copy number detected for both tissues.

Cytochrome C (***CYCS***) encodes a small soluble electron carrier hemeprotein. It plays a role in transferring the electrons from complex III to complex IV and facilitating cell energy production. Under normal conditions, this protein is located inside the mitochondria. Releasing it into the cytoplasm occurs during noninflammatory cell apoptosis (Eleftheriadis et al., 2016).

Another gene under the study, *ND2* is a mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 2. This gene involves mitochondrial electron transport, NADH to ubiquinone, and mitochondrial respiratory chain complex I assembly (Stelzer et al., 2016). *ND2* also plays a critical role in controlling the production of mitochondrial ROS (Zhang et al., 2016). A study of the chicken *ND2* gene showed heteroplasmy related to tissue and developmental stage (Yang et al., 2020).

Manganese superoxidase dismutase (***MnSOD***) encodes an enzyme that protects the mitochondria from oxidative damage. Most studies reporting the downregulation of *MnSOD* relate this to the disease state. The upregulation of the *MnSOD* mRNA leads to an increased enzyme level. Since the manganese superoxidase dismutase enzyme is responsible for controlling the level of superoxidate anion (O_2^-) in mitochondria, increased activity of *MnSOD* leads to H_2O_2 accumulation. The H_2O_2 damages the cells (Li and Zhou, 2011). The *cMnSOD* is another gene whose expression is known to be related to tissue and chicken developmental stage. The highest expression was detected in the heart, the lowest expression was in the lung, testis, and thymus. Differential expression of *cMnSOD* in heart tissue was the highest on d 13 of embryo development and decreased after that to d 2 posthatch (Kong et al., 2003).

Citrate synthase (***CS***) is a protein-coding gene which product catalyzes the synthesis of citrate from oxaloacetate and acetyl coenzyme A. The protein itself is encoded in the nucleus and subsequently transported to the mitochondrial matrix (Stelzer et al., 2016). Citrate synthase is also known as a biomarker associated with mitochondrial content (Hakamata et al., 2018).

Eosinophil peroxidase (***EPX***) is coding a protein found in human and mouse cytoplasmic granules of eosinophils. Eosinophils promote gut microbiome homeostasis and protection against gastrointestinal infections. *EPX* shows antibacterial activity against gram-negative bacteria (Percopo et al., 2019). The gene network of the analyzed genes is presented in Figure 5. Genes responsible for mitochondrial biogenesis (*NRF1, TFAM*), oxidative metabolism (*CS*), mitochondrial superoxide generation (*MnSOD*), and electron transport chain (*CYCS*) are grouped together.

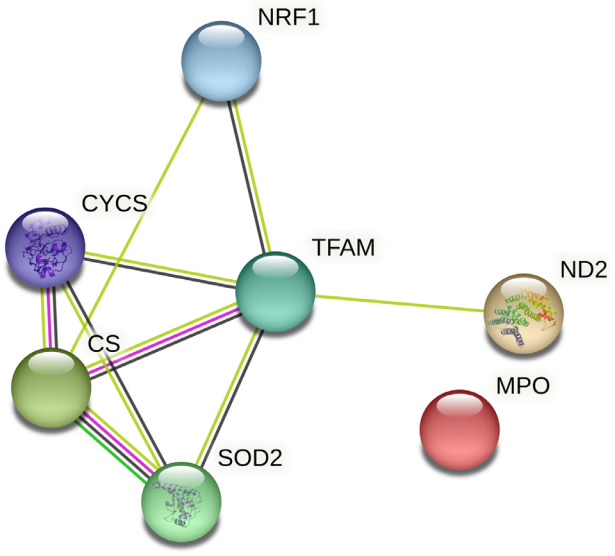

Figure 5. Analysis of the relationship between proteins encoded by genes selected for mitochondrial expression analysis. Lines of interactions according to STING-software: pink—experimentally determined; dark green—gene neighborhood; light green—text mining.

The enhancement of oxidative metabolism through mitochondria biogenesis might be consider a beneficial effect. In the study performed by Kikusato et al., 2016 in impact of oleuropein on avian muscle cells in vitro was analyzed. The study showed an induced mitochondrial biogenesis via upregulation of *NRF1* and *TFAM* genes upon oleuropein treatment. The same study showed also decrease of mitochondrial superoxide generation by upregulation of *MnSOD* mRNA level upon oleuropein treatment. Upregulation of antioxidant enzyme (*MnSOD*) and mitochondrial function genes (*NRF1, TFAM*) upon Bacillus probiotic supplementation in meat from broiler chickens was reported by Bai et al. (2016). The authors showed beneficial impact of Bacillus probiotic supplementation on antioxidant capacity and oxidative stability in meat samples subjected the storage at 4°C.

The direction of gene expression change observed in the current study in the cecal mucosa from chickens administered XOS4 and MOS3 in ovo follows the same upregulated path. It might be speculated that XOS4 and MOS3 supplied in ovo modulate chicken gut microbiota increasing the production of butyrate or bile acid which has an impact on induced mitochondrial biogenesis.

The analysis done for cecal tonsils shows quite opposite picture. The entire set of analyzed genes in all experimental groups shows negative regulation. The explanation of this phenomenon might be as follows. Cecal tonsils are the largest lymphoid aggregates of the lymphoid tissue associated with the chicken intestines. They are involved in the immune responses against bacterial and viral pathogens. They are part of immune system associated with gastrointestinal track. Immune response against environmental stimulus needs energy supply. Mitochondrion plays a significant role of the immune cells in particular when comes to the energy supply (Su et al., 2021). However as presented by all our previous studies, administration of bioactive substances in ovo causes downregulation of the gene expression in cecal tonsils (Sławinska et al., 2014; Dunislawska et al., 2017) thus indicating an oral tolerance. Adult chickens which received prebiotic GOS in ovo, showed downregulation of 286 genes from 378 differentially expressed genes (**DEGs**) in cecal tonsils (Slawinska et al., 2016). Therefore the activity of the immune system and related to that requirements for the energy supply are minimal.

To the best of our knowledge, this is the first report of an impact of early-life microbiota modulation by prebiotics on mitochondria in immune-related tissues of adult chicken. In is yet another in depth study on the effects of cross talk between microbiota and host organisms upon early life administration of prebiotics in chicken. What is more important, most of the available literature related to microbiota-mitochondria cross talk concerns liver or muscle tissue in human or model organism. However, as these study indicate, the impact of early stimulation of the intestinal microbiota in chickens on mitochondria in intestinal tissues can be considered an important research direction that requires further

analysis. We have shown that under the influence of XOS and MOS prebiotics, the expression of genes is functionally closely related to mitochondria changes.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in the present study.

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The effect of sodium butyrate administered in ovo on the health status and intestinal response in broiler chicken

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ABSTRACT A healthy gut is one of the main factors influencing bird response. Over the years, efforts have been made to improve intestinal health. One of the supporting methods may be enriching the diet with bioactive ingredients, including sodium butyrate (**SB**). One of the possible ways of administering such supplementation is in ovo technology. Over the years, research has shown that administering bioactive substances this way has a positive effect on the health status of chickens. The current study aimed to modify the gut microbiota of broiler chickens by in ovo stimulation on d 12 of egg incubation with SB and to determine the changes occurring in intestines. One thousand eggs were incubated and injected with 0.1, 0.3, or 0.5% SB on d 12 of incubation. The control group was injected with physiological saline. Samples collected for analysis were obtained postmortem from 42-day-old ROSS 308 broiler chickens. Growth performance parameters were also monitored during broiler rearing. Gene expression analysis showed significant changes in the levels of *IL4, IFNγ, AvBD1, TJAP* and *MUC6* genes in the ileum. However, the *IL8, MUC2* and *MUC6* genes were significantly expressed in the cecal mucosa. These changes depended on the administered dose of butyrate. There was no effect of in ovo administration of various doses of SB on digesta pH, SCFA level and histological parameters. However, a significant increase in *Bifidobacterium* bacteria was detected in the ileum after administration of a dose of 0.5% SB and in the cecum after administration of a dose of 0.3%. Administration of SB in ovo has the potential to support intestinal health in poultry. The effects depend on the administered dose, while the results indicate a dose of 0.3% as the most optimal.

Key words: gene expression, immune response, in ovo technology, microbiome, postbiotic

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INTRODUCTION

The digestion and absorption of nutrients by a healthy intestine support the growth and production of poultry. The importance of gastrointestinal tract health in poultry has been increasingly reported due to its benefits to their overall health and performance. Any intestinal damage and disease had a negative impact on the absorption and digestion of nutrients (Elnesr et al., 2020). Intestinal mucosa plays a significant role in providing an active barrier for the host's internal tissues from pathogens and mechanical damage during digestion. Therefore, mucosa represents the first line of defense (Duangnumsawang et al., 2021a). Pathogenic bacteria and other microorganisms trying to get from the outside to the host's body encounter a layer of intestinal mucus. Owing to its gel-like structure, the mucus captures these microorganisms and blocks their further penetration into the host (Forder et al., 2007). Hence, poultry supplementation is justified in improving intestinal health. The substance that acts in this direction is sodium butyrate (**SB**). It has been reported that it plays

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an essential role in lowering intestinal pH, controlling the colonization and growth of harmful microorganisms, stimulating the growth of absorptive cells in the intestine, and supporting birds' growth performance (Elnesr et al., 2020). Butyrate is one of the 3 main short-chain fatty acids (**SCFA**), and its functions include providing energy, balancing the intestinal microbiota, and mediating the immune response (Deng et al., 2023). According to literature reports, SB improves poultry production parameters and stimulates dynamic changes in the intestinal microbiota (Wan et al., 2022).

The study aimed to determine the effect of SB delivered in 3 different concentrations in ovo on broiler chickens' health status and intestinal response. The innovative nature of the research is the method of administering the substance already during embryonic development. Previous studies have clearly proven that the administration of bioactive substances in ovo on d 12 of egg incubation has immunostimulating and immunomodulatory potential (Siwek et al., 2018).

MATERIAL AND METHODS

Experimental Setup and Housing

Ross 308 broiler chicken hatching eggs (n = 1,000) were obtained from the Poultry Hatchery (Kuyavian-Pomeranian Voivodship Poland). All eggs were incubated as described by Biesek et al. (2023). On d 12 of incubation, eggs were randomly divided into 4 groups of 250 eggs each, which were injected into the air chamber with 0.2 mL of physiological saline (control) or with 0.1, 0.3, or 0.5% SB (110.09 g/mol, Merck Life Science, Poland) solution in physiological saline. The eggs were incubated for 21 d.

After hatching, 60 chicks with an average body weight of 50.99 g were selected from each group for rearing. Each group was kept in 5 replicates, 12 chicks were provided in each replicate. On the first day of rearing, the temperature was 30°C, then lowered to 20°C in the last week of rearing. The chickens were provided with an additional heat source with a temperature of 2°C higher than in the building for 4 wk. The humidity was approximately 60%. The building was prepared 24 h before the rearing started. Chopped wheat straw was used as bedding. The lighting program included 18 h of light and 6 h of darkness. In the first and last 3 d of rearing, the light was extended to 23 h. The chickens had free access to fresh water and feed. A bell-shaped drinker was placed in each pen, and a feeder was on the pen's walls. Three feeding phases were used: starter feed from d 1 to d 14, grower feed from d 15 to d 35, and finisher feed from d 36 to d 42 of rearing.

Bird deaths were recorded during rearing, and viability was calculated according to Biesek et al. (2022). The birds were weighed on days 1, 14, 35, and 42 of rearing, while feed intake was recorded daily. The production efficiency factors were calculated:

European Production Efficiency Factor

$$\left(EPEF = \frac{viability\ (\%) \times BW(kg)}{age(days)\ \times FCR\left(\frac{kg\ feed}{kg\ gain}\right)} \times\ 100 \right);$$

European Broiler Index

$$\left(EBI = \frac{viability\ (\%)\ \times ADG\left(\frac{g}{\frac{chick}{day}}\right)}{FCR\ \left(\frac{kg\ feed}{kg\ gain}\right) \times\ 10} \right).$$

Samples Collection

After d 42, 10 broiler chickens were randomly selected from each group. The chickens were stunned by percussive blows to the head, which involved delivering firm and precise impacts to the head, resulting in severe damage to the brain. This procedure was conducted following relevant regulations: Council Regulation (EC) No 1099/2009 of 24 September 2009 concerning the protection of animals at the time of killing (mechanical methods), and Directive No. 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes (Annex IV - methods of animal killing). Decapitation was executed by severing the head between the occipital condyle and the first cervical vertebra, leading to swift bleeding of the carcass.

After sacrifice, the ileal and cecal mucosa samples for RNA isolation were immediately placed in a stabilization buffer (FixRNA, EURx, Gdansk, Poland), while intestinal contents for analysis of bacteria abundance were placed on dry ice and stored at -80°C pending further analysis. Blood was collected into heparinized tubes and centrifuged at 3,350 rpm for 10 min. Plasma samples were taken and stored at -40°C until further biochemical analyses. Digesta samples for the analysis of SCFA concentrations were taken from the distal part of the ileum and ceca, frozen in dry ice, and stored at -20°C. Small pieces of ileum and cecum were also taken, rinsed with physiological saline, and fixed in 10% neutral-buffered formalin for histological examination.

Analyses of Basic Biochemical Blood Parameters

Biochemical parameters of blood plasma were analyzed spectrophotometrically using a MAXMAT PL analyzer (Erba Diagnostics SARL, Montpellier, France) using diagnostic kit of ELITech Clinical Systems SAS (Sees, France). Sodium and potassium concentrations were measured on EasyLyte Na/K Analyzer (Medica, Bedford, MA).

Bacterial DNA Isolation and Analysis of Bacterial Abundance

To isolate bacterial DNA, approximately 200 mg of intestinal content was taken. The weighed samples were

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Table 1. Bacterial primer sequences used in qPCR (F - forward primer; R - reverse primer).

Bacteria	Primer sequences (Forward/Reverse)	Reference
Universal bacteria	F: ACTCCTACGGGAGGCAGCAGT R: GTATTACCGCGGCTGCTGGCAC	Christensen et al., 2014
Bifidobacterium spp.	F: GCGTGCTTAACACATGCAAGTC R: CACCCGTTTCCAGGAGCTATT	Christensen et al., 2014
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: CGGGTAACGTCAATGAGCAAA	Huijsdens et al., 2002
Faecalibacterium prausnitzii	F: ACCATGAGAGCCGGGGGG R: GGTTACCTTGTTACGACTT	Lund et al., 2010
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Christensen et al., 2014

lysed and purified using the GeneMATRIX Stool DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions. The storage temperature for the DNA samples was -20°C. The extracted DNA was subjected to quantitative and qualitative evaluation using a spectrophotometric method using NanoDrop2000 (Thermo Scientific Nanodrop Products, Wilmington, NC). The relative abundances of the examined bacteria (Table 1) in the intestinal content were determined by quantitative PCR (**qPCR**) performed on the LightCycler 480 II system (Roche-Diagnostics, Basel, Switzerland). The qPCR reactions mixture contained SG onTaq qPCR Master Mix (2x) (EURx, Gdansk, Poland), 1 μM of each primer specific to 16S rRNA (synthesized by Sigma-Aldrich, Schnelldorf, Germany), and ca. 20 ng of bacterial DNA template. The thermal profile of the specific qPCR reaction was performed according to the instructions included with the bacterial DNA isolation kit: initial denaturation at 95°C for 15 min, followed by 40 cycles of amplification consisting of denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The fluorescence was measured at the end of each extension step. The qPCR efficiency for each pair of bacterial primers was calculated in the LightCycler 480 II software from a standard curve prepared in 5 dilutions (1x, 0.5x, 0.25x, 0.125x, and 0.0625x) of pooled bacterial DNA template. The relative abundances of the bacteria were calculated according to Christensen et al. (2014) as follows:

$$\text{Relative abundance}[\%] = (\text{E universal})^{\text{Ct universal}}/(\text{E target})^{\text{Ct target}}$$

where "E universal" is the efficiency of qPCR with primers for all bacteria, "Ct universal" is the Ct values for reaction with primers for all bacteria, "E target" is the efficiency of qPCR with primers specific for target bacteria, and "Ct target" is the Ct values for reaction with primers for target bacteria.

Total RNA Isolation and Analysis of Gene Expression

RNA was isolated from about 120 mg of intestinal mucosa. The RNA isolation procedure was performed according to the manufacturer protocol for the Universal RNA Purification Kit (EURx, Gdansk, Poland). After isolation, each RNA sample was assessed quantitatively and qualitatively using NanoDrop 2000 (Thermo Scientific Products, Wilmington, NC). Gene expression analysis was performed for the following gene panels: immune-related genes (*IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IFNγ*), host defense peptide genes (*AvBD1, CATHL2*), and barrier function genes (*TJAP, CLDN, MUC2, MUC6*). Normalization of the expression of the target genes was performed with the geometric mean of 2 housekeeping genes: *ACTB* and *G6PDH*. Primer sequences for all genes are presented in Table 2. cDNA was synthesized using the smart First Strand cDNA Synthesis kit (EURx, Gdansk, Poland). qPCR reactions were performed using LightCycler 480 II. qPCR mixture contained SG onTaq qPCR Master Mix (2x) (EURx, Gdansk, Poland), 1 μM of each primer (synthesized by Merck Darmstadt, Germany), and 70 ng of cDNA. The thermal program was carried out in a LightCycler 480 instrument II (Roche Diagnostics, Basel, Switzerland). The annealing temperature for all genes was 58°C (except IL12 - the temperature was 62°C). Melting curves were generated to test for the specificity of reactions at the end of the thermal cycling. Each qPCR reaction was performed in duplicate technical repetitions. Relative gene expression was calculated by the $^{\Delta\Delta}C_T$ algorithm, and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula according to Livak and Schmittgen (2001).

Digesta pH and SCFA Analysis

Digesta pH was measured using a SevenMulti pH-meter (Mettler-Toledo, Warsaw, Poland) after mixing with ultra-pure water in a 1:2 ratio (w/v). Then, a few drops of 1 M NaOH were added to adjust the pH to 8.2 to convert SCFA to their sodium salts. After centrifugation (10 min, 1,800 *g*, room temperature), the supernatants were stored at -20°C until further analysis. SCFA concentrations were analyzed as described by Barszcz et al. 2011 using an HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany). Isocaproic acid was used as an internal standard.

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Table 2. Gene primer sequences used in RT-qPCR (F - forward primer; R - reverse primer).

Gene	Name	Sequences	Reference
Reference genes			
ACTB	Actin - beta	F: CACAGATCATGTTTGAGACCTT R: CATCACAATACCAGTGGTACG	Slawinska et al., 2019
G6PDH	Glucose 6 phosphate dehydrogenase	F: CGGGAACCAAATGCACTTCGT R: GGCTGCCGTAGAGGTATGGGA	Slawinska et al., 2019
Immune-related genes			
IL-1β	Interleukin 1 beta	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	Slawinska et al., 2019
IL-2	Interleukin 2	F: GCTTATGGAGCATCTCTATCATCA R: TTGGGCAGGTTGAGGTTGTT	Slawinska et al., 2019
IL-4	Interleukin 4	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	Biesek et al., 2021
IL-6	Interleukin 6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	Sławinska et al., 2014
IL-8	Interleukin 8	F: AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	Sławinska et al., 2014
IL-10	Interleukin 10	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG	Biesek et al., 2021
IL-12	Interleukin 12	F: TTGCCGAAGAGCACCAGCCG R: CGGTGTGCTCCAGGTCTTGGG	Slawinska et al., 2019
IFNγ	Gamma interferon	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	Biesek et al., 2021
Host defense peptide genes			
AvBD1	Avian beta defensin 1	F: AAACCATTGTCAGCCCTGTG R: TTCCTTAGAGCCTGGGAGGAT	Slawinska et al., 2019
CATHL2	Cathelicidin 2	F: AGGAGAATGGGGTCATCAGG R: GGATCTTTCTCAGGAAGCGG	Slawinska et al., 2019
Barrier function genes			
CLDN1	Claudin 1	F: TCTTCATCATTGCAGGTCTGTC R: AACGGGTGTGAAAGGGTCAT	Slawinska et al., 2019
TJAP	Tight junction associated protein 1	F: AGGAAGCGATGAATCCCTGTT R: TCACTCAGATGCCAGATCCAA	Slawinska et al., 2019
MUC2	Mucin 2	F: ATGCGATGTTAACACAGGACTC R: GTGGAGCACAGCAGACTTTG	Daneshmand et al., 2019
MUC6	Mucin 6	F: TTCAACATTCAGTTCCGCCG R: TTGATGACACCGACACTCCT	Slawinska et al., 2019

Histological Analyses of Ileum and Cecum

Formalin-fixed samples were dehydrated in graded ethanol concentrations, cleared in xylene, and embedded in paraffin. Then, the specimens were sliced in 4.5 μm sections using a rotary microtome HM355S (Thermo Shandon Limited, Runcorn, UK) equipped with the Cool-Cut system and section transfer system on a wet tray. The sections were dewaxed, rehydrated, and stained using the alcian blue pH 2.5-periodic acid-Schiff's reagent (**AB-PAS**) technique to distinguish neutral and acidic mucins in goblet cells. The acidic chemotype of mucins was further distinguished into sulfomucins and sialomucins with the high iron diamine-alcian blue pH 2.5 (HID-AB) staining (SPICER, 1965). Various goblet cell types were evaluated at 20x objective using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) and ImageJ 1.47v program (National Institute of Mental Health, Bethesda, MD). For the quantification, all goblet cells on the villi area (ileum) and crypt area (cecum) were counted on 8-10 images per bird. On average, 187 goblet cells were counted on each image of the ileal section and 36 cells on each image of the cecal section. The number of cells of neutral and acidic mucin chemotypes was expressed in percentage of total goblet cell count, while the number of sulfomucin- and sialomucin-containing cells was expressed in percentage of total acidic goblet cell count. The mixed-type (sulfo-sialomucins) cells were scarcely detected and counted as sulfomucin (black) or sialomucin (blue) cells, depending on the dominant color.

Intestinal sections stained with AB-PAS were further used for the histological measurements. In the ileum, villus height, villus width, crypt depth, villus height to crypt depth (**VH/CD**) ratio, and tunica muscularis thickness were determined. Also, villus surface area was calculated using the formula of Law et al., 2007. In the cecum, crypt depth and muscular layer thickness were measured. For each bird, at least 10 measurements of each parameter were done and individual means were calculated. All histological measurements were performed using a Zeiss Axio Star Plus (Carl Zeiss, Göttingen, Germany) light microscope and AxioVision 4.8.2.0 (Carl Zeiss MicroImaging GmbH) image analysis software.

Statistical Analysis

The collected data was verified in a statistical program (Statistica, 13.3, TIBCO, Statsoft, Kraków, Poland). Normal distribution and sample homogeneity were analyzed. Tukey's test verified statistically significant differences between the groups, assuming $P < 0.05$.

Data were analyzed by one-way analysis of variance followed by Duncan's *post hoc* test using the

Table 3. Growth performance of broiler chickens.

Item n: 5 pens each pen: 12 birds	Sodium butyrate dose (%)				SEM	*P*-value
	0.0	0.1	0.3	0.5		
Viability (%)	93.33	96.67	95.00	96.67	1.415	0.838
Body weight (g)						
1 d	50.55	50.45	51.22	51.72	0.225	0.149
14 d	484.20	466.25	498.02	484.59	9.187	0.714
35 d	2050.56	1960.63	2053.10	1988.64	32.639	0.718
42 d	2711.91	2632.52	2766.31	2579.63	45.683	0.514
Growth rate (%)						
1 – 14 d	162.06	160.37	162.61	161.34	0.741	0.764
15 – 35 d	123.60	123.42	121.86	121.46	0.805	0.749
36 – 42 d	27.84	29.43	29.64	25.48	0.817	0.255
Total	192.67	192.47	192.70	192.04	0.139	0.328
BWG (g)						
1 – 14 d	433.65	415.80	446.80	432.87	9.166	0.729
15 – 35 d	1566.36	1494.38	1555.08	1504.05	26.005	0.729
36 – 42 d	661.35	671.89	713.21	590.99	22.327	0.288
Total	2661.36	2582.07	2715.09	2527.91	45.686	0.512
ADBWG (g)						
1 – 14 d	30.98	29.70	31.91	30.92	0.655	0.729
15 – 35 d	74.59	71.16	74.05	71.62	1.238	0.729
36 – 42 d	94.48	95.98	101.89	84.43	3.190	0.288
Total	63.37	61.48	64.65	60.19	1.088	0.512
FI (g)						
1 – 14 d	597.75	600.58	610.08	620.22	4.591	0.314
15 – 35 d	2545.86	2499.50	2591.39	2594.24	22.637	0.431
36 – 42 d	1562.43	1497.14	1528.28	1530.19	24.819	0.857
Total	4897.70	4710.23	4808.17	4812.23	76.451	0.881
ADFI (g)						
1 – 14 d	42.70	42.90	43.58	44.30	0.328	0.314
15 – 35 d	121.23	119.02	123.40	123.54	1.078	0.431
36 – 42 d	223.20	213.88	218.33	218.60	3.546	0.857
Total	116.61	112.15	114.48	114.58	1.820	0.881
FCR (kg/kg)						
1 – 14 d	1.38	1.48	1.37	1.44	0.033	0.664
15 – 35 d	1.63	1.68	1.67	1.74	0.026	0.568
36 – 42 d	2.37	2.26	2.14	2.76	0.106	0.190
Total	1.84	1.83	1.77	1.92	0.029	0.346
EPEF	327.15	333.16	352.82	313.31	8.782	0.486
EBI	321.02	326.78	346.26	307.04	8.657	0.480

BWG, body weight gain; ADBWG, average daily body weight gain; FI, feed intake; ADFI, average daily feed intake; FCR, feed conversion ratio; EPEF, European production efficiency factor; EBI, European broiler index;
SEM: standard error of the mean.

Statgraphics Centurion XVI ver. 16.1.03 statistical package (StatPoint Technologies, Inc., Warrenton, VA). The level of significance was set at $P \leq 0.05$.

Statistical differences for gene expression data and the relative abundance of bacteria were determined using a one-way ANOVA test where the differentiating factor was the dose of SB administrated in ovo on the day 12 of egg incubation. Post hoc intergroup comparisons were performed using the Tukey HSD test. The calculations for these data sets were prepared using SAS 9.4 software (SAS Institute Inc., Cary, NC).

RESULTS

Growth Performance

When analyzing body weight and gains, feed intake, and feed conversion efficiency (Table 3), statistically significant differences were not shown ($P > 0.05$). In all groups, the body weight gain was similar, from 2527.91 to 2715.09 g, and the FCR was 1.77 to 1.92 kg per kg of body weight gain. Also, no effect on the European Production Efficiency Factor and European Broiler Index was found ($P = 0.486$; $P = 0.480$, respectively).

Blood Biochemistry

In ovo stimulation with SB did not affect the biochemical blood parameters of chickens, except for chloride and uric acid concentrations (Table 4). Birds stimulated with 0.1% and 0.5% SB had significantly higher chloride concentrations than birds from the control group. Chickens from the group treated with 0.1% SB had also higher chloride concentration than birds treated with 0.3% SB ($P = 0.043$). Birds stimulated with 0.5% SB had greater concentration of uric acid in the blood than those from the control and 0.1% SB groups ($P = 0.045$).

Bacterial Abundance

Bacterial abundance in the ileum was presented in Figure 1, while in the cecum in Figure 2. There were no significant changes in the ileum in the case of

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Table 4. Biochemical blood parameters of chickens stimulated in ovo with sodium butyrate of different concentrations.

	Sodium butyrate (%)					
Parameter	0.0	0.1	0.3	0.5	SEM	*P*
Albumin, g/l	15.0	15.8	18.0	15.0	0.79	0.503
ALP, U/l	4042	5412	3807	4854	301.8	0.214
ALT, U/l	20.5	27.7	25.1	33.9	2.24	0.199
Amylase, U/l	537	606	606	637	28.0	0.648
AST, U/l	364	331	313	285	14.5	0.283
Total protein, g/l	35.8	34.9	37.6	37.2	0.63	0.413
Bilirubin, μmol/l	3.31	3.35	3.71	5.11	0.33	0.181
Chloride, mmol/l	178[a]	215[c]	184[ab]	209[bc]	5.7	0.043
Cholesterol, mmol/l	2.77	2.82	2.91	2.65	0.053	0.386
Cholinesterase, U/l	890	960	944	1373	100.7	0.305
CK, U/l	32250	28375	25845	23005	2681.7	0.674
Phosphorus, mmol/l	1.88	1.83	1.84	1.86	0.020	0.766
GGTP, U/l	20.6	20.1	25.7	21.9	1.23	0.372
Glucose, mmol/l	14.55	14.51	14.98	14.58	0.196	0.827
HDL, mmol/l	1.79	1.82	1.81	1.86	0.038	0.942
Creatinine, μmol/l	21.10	19.43	22.93	24.36	1.513	0.696
Uric acid, μmol/l	205[a]	183[a]	226[ab]	308[b]	17.0	0.045
LDH, U/l	2091	1895	1892	1866	112.4	0.895
LDL, mmol/l	1.08	1.13	1.13	1.15	0.035	0.930
Lipase, U/l	16.15	13.68	12.00	15.96	0.893	0.306
Magnesium, mmol/l	1.27	1.28	1.30	1.18	0.021	0.205
Urea, mmol/l	0.44	0.52	0.40	0.36	0.042	0.595
Triglycerides, mmol/l	0.53	0.61	0.63	0.76	0.036	0.146
Calcium, mmol/l	2.77	2.62	2.73	2.59	0.079	0.844
Iron, μmol/l	12.49	11.86	13.65	15.27	0.682	0.313
Sodium, mmol/l	145.3	146.5	147.9	146.2	0.40	0.124
Potassium, mmol/l	6.62	6.42	6.43	6.68	0.088	0.653

[a,b,c]means in columns marked with different letters differ significantly at P ≤ 0.05; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; GGTP, γ-glutamyltransferase; HDL, high density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; SEM, standard error of the mean.

Lactobacillus spp. and *Faecalibacterium prausnitzii* for any studied groups. In the case of *Bifidobacterium* spp., a significant increase in abundance was noted for the 0.3% SB (3.176E-09) and 0.5% SB (8.688E-09) groups compared to the control (6.293E-10). Additionally, in the ileum there was a significant decrease in *Escherichia coli* bacteria by approximately 20 times in the 0.1% SB group (3.35E-05) and an approximately nine times decrease in the abundance in the 0.3% SB group (7.75E-05) compared to the control group (6.82E-04).

In the cecum, the abundance of *Lactobacillus* spp. decreased approximately 2 times in each tested group compared to the control (3.84E-02). In birds treated with 0.1% SB (5.07E-04) and 0.3% SB (6.04E-04), the abundance of *Bifidobacterium* spp. increased more than 2 times compared to the control (2.35E-04). SB concentration had no significant effect on the abundance of *Escherichia coli.* Moreover, 0.1 and 0.3% SB increased the abundance of *Faecalibacterium prausnitzii* compared to the control group.

Gene Expression

Significant changes between groups in the ileum occurred for the *IL-4, IFNγ, AvBD1, CATHL2,* and *MUC6* genes. Treatment with 0.1% SB caused a significant downregulation of *IL-4, IFNγ,* and *MUC6* gene expression. Downregulation also occurred for the *CATHL2* and *MUC6* genes in the 0.5% SB group relative to the control. *AvBD1* was the only one that showed a significant increase in every SB-treated group compared to the control. Its expression level increased 4 times in the 0.1% SB group (3.77) compared to the control and almost 3 times decreased in the 0.3% group (1.46) compared to the 0.1% SB (3.77). Figures 3, 4, and 5 present all ileum gene expression results.

In the cecum, there was a decrease in *MUC6* and an increase in *MUC2* expression in the groups treated with 0.1% and 0.3% SB as compared to the control. In ovo treatment with 0.1% SB significantly increased the expression of *IL-8* as compared to other groups, while with 0.5% upregulated *IL-1β* expression. Figures 6, 7, and 8 present all cecum gene expression results.

Digesta pH and SCFA

In the ileum of chickens, there was no effect of in ovo treatment with SB of different concentrations on digesta pH and SCFA levels. In the cecum, stimulation with 0.1% and 0.3% SB significantly increased propionic acid concentration as compared to the control and 0.5% SB group ($P = 0.020$). There was also an effect on butyric acid concentration, which was significantly greater in birds treated with 0.1% SB than in those treated with 0.3% and 0.5% SB ($P = 0.014$). Other SCFA and cecal digesta pH were unaffected by in ovo stimulation with SB (Table 5).

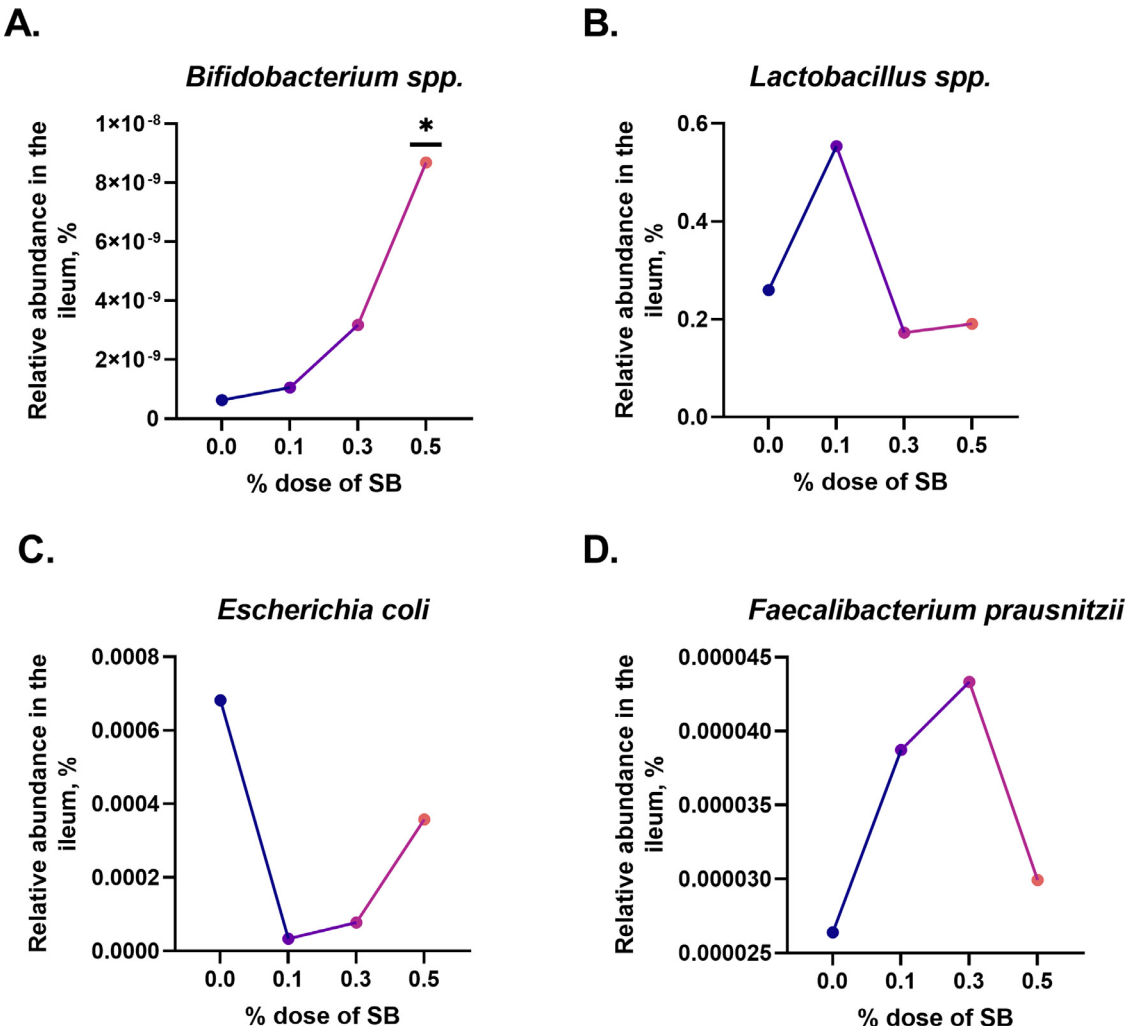

Figure 1. The relative abundance of bacteria in the ileum content in ROSS 308 chickens (n = 8) stimulated in ovo on d 12 of egg incubation with sodium butyrate (SB). The x-axis shows percentage doses of SB (0.0, 0.1, 0.3, 0.5). The y-axis indicates the relative abundance of bacteria: (A) *Bifidobacterium spp.*, (B) *Lactobacillus spp.*, (C) *Escherichia coli*, (D) *Faecalibacterium prausnitzii*. The relative abundance of the bacteria was calculated using formula: $E_{universal}^{Ct\ universal}/E_{target}^{Ct\ target}$ where E means efficiency of the qPCR reaction and Ct is the threshold cycle of the reaction. The effect of SB stimulation on the relative abundance of bacteria was determined using one-way ANOVA. Intergroup differences were assessed using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

Intestinal Morphology and Mucin Chemotypes in Goblet Cells

There was no effect of SB concentration administered in ovo on villus height, villus surface area, crypt depth, VH/CD ratio, and tunica muscularis thickness in the ileum of broiler chickens (Table 6). In the cecum, in ovo treatment with SB affected crypt depth, which was significantly greater in birds treated with 0.1% SB than in those treated with 0.3% SB. Administration of 0.5% SB significantly increased crypt depth in comparison with the control and 0.3% groups ($P = 0.002$).

In the ileum and cecum of broiler chickens, only goblet cells containing acidic mucins were found (Table 6), and their population was much greater in the former segment (Figure 9). In both parts of the gut, acidic goblet cells with sulfomucins (black-stained) predominated. Sialomucins-containing (blue-stained) goblet cells were found only along the ileal villi and were sparsely distributed along the cecal crypts (Figure 10). Neither in the ileum nor in the cecum of chickens mucin chemotypes in goblet cells were affected by SB concentration.

DISCUSSION

The aim of this study was to analyse the effect of sodium butyrate administered in ovo during embryonic development on the intestinal health of broiler chickens. The rationale for using 3 different doses was based on literature data where sodium butyrate was an additive to poultry nutrition (Salmanzadeh et al., 2014). In this study demonstrated the effect of the administration of SB on molecular and phenotypic changes in the intestines. The cecum is one of the most studied sections of the digestive tract in poultry. This is the section of the intestines where most of the processes involved in fermentation occur. The cecum is colonized by a diverse microbiome, making it metabolically crucial to the organism. The bacteria responsible for metabolic activity are primarily *Bacillus, Lactobacillus, Clostridium, Faecalibacterium,*

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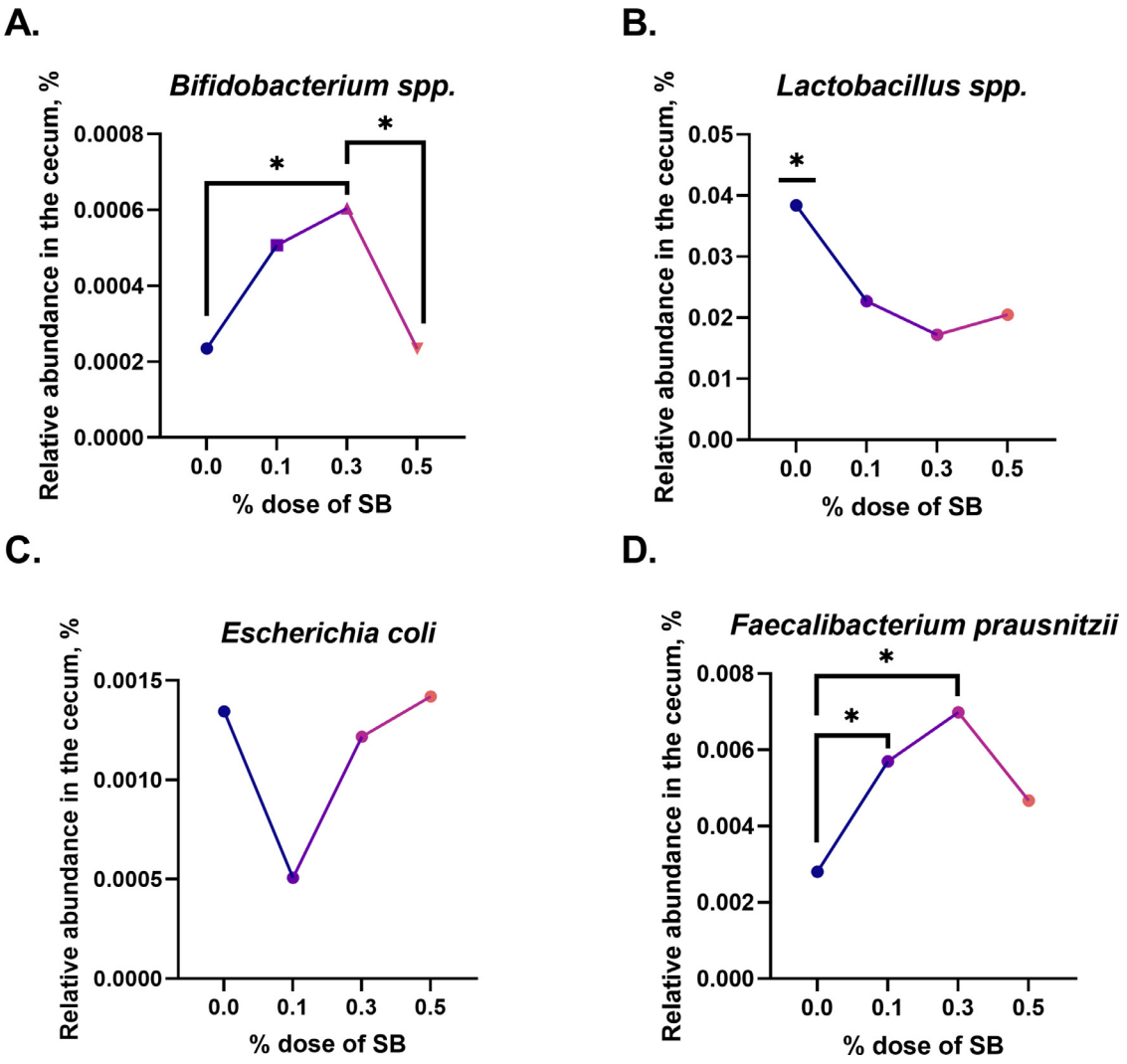

Figure 2. The relative abundance of bacteria in the cecal content in ROSS 308 chickens (n = 8) stimulated in ovo on d 12 of egg incubation with sodium butyrate (SB). The x-axis shows percentage doses of SB (0.0, 0.1, 0.3, 0.5). The y-axis indicates the relative abundance of bacteria: (A) *Bifidobacterium spp.*, (B) *Lactobacillus spp.*, (C) *Escherichia coli*, (D) *Faecalibacterium prausnitzii*. The relative abundance of the bacteria was calculated using formula: $E_{universal}^{Ct\ universal}/E_{target}^{Ct\ target}$ where E means efficiency of the qPCR reaction and Ct is the threshold cycle of the reaction. The effect of SB stimulation on the relative abundance of bacteria was determined using one-way ANOVA. Intergroup differences were assessed using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

Ruminococcus, Streptococcus. They support fermentation processes and produce SCFA including butyrate (Borda-Molina et al., 2021). Despite the low diversity of the microbiota, the ileum is the main section where nutrient absorption occurs (Lv et al., 2021).

In the current study, SB had no effect on body weight gain, growth rate, or FCR. The lack of differences indicate that in ovo treatment with SB is safe for birds and does not impair their growth and feed utilization. Chickens remained healthy throughout the study, which was confirmed by very high viability and biochemical blood parameters, which were similar to those obtained in other studies (Chodkowska et al., 2022; Di Gregorio et al., 2023) No effect on aminotransferases and cholinesterase activities, as well as on total protein, albumin, bilirubin, and cholesterol concentrations indicated that in ovo administration of SB had no detrimental effect on the liver of chickens. Pancreas cells also were not damaged by this treatment, which was shown particularly by the lack of effect on amylase and lipase activities (Chodkowska et al., 2022). Similar concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and PO_4^{3-} in blood indicated an appropriate electrolyte balance in chickens. However, Cl^- concentrations were greater in 0.1% and 0.5% SB groups. For this experiment, SB solutions were prepared in physiological saline (0.9% NaCl), and each egg was injected with the same dose (0.2 mL). The effect of SB on Cl^- concentration in blood without the impact on Na^+ level may suggest disturbances of acid-base balance and be related to pancreatic fluid secretion (Evans, 2009) To confirm this, more in-depth research should be performed, including measurements of bicarbonate concentration in blood and other than amylase and lipase activities markers of pancreas physiology.

In birds, uric acid is the main end product of nitrogen metabolism, and its increase in the blood may indicate

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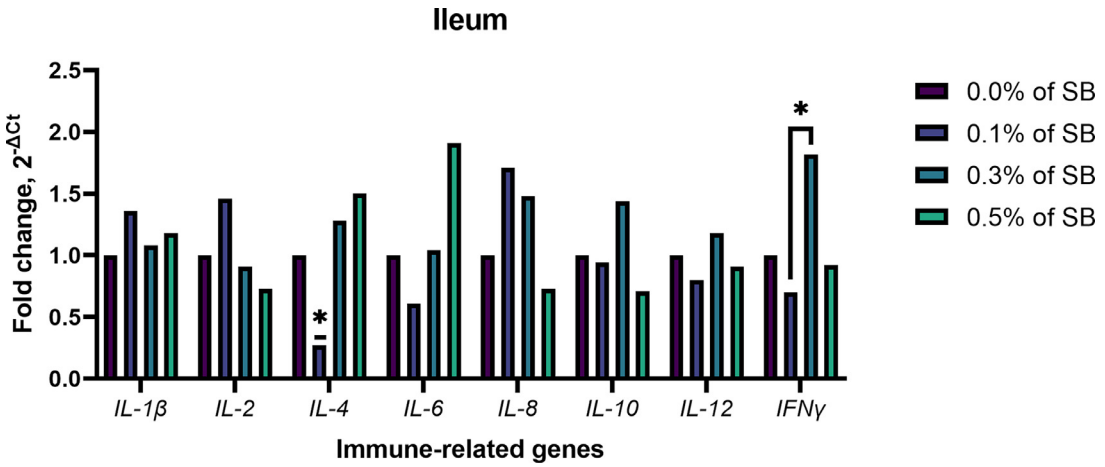

Figure 3. The relative mRNA expression of immune-related genes in the ileum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (SB). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after in ovo stimulation with SB (n = 8). The relative gene expression (**FC**—fold change) was calculated with the delta delta threshold cycle (**ddCt**) formula, and the fold change (FC) was calculated as follows: $FC = 2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, and 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 7 (GraphPad, La Jolla, CA).

renal insufficiency (Bogin et al., 1996). The highest level of uric acid in chickens treated with 0.5% SB suggests that this concentration is too high for the in ovo stimulation. On the other hand, this result might be related to bacterial uricase activity and uric acid secretion and excretion in the cecum, which plays an important role in these processes (Scanes & Pierzchala-Koziec, 2014). Higher concentration of uric acid in the blood of chickens may indicate its reduced degradation to ammonia in the cecum. Studies on laying hens showed that SB increased uric acid concentration and reduced uricase activity in the fermentation broth of cecal content and that bacterial decomposition of amino acids might play a role in SB-mediated reduction of ammonia emission (Zhong et al., 2022) This effect of in ovo administration of 0.5% SB would be very beneficial from the point of view of environmental protection.

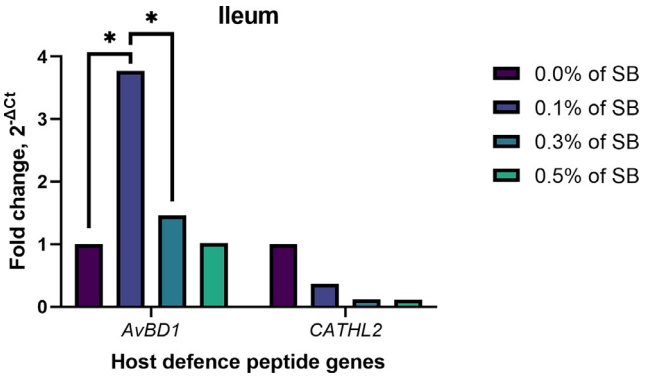

Figure 4. The relative mRNA expression of host defence peptide genes in the ileum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (SB). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after in ovo stimulation with SB (n = 8). The relative gene expression (FC—fold change) was calculated with the delta delta threshold cycle (ddCt) formula and the fold change (**FC**) was calculated as follows: $FC = 2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, and 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

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Bawish et al., 2023 showed that the use of SB as a feed additive has a beneficial effect on the growth of intestinal villi and on increasing the absorptive surface in the intestines. Moreover, the growth of intestinal villi promoted an increase in the abundance of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. In the present study, there was no effect of SB on ileal villi height and villi surface area but the abundance of *Bifidobacterium* increased, while that of *E. coli* decreased. In the cecum, decreased abundance of *Lactobacillus* may be associated with a simultaneous increase in *Faecalibacterium prausnitzii*. The observed changes may be attributed to the increased depth of cecal crypts caused by in ovo SB stimulation. The enlargement of crypt depth is associated with higher proliferation rate of epithelial cells (Choct, 2009; Barszcz and Skomiał, 2011; Xue et al., 2018) and increases the space for colonization by bacteria. Increasing the abundance of one group of bacteria could have blocked the availability of the binding sites and inhibited colonization by another bacteria (Ducarmon et al., 2022; Makowski et al., 2022).

The described study showed an increase in the expression of genes related to the immune response. A significant up regulation of *IL1β* expression occurred in the cecum and *IL4* in the ileum. *IL1β* is a pro-inflammatory cytokine with protective functions. *IL1β* secreted into the intestinal lumen is a key mediator in the process associated with intestinal inflammation. *IL1β* expression is modulated by commensal gut microbiota (Samuel, 2001). SB has an anti-inflammatory effects, which are

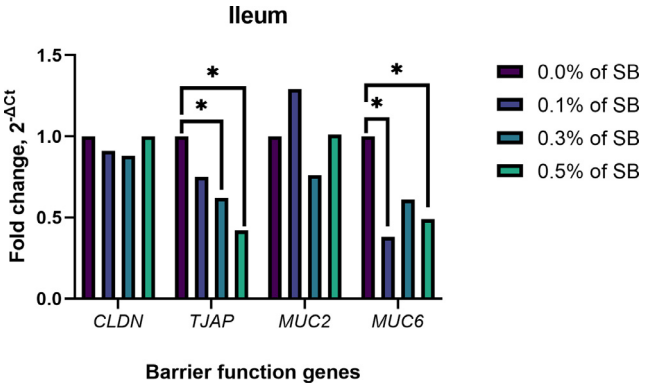

Figure 5. The relative mRNA expression of barrier function genes in the ileum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (**SB**). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after in ovo stimulation with SB (n = 8). The relative gene expression (FC—fold change) was calculated with the delta delta threshold cycle (**ddCt**) formula and the fold change (**FC**) was calculated as follows: FC = $2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, and 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

associated with stimulation of mucin and host defense peptides synthesis and reduction of the intestinal permeability. In the caecum, there was a phenomenon in which the expression of *MUC2* increased, but at the same time, the expression of *MUC6* decreased in the 0.1% SB-treated group. A similar phenomenon was described by (Melhem et al., 2021), who found that *TNF-α* increased the expression of *MUC2*, but did not affect the expression of *MUC6*. This may be related to the fact that *IL-1β* stimulates *MUC2* mRNA expression. The expression of *IL-1β* and *MUC2* in our experiment is indeed significantly higher in the 0.5% SB group. Butyric acid, obtained in the process of fermentation of dietary carbohydrates by intestinal bacteria, is a known inducer of host defense peptides (**HDP**) in both humans and animals. Butyrate has been shown to provide protection against infection, in part through the induction of HDP (Yang et al., 2022). In the ileum, there was a difference in the expression between 2 HDP genes - avian beta defensin 1 (**AvBD1**) and cathelicidin 2 (**CATHL2**). The first gene showed increased expression under SB stimulation, while the expression of the second gene was noticeably decreased. Similar results were obtained by Yang et al., 2022 who demonstrated that SB together with quercetin synergistically increased the expression of *AvBD1, AvBD2, AvBD3, AvBD4, AvBD6,* and *AvBD7* and *AvBD9*, but did not affect the expression of *CATHB1* in HTC macrophages. The observed different magnitude of induction for 2 different HDP genes may indicate an effect specific to each individual gene.

In ovo, treatment with SB had no effect on SCFA concentration in the ileum but affected the cecum of chickens. Stimulation with 0.1% and 0.3% SB increased propionic acid concentration, which suggests a growth of *Bacteroidetes, Megasphaera* spp., or other propionate producers that may utilize 3 different pathways for its production, that is, succinate, acrylate, and propanediol pathway (Flint et al., 2015).

Administration of 0.1% SB also increased butyric acid concentration in the cecum. This result can be ascribed to a greater population of *F. prausnitzii*, abundant butyrate-producing species belonging to the *Ruminococcaceae* (clotridial cluster IV) (Flint et al., 2015). As butyric acid exerts a trophic effect on the epithelium (Topping and Clifton, 2001), its higher concentrations explain the deeper crypts found in the cecum of birds stimulated in ovo with 0.1% SB. It remains unclear what mechanism was responsible for the crypt enlargement in the cecum of birds treated with 0.5% SB, since there was no increase in butyric acid concentration and *F.*

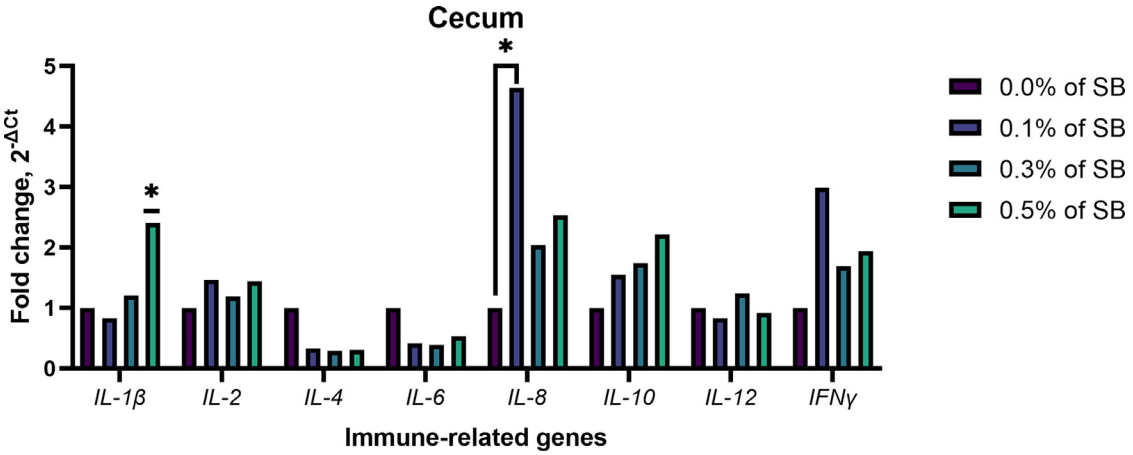

Figure 6. The relative mRNA expression of immune-related genes in the cecum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (**SB**). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after in ovo stimulation with SB (n = 8). The relative gene expression (FC—fold change) was calculated with the delta delta threshold cycle (ddCt) formula and the fold change (**FC**) was calculated as follows: FC = $2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

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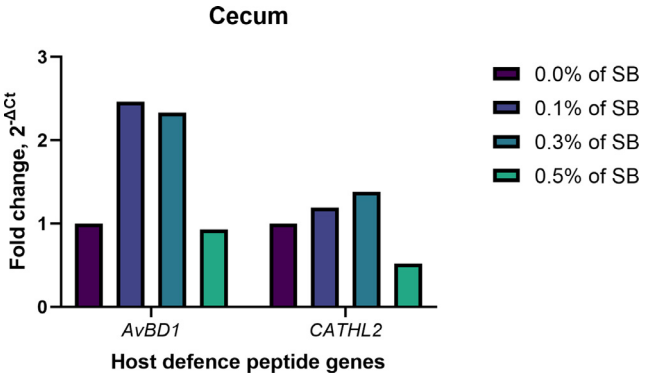

Figure 7. The relative mRNA expression of host defence peptide genes in the cecum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (**SB**). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after *in ovo* stimulation with SB (n = 8). The relative gene expression (FC—fold change) was calculated with the delta delta threshold cycle (ddCt) formula, and the fold change (**FC**) was calculated as follows: $FC = 2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, and 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

prausnitzii abundance. The latter was even slightly lower than in the 0.1% SB group. Probably, at the highest concentration applied, the trophic effect of SB itself was revealed. However, such an effect was not observed in the ileum. Comparison of the effects, which were exerted by SB on the ileal and cecal SCFA and mucosa, suggests a greater specificity of SB toward the latter. This could be explained by higher SB sensitivity of

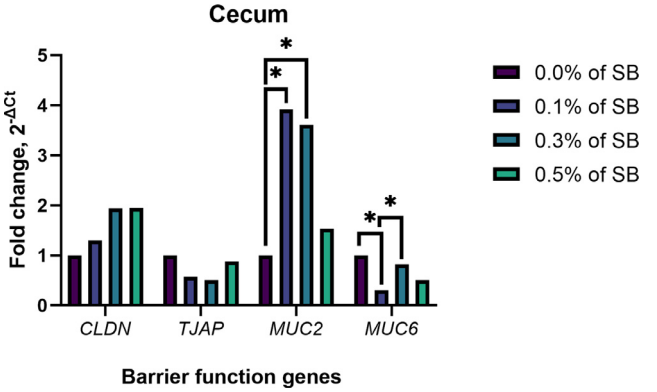

Figure 8. The relative mRNA expression of barrier function genes in the cecum mucosa of ROS 308 chickens stimulated in ovo on d 12 of egg incubation with sodium butyrate (**SB**). The x-axis shows a list of gene targets. The y-axis indicates the relative mRNA abundance of the genes after in ovo stimulation with SB (n = 8). The relative gene expression (FC—fold change) was calculated with the delta delta threshold cycle (ddCt) formula, and the fold change (**FC**) was calculated as follows: $FC = 2^{-\Delta\Delta Ct}$. To assess the impact of different doses of SB (0.0, 0.1, 0.3, and 0.5%) on the kinetics of target genes, one-way ANOVA was employed, followed by multiple intergroup comparisons using the Tukey HSD test. Significant differences ($p < 0.05$) are labeled with an asterisk (*). Calculations were performed based on normalized dCT data. Figures were prepared by using GraphPad Prism 9 (GraphPad, La Jolla, CA).

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Table 5. Intestinal digesta pH and SCFA concentrations in the ileum and cecum of broiler chickens stimulated in ovo with sodium butyrate of different concentrations.

	Sodium butyrate (%)					
Parameter	0	0.1	0.3	0.5	SEM	*P*
Ileum						
pH	7.65	7.81	7.55	7.14	0.137	0.357
Aacetic acid	7.74	9.81	10.99	11.61	0.995	0.550
Propionic acid	0.46	0.64	0.58	0.81	0.073	0.404
Isobutyric acid	0.24	0.3	0.22	0.17	0.027	0.356
Butyric acid	0.16	0.18	0.15	0.11	0.017	0.476
Isovaleric acid	0.21	0.3	0.33	0.38	0.036	0.372
Valeric acid	0.14	0.17	0.11	0.22	0.021	0.343
Total SCFA	8.95	11.42	12.37	13.29	1.148	0.592
Cecum						
pH	7.48	7.32	7.14	7.40	0.062	0.254
Aacetic acid	45.95	58.22	44.71	47.35	2.243	0.121
Propionic acid	11.99[a]	19.00[b]	18.59[b]	12.71[a]	1.060	0.020
Isobutyric acid	1.04	0.98	1.00	1.33	0.076	0.327
Butyric acid	10.58[ab]	12.51[b]	8.92[a]	7.51[a]	0.595	0.014
Isovaleric acid	0.92	0.86	1.08	1.42	0.111	0.280
Valeric acid	0.91	1.21	1.18	0.97	0.050	0.071
Total SCFA	71.38	92.78	75.48	71.30	3.460	0.082

[a,b,c]means in columns marked with different letters differ significantly at P ≤ 0.05; SEM, standard error of the mean.

epithelial and bacterial cells in the cecum. Goblet cells synthesize and secrete mucus, which covers the gastrointestinal epithelium. Mucus is the first line of defence in the gastrointestinal tract and is composed of mucins that are divided into neutral and acidic chemotypes. The latter is further classified into sulfomucins and sialomucins (Deplancke & Gaskins, 2001). It is thought that acidic mucins, particularly sulfomucins, protect against bacterial translocation because they are less degradable by bacteria and host proteases (Deplancke & Gaskins, 2001). This mucin chemotype is abundant in these gut

Table 6. Morphometric characteristics and mucin chemotypes in goblets cells of the ileum and cecum of broiler chickens treated in ovo with sodium butyrate of different concentrations.

	Sodium butyrate (%)					
Parameter	0	0.1	0.3	0.5	SEM	*P*
Ileum						
Villus height, μm	701	805	776	650	25.1	0.112
Villus surface area, mm^2	0.51	0.53	0.61	0.47	0.024	0.202
Crypt depth, μm	198	220	217	198	4.5	0.165
VH/CD	3.58	3.69	3.68	3.33	0.141	0.801
Muscular layer thickness, μm	313	305	296	300	9.2	0.932
AB-PAS staining						
neutral mucins, %	-	-	-	-	-	-
acidic mucin, %	100	100	100	100	-	-
HID-AB staining						
sulfomucins, %	88.6	88.4	85	78.8	2.02	0.310
sialomucins, %	11.4	11.6	15	21.2	2.02	0.310
Cecum						
Crypt depth, μm	387[ab]	425[bc]	337[a]	455[c]	12.2	0.002
Muscular layer thickness, μm	387	376	317	412	13.3	0.072
AB-PAS staining						
neutral mucins, %	-	-	-	-	-	-
acidic mucins, %	100	100	100	100	-	-
HID-AB staining						
sulfomucins, %	98.7	98.9	98.8	98.6	0.39	0.995
sialomucins, %	1.3	1.1	1.2	1.4	0.39	0.995

[a,b,c]means in columns marked with different letters differ significantly at $P \leq 0.05$. VH/CD, villus height to crypt depth ratio; AB-PAS, alcian blue-periodic acid-Schiff's reagent staining; HID-AB, high iron diamine-alcian blue staining.

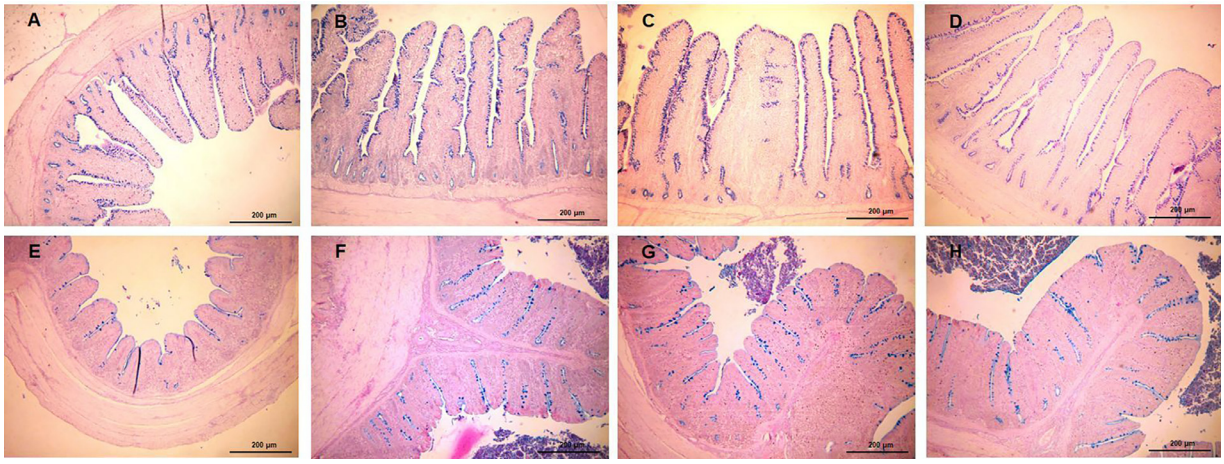

Figure 9. Ileal (A – D) and cecal (E – H) sections of broiler chickens after staining with alcian blue (pH 2.5)-periodic acid-Schiff's reagent technique. A, E – control group; B, F – group treated with 0.1% sodium butyrate; C, G – group treated with 0.3% sodium butyrate; D, H – group treated with 0.5% sodium butyrate. The pictures were taken under the light microscope Zeiss Axio Star Plus (Carl Zeiss, Göttingen, Germany) at 20x objective. In both segments, only goblet cells with acidic mucins (blue-stained) were found. Goblet cells with neutral mucins (magenta-stained) were not found.

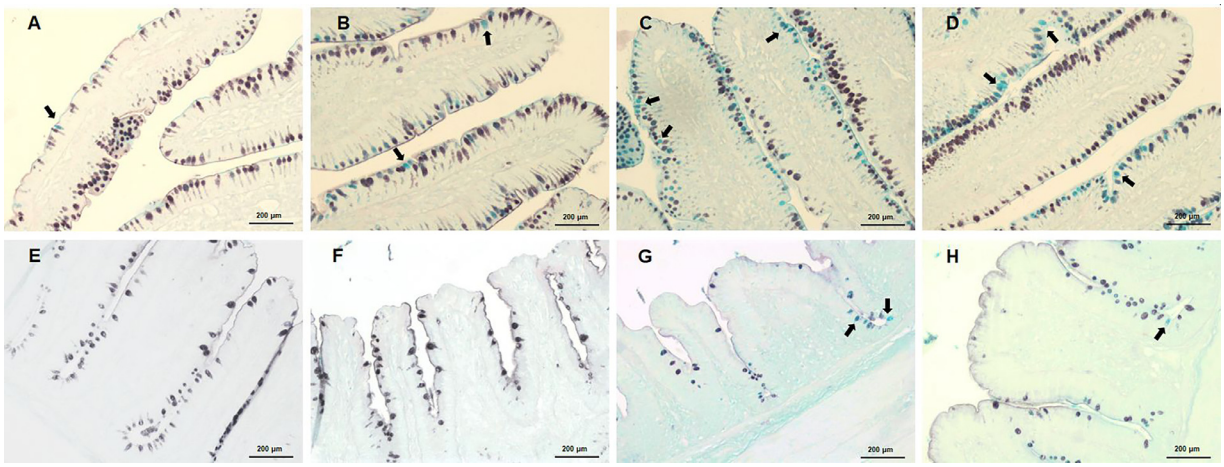

Figure 10. Ileal (**A – D**) and cecal (**E – H**) sections of broiler chickens after staining with high irone diamine-alcian blue (pH 2.5) technique. **A, E** – control group; **B, F** – group treated with 0.1% sodium butyrate; **C, G** – group treated with 0.3% sodium butyrate; **D, H** – group treated with 0.5% sodium butyrate. The pictures were taken using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) at 20x objective. In both segments, goblet cells with sulfomucins (black-stained) predominated. Goblet cells secreting sialomucins (blue-stained) were found only along the ileal villi and were sparsely distributed along the cecal crypts.

regions, which are densely colonized by microbiota. This was also found in the current research, which showed that cells with sulfomucins constituted almost 100% of acidic goblet cells found in the cecal mucosa and 85% in the ileal mucosa of chickens. Goblet cells develop and mature rapidly after hatch in response to environmental factors, intestinal microbiota, and nutritional factors (Duangnumsawang et al., 2021a). The synthesis of sialomucins may indicate goblet cell maturation after hatch. As goblet cells migrate up the villus, the sialylation of mucins increases (Duangnumsawang et al., 2021a), which was also shown in the current research as in the ileum of chickens sialomucins were absent in the crypts and lower parts of villi and started to appear from the mid-height of the villi.

Wu et al. (2018) demonstrated that the use of SB as feed additive (0.2-1 g/kg) increased goblet cell density and mucus secretion in the jejunum and ileum of chickens. The number of acidic goblet cells per villus of the small intestine was also increased by dietary supplementation with 0.5 to 1 g/kg SB (Sikandar et al., 2017). Despite butyrate may regulate differentiation of goblet cells and modulate mucus production (Duangnumsawang et al., 2021a), in the present study, there was no effect on the percentage of different goblet cell chemotypes, even in the cecum of broilers, where butyric acid concentration and crypt depth were increased by 0.1% SB treatment. The lack of SB effect on mucin histochemistry is difficult to explain. It is possible that SB administered in ovo and SB used as feed additive differ in their

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effects on the chicken gut due to the duration of its availability for developing organs and tissues. In-feed administration ensures a constant delivery of SB throughout the 42 d-long production cycle, while in ovo it was administered once during 12 d of incubation. It was sufficient for altering crypt depth but not the mucin chemotypes in goblet cells.

CONCLUSIONS

Based on the results obtained, it can be concluded that the administration of sodium butyrate during embryonic development on the d 12 of egg incubation has supplementation potential in broiler production. Studies have shown that the dose used affects the intestinal response at the level of changes in the intestinal microbiota and changes in the expression of genes related to the immune response and the intestinal barrier. It can be concluded that sodium butyrate administered at a dose of 0.3% in ovo has the greatest potential in this direction.

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DISCLOSURES

The authors declare no conflicts of interest.

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Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Autora rozprawy doktorskiej

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OŚWIADCZENIE

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   b) opracowanie manuskryptu
   c) korekta manuskryptu po recenzjach

2. Aleksandra Bełdowska, Maria Siwek, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Kamil Gawin, Aleksandra Dunisławska, "Impact of *in ovo* administration of xylo-and mannooligosaccharides on broiler chicken gut health", Poultry Science, 2024, 103, 12, 104261, https://doi.org/10.1016/j.psj.2024.104261, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) przeprowadzenie stymulacji *in ovo*
   b) zebranie materiału biologicznego
   c) wykonanie analiz molekularnych
   d) analiza danych
   e) opracowanie statystyczne
   f) przygotowanie manuskryptu
   g) korekta manuskryptu po recenzjach

3. Aleksandra Dunisławska, Aleksandra Bełdowska, Olha Yatsenko, Maria Siwek, "Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens", Poultry Science, 2023, 102, 6, 102663, https://doi.org/10.1016/j.psj.2023.102663, MNiSW: 140, Impact Factor: 4,40.
   Wykonane zadania w ramach artykułu:
   a) pobranie materiału biologicznego
   b) analiza molekularna
   c) analiza i opracowanie danych
   d) przygotowanie manuskryptu
   e) udział w procesie redakcyjnym

---

* *W przypadku prac dwu- lub wieloautorskich wymagane są oświadczenia kandydata do stopnia doktora oraz współautorów, wskazujące na ich merytoryczny wkład w powstanie każdej pracy (np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań – np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie ankiet itp., wykonanie analizy wyników, przygotowanie manuskryptu artykułu i inne). Określenie wkładu danego autora, w tym kandydata do stopnia doktora, powinno być na tyle precyzyjne, aby umożliwić dokładna ocenę jego udziału i roli w powstaniu każdej pracy.*

4. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated in ovo on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) udział w opracowaniu koncepcji doświadczenia
   b) przeprowadzenie stymulacji *in ovo*
   c) pobranie materiału biologicznego
   d) wykonanie analiz molekularnych
   e) analiza danych
   f) opracowanie manuskryptu
   g) korekta manuskryptu po recenzjach

Bydgoszcz 18.10.2024
miejscowość, data

Bełdowska
Podpis Autora rozprawy doktorskiej

Dunisławska
Podpis promotora

Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Współautora

dr hab. inż. Aleksandra Dunisławska, prof. PBŚ
(tytuł zawodowy, imiona i nazwisko współautora)

Politechnika Bydgoska im. Jana i Jędrzeja Śniadeckich, Wydział Hodowli i Biologii Zwierząt, Katedra Biotechnologii i Genetyki Zwierząt, ul. Mazowiecka 28, 85-084 Bydgoszcz
(miejsce pracy/afiliacja)

OŚWIADCZENIE

Oświadczam, iż mój wkład autorski w niżej ~~wymienionym~~/wymienionych ~~artykule~~/artykułach ~~naukowym~~/naukowych był następujący*:

1. Aleksandra Bełdowska, Marcin Barszcz, Aleksandra Dunisławska, "State of the art in research on the gut-liver and gut-brain axis in poultry", Journal of Animal Science and Biotechnology, 2023, 14, 37, https://doi.org/10.1186/s40104-023-00853-0, MNiSW: 200, Impact Factor: 7,00.
   Wykonane zadania w ramach artykułu:
   a) opracowanie koncepcji artykułu
   b) korekta manuskryptu

2. Aleksandra Bełdowska, Maria Siwek, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Kamil Gawin, Aleksandra Dunisławska, "Impact of *in ovo* administration of xylo-and mannooligosaccharides on broiler chicken gut health", Poultry Science, 2024, 103, 12, 104261, https://doi.org/10.1016/j.psj.2024.104261, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) opracowanie koncepcji analiz oraz metodyki doświadczenia
   b) przeprowadzenie stymulacji *in ovo*
   c) pobranie materiału biologicznego
   d) korekta manuskryptu

3. Aleksandra Dunisławska, Aleksandra Bełdowska, Olha Yatsenko, Maria Siwek, "Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens", Poultry Science, 2023, 102, 6, 102663, https://doi.org/10.1016/j.psj.2023.102663, MNiSW: 140, Impact Factor: 4,40.
   Wykonane zadania w ramach artykułu:
   a) opracowanie koncepcji badań
   b) zdobycie finansowania badań
   c) opracowanie metodyczne analiz
   d) analiza danych
   e) udział w opracowaniu manuskryptu oraz w procesie redakcyjnym

4. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated *in*

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ovo on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
Wykonane zadania w ramach artykułu:

a) zdobycie finansowania badań
b) opracowanie koncepcji doświadczenia
c) przepracowanie stymulacji *in ovo*
d) pobranie materiału biologicznego
e) korekta manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wyżej ~~wymienionej~~/wymienionych ~~pracy~~/prac przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Bydgoszcz, 18.10.2024
miejscowość, data

podpis Współautora

Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Współautora

dr inż. Marcin Barszcz
(tytuł zawodowy, imiona i nazwisko współautora)

Instytut Fizjologii i Żywienia Zwierząt im. Jana Kielanowskiego PAN, ul. Instytucka 3, 05-110 Jabłonna
(miejsce pracy/afiliacja)

OŚWIADCZENIE

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   Wykonane zadania w ramach artykułu:
   a) współudział w opracowaniu koncepcji pracy
   b) korekta manuskryptu

2. Aleksandra Bełdowska, Maria Siwek, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Kamil Gawin, Aleksandra Dunisławska, "Impact of in ovo administration of xylo-and mannooligosaccharides on broiler chicken gut health", Poultry Science, 2024, 103, 12, 104261, https://doi.org/10.1016/j.psj.2024.104261, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) udział w pobieraniu prób
   b) przeprowadzenie analizy aktywności beta-glukuronidazy
   c) przeprowadzenie analizy statystycznej danych z analizy SCFA, amoniaku i beta-glukuronidazy, korekta manuskryptu

3. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated in ovo on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) udział w kolekcji prób
   b) przeprowadzenie analizy komórek kubkowych
   c) statystyczna analiza danych biochemicznych i histologicznych
   d) korekta manuskryptu

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Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Jednocześnie wyrażam zgodę na przedłożenie wyżej ~~wymienionej~~/wymienionych ~~pracy~~/prac przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Jabłonna, 17.10.2024 r.
miejscowość, data

podpis Współautora

Z.16.2021.2022

Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Współautora

prof. dr hab. inż. Maria Siwek - Gapińska
(tytuł zawodowy, imiona i nazwisko współautora)

Politechnika Bydgoska im. Jana i Jędrzeja Śniadeckich, Wydział Hodowli i Biologii Zwierząt, Katedra Biotechnologii i Genetyki Zwierząt, ul. Mazowiecka 28, 85-084 Bydgoszcz
(miejsce pracy/afiliacja)

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Oświadczam, iż mój wkład autorski w niżej ~~wymienionym~~/wymienionych ~~artykule~~/artykułach ~~naukowym~~/naukowych był następujący*:

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   Wykonane zadania w ramach artykułu:
   a) opracowanie metodyki doświadczenia
   b) pobranie materiału biologicznego
   c) zdobycie finansowania badań
   d) korekta mansukryptu

2. Aleksandra Dunisławska, Aleksandra Bełdowska, Olha Yatsenko, Maria Siwek, "Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens", Poultry Science, 2023, 102, 6, 102663, https://doi.org/10.1016/j.psj.2023.102663, MNiSW: 140, Impact Factor: 4,40.
   Wykonane zadania w ramach artykułu:
   a) udział w opracowaniu manuskryptu
   b) udostępnienie materiału biologicznego do badań

Jednocześnie wyrażam zgodę na przedłożenie wyżej ~~wymienionej~~/wymienionych ~~pracy~~/prac przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Bydgoszcz 22.10.2024
miejscowość, data

Maria G.
podpis Współautora

* *W przypadku prac dwu- lub wieloautorskich wymagane są oświadczenia kandydata do stopnia doktora oraz współautorów, wskazujące na ich merytoryczny wkład w powstanie każdej pracy (np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań – np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie ankiet itp., wykonanie analizy wyników, przygotowanie manuskryptu artykułu i inne). Określenie wkładu danego autora, w tym kandydata do stopnia doktora, powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Z.16.2021.2022

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Oświadczenie Współautora

dr hab. inż. Jakub Biesek, prof. PBŚ
(tytuł zawodowy, imiona i nazwisko współautora)

Politechnika Bydgoska im. Jana i Jędrzeja Śniadeckich, Wydział Hodowli i Biologii Zwierząt, Katedra Hodowli i Żywienia Zwierząt, ul. Mazowiecka 28, 85-084 Bydgoszcz
(miejsce pracy/afiliacja)

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   Wykonane zadania w ramach artykułu:
   a) przeprowadzenie doświadczenia fermowego
   b) zebranie i analiza danych produkcyjnych

2. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated *in ovo* on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
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Bydgoszcz, 18.10.2024
miejscowość, data

J. Biesek
podpis Współautora

* *W przypadku prac dwu- lub wieloautorskich wymagane są oświadczenia kandydata do stopnia doktora oraz współautorów, wskazujące na ich merytoryczny wkład w powstanie każdej pracy (np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań – np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie ankiet itp., wykonanie analizy wyników, przygotowanie manuskryptu artykułu i inne). Określenie wkładu danego autora, w tym kandydata do stopnia doktora, powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

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Oświadczenie Współautora

dr hab. Anna Tuśnio, profesor instytutu
(tytuł zawodowy, imiona i nazwisko współautora)

Instytut Fizjologii i Żywienia Zwierząt im. Jana Kielanowskiego PAN, ul. Instytucka 3, 05-110 Jabłonna
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   Wykonane zadania w ramach artykułu:
   a) udział w pobieraniu prób
   b) oznaczenie stężenia amoniaku
   c) korekta manuskryptu

2. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated in ovo on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) wykonanie pomiarów histologicznych jelit
   b) korekta manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie wyżej ~~wymienionej~~/wymienionych ~~pracy~~/prac przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Jabłonna, 17.10.2024
miejscowość, data

Anna Tuśnio
podpis Współautora

* *W przypadku prac dwu- lub wieloautorskich wymagane są oświadczenia kandydata do stopnia doktora oraz współautorów, wskazujące na ich merytoryczny wkład w powstanie każdej pracy (np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań – np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie ankiet itp., wykonanie analizy wyników, przygotowanie manuskryptu artykułu i inne). Określenie wkładu danego autora, w tym kandydata do stopnia doktora, powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Z.16.2021.2022

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Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Współautora

mgr Kamil Gawin
(tytuł zawodowy, imiona i nazwisko współautora)

Instytut Fizjologii i Żywienia Zwierząt im. Jana Kielanowskiego PAN, ul. Instytucka 3, 05-110 Jabłonna
(miejsce pracy/afiliacja)

OŚWIADCZENIE

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   Wykonane zadania w ramach artykułu:
   a) udział w pobieraniu prób
   b) oznaczenie stężeń SCFA

2. Aleksandra Bełdowska, Elżbieta Pietrzak, Jakub Biesek, Marcin Barszcz, Anna Tuśnio, Adrianna Konopka, Kamil Gawin, Aleksandra Dunisławska, "The effect of sodium butyrate administrated in ovo on the health status and intestinal response in broiler chicken", Poultry Science, 2024, 103, 10, 104108, https://doi.org/10.1016/j.psj.2024.104108, MNiSW: 140, Impact Factor: 3,80.
   Wykonane zadania w ramach artykułu:
   a) udział w kolekcji prób
   b) wykonanie analizy SCFA

Jednocześnie wyrażam zgodę na przedłożenie wyżej ~~wymienionej~~/wymienionych ~~pracy~~/prac przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Jabłonna, 17.10.2024
miejscowość, data

K.Gawin
podpis Współautora

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Z.16.2021.2022

Załącznik nr 3 do
Instrukcji drukowania, gromadzenia, rejestrowania i udostępniania rozpraw doktorskich przez rady naukowe dyscyplin (dyscyplin artystycznych) prowadzących postępowanie w sprawie nadania stopnia naukowego doktora

Oświadczenie Współautora

dr Olha Yatsenko
(tytuł zawodowy, imiona i nazwisko współautora)

Politechnika Bydgoska im. Jana i Jędrzeja Śniadeckich, Wydział Hodowli i Biologii Zwierząt, Katedra Biotechnologii i Genetyki Zwierząt, ul. Mazowiecka 28, 85-084 Bydgoszcz
(miejsce pracy/afiliacja)

OŚWIADCZENIE

Oświadczam, iż mój wkład autorski w niżej wymienionym/~~wymienionych~~ artykule/~~artykułach~~ naukowym/~~naukowych~~ był następujący*:

1. Aleksandra Dunisławska, Aleksandra Bełdowska, Olha Yatsenko, Maria Siwek, "Effect of prebiotics administered during embryo development on mitochondria in intestinal and immune tissues of adult broiler chickens", Poultry Science, 2023, 102, 6, 102663, https://doi.org/10.1016/j.psj.2023.102663, MNiSW: 140, Impact Factor: 4,40.
   Wykonane zadania w ramach artykułu:
   a) pobranie materiału biologicznego
   b) analiza molekularna

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Bydgoszcz, 18.10.2024
miejscowość, data

Olha Yatsenko
podpis Współautora

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Oświadczenie Współautora

dr inż. Elżbieta Pietrzak
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   Wykonane zadania w ramach artykułu:
   a) przeprowadzenie stymulacji *in ovo*
   b) analiza statystyczna

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej/~~wymienionych~~ pracy/~~prac~~ przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

Bydgoszcz, 18.10.2024
miejscowość, data

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(miejsce pracy/afiliacja)

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   Wykonane zadania w ramach artykułu:
   a) udział w kolekcji prób
   b) wykonanie analizy wskaźników biochemicznych krwi kurcząt

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej/~~wymienionych~~ pracy/~~prac~~ przez mgr inż. Aleksandrę Bełdowską jako część rozprawy doktorskiej opartej na zbiorze opublikowanych i powiązanych tematycznie artykułów naukowych.

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