Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

ROZPRAWA DOKTORSKA

lek. Kamil Bar

Odrębności bakteryjnej mikrobioty dróg oddechowych dzieci chorujących na astmę oskrzelową

Promotor: dr hab. n. med. Barbara Sozańska, prof. UM

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Spis treści

1. WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

Niniejszy cykl obejmuje dwie prace (obie pierwszoautorskie) o łącznym IF : **9,89** oraz punktacji MNiSW : **180pkt**

1. Bar, K.; Żebrowska, P.; Łaczmański, Ł.; Sozańska, B. Airway Bacterial Biodiversity in Exhaled Breath Condensates of Asthmatic Children—Does It Differ from the Healthy Ones? *J. Clin. Med.* **2022**, *11*, 6774. https://doi.org/10.3390/jcm11226774

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2. Bar, K.; Litera-Bar, M.; Sozańska, B. Bacterial Microbiota of Asthmatic Children and Preschool Wheezers' Airways—What Do We Know? *Microorganisms* **2023**, *11*, 1154. https://doi.org/10.3390/microorganisms11051154

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2. STRESZCZENIE

Wstęp: Etiologia astmy oskrzelowej nie została w pełni wyjaśniona. Wyniki badań naukowych ostatnich lat podkreślają znaczenie mikrobiomu w rozwoju i kontroli choroby. Nowe sposoby oznaczania drobnoustrojów przy pomocy metod genetycznych umożliwiły znaczny rozwój badań w tym zakresie, jednak wiedza na temat mikrobioty dolnych dróg oddechowych u dzieci jest bardzo mocno ograniczona.

Cele: Głównymi celami pracy doktorskiej były: 1) ocena różnic pomiędzy mikrobiotą bakteryjną dróg oddechowych dzieci chorujących na astmę oskrzelową w porównaniu do ich zdrowych rówieśników, 2) ocena różnicy składu mikrobioty pomiędzy górnymi a dolnymi drogami oddechowymi, 3) porównanie mikrobioty bakteryjnej dzieci z dotychczas uzyskanymi wynikami w grupie dorosłych, 4) próba oceny zmiany mikrobiomu w zależności od wieku pacjenta, 5) ocena czynników zewnętrznych i środowiskowych mogących wpływać na bakteryjną mikrobiotę dróg oddechowych

Materiał i metody: W badaniu wzięło udział 38 dzieci, 19 astmatyków i 19 zdrowych rówieśników, w wieku 6-17 lat. Do kryteriów wykluczających włączenie do badania należała jakakolwiek infekcja i/lub antybiotykoterapia na 30 dni przed rekrutacją oraz choroby przewlekłe poza astmą i chorobami alergicznymi w grupie astmatyków. Od każdego z pacjentów pobrano wymaz z jamy ustno-gardłowej oraz kondensat powietrza wydychanego, przy pomocy zestawów RTube. Z pobranych materiałów wyizolowano materiał genetyczny bakterii metodą ekstrakcji DNA, a następnie amplifikowano 16S rRNA bakteryjne przy pomocy metody PCR. Uzyskany materiał zostawał poddany sekwencjonowaniu nowej generacji.

Wyniki: Wymazy z jamy ustno-gardłowej charakteryzują się większą alfa-różnorodnością w stosunku do kondensatów powietrza wydychanego. Mikrobiota bakteryjna dolnych dróg oddechowych astmatyków cechuje się większą alfa-różnorodnością w porównaniu do zdrowych rówieśników. Wykazano ujemną korelację wieku z alfa-różnorodnością w materiale z kondensatów powietrza wydychanego. Do czynników wpływających na różnice w materiale dolnych dróg oddechowych astmatyków w kontekście beta-różnorodności należały narażenie na bierne palenie, dodatni wywiad rodzinny w kierunku chorób alergicznych, codzienne przyjmowanie leków przeciwastmatycznych oraz miejsce zamieszkania (miasto/wieś). W przypadku obu części układu oddechowego dominującymi typami bakterii były Firmicutes,

Proteobacteria i Actinobacteriota. W obrębie klas stężenie Gammaproteobacteria i Bacilli było większe w materiale z kondensatów w grupie dzieci zdrowych, niż u astmatyków.

Wnioski :

1. Mikrobiota bakteryjna górnych dróg oddechowych u astmatyków w populacji dziecięcej nie różni się istotnie w stosunku do dzieci zdrowych

2. Mikrobiota bakteryjna w uzyskanych próbkach z dolnych i górnych dróg oddechowych różni się między sobą

3. Bakteryjna mikrobiota dolnych dróg oddechowych astmatyków w populacji dziecięcej charakteryzuje się większą alfa-różnorodnością oraz beta-różnorodnością względem dzieci zdrowych

4. Mikrobiota bakteryjna dolnych dróg oddechowych dzieci urodzonych siłami natury cechuje się większą alfa-różnorodnością od urodzonych drogą cięcia cesarskiego

5. Astmatyków z dodatnim wywiadem rodzinnym w kierunku chorób alergicznych cechuje większa alfa-różnorodność w obrębie kondensatów powietrza wydychanego w stosunku astmatyków bez takiego czynnika

6. Najliczniej prezentowanymi typami bakterii w materiale z górnych i dolnych dróg oddechowych były Firmicutes, Proteobacteria i Actinobacteriota

7. Liczebność klas Gammaproteobacteria i Bacilli była statystycznie niższa w kondensatach powietrza wydychanego u astmatyków w porównaniu do dzieci zdrowych

8. W obrębie kondensatów powietrza wydychanego astmatyków do czynników wpływających na wyodrębnienie różnić w zakresie beta-bioróżnorodności pomiędzy grupami należały ekspozycja na dym tytoniowy, obciążenie rodzinne chorobami alergicznymi, przyjmowanie leków przeciwastmatycznych oraz miejsce zamieszkania (miasto/wieś).

9. Kondensat powietrza wydychanego może stanowić alternatywę dla badań mikrobiomu dolnych dróg oddechowych u dzieci w stosunku do obecnie przyjętych metod inwazyjnych

3. SUMMARY

Introduction: Asthma etiology still remains uncertain. Recent studies have indicated the meaning of microbiome in disease development and control. The new methods of microorganisms detection with genetic methods allowed for a vast increase in microbial studies, however the knowledge about lower airways microbiota among children is very scarce.

Aims: The main aims of the study were: 1) assessment of differences between bacterial microbiota of asthmatic children's airways in comparison to healthy children, 2) assessment of microbiota composition differences between upper and lower airways, 3) comparison of children microbiota with data acquired from studies on adults, 4) assessment of microbiota changes depending on the age of children, 5) assessment of external and environmental factors that can influence bacterial microbiota of airways

Materials and methods: 38 children, 19 asthmatics and 19 healthy peers, aged 6-17 years were enrolled. The exclusion criteria were any infection or/and antibiotic therapy 30 days prior to enrollment and any chronic disease, except asthma and allergic diseases in the asthmatic group. From each participant, oropharyngeal swab and exhaled breath condensate, with use of RTube kit, were acquired. From samples bacterial DNA was extracted, followed by bacterial 16S rRNA PCR amplification. Acquired material has undergone new generation sequencing.

Results: Oropharyngeal swabs are characterized by higher alpha-diversity than exhaled breath condensates. The bacterial microbiota of asthmatics' lower airways has higher alpha-diversity than that of healthy peers. Age and alpha-diversity have been negatively correlated in exhaled breath condensates samples. Factors influencing differences in asthmatics' lower airways material were second-hand smoking, family burden of allergic diseases, asthma medications daily intake and place of living (village/town). In both airways samples dominant phyla were Firmicutes, Proteobacteria and Actinobacteria. Class Gammaproteobacteria and Bacilli higher abundance were found among the healthy group, compared to asthmatics.

Conclusions :

1. Bacterial microbiota of pediatric asthmatics upper airways does not alter compared to healthy peers
2. Bacterial microbiota of upper and lower airways samples does differ
3. Bacterial microbiota of pediatric asthmatics lower airways is characterized by higher alpha-diversity and beta-diversity, compared to healthy group
4. Bacterial microbiota of children born naturally lower airways is characterized by higher alpha-diversity than those born by cesarean section
5. Asthmatics with a family burden of allergic diseases have higher alpha-diversity in ehxaled breath condensates compared to asthmatics without such a burden
6. The most abundant phyla in both upper and lower airways were Firmicutes, Proteobacteria and Actinobacteriota
7. Class Gammaproteobacteria and Bacilli abundance was statistically lower in exhaled breath condensates of asthmatics compared to healthy children
8. Factors that allowed for distinguishing differences in beta-diversity of asthmatics exhaled breath condensates were second-hand smoking, the burden of allergic diseases, asthma medications intake and place of living (town/village)
9. Exhaled breath condensate can be an alternative to invasive methods of lower airways microbiome assessment

4. WSTĘP

Astma to choroba o niejednorodnej etiologii związana z przewlekłym stanem zapalnym w obrębie dróg oddechowych [1]. Według definicji GINA charakteryzuje się upośledzeniem przepływu wydechowego powietrza z towarzyszącymi objawami, takimi jak duszność, kaszel, czy ból w klatce piersiowej [1]. Jest to najczęstsza przewlekła choroba dolnych dróg oddechowych u dzieci [2]. Obecnie wskazuje się na znaczący udział predyspozycji genetycznej do rozwoju choroby, a także zmian epigenetycznych w odpowiedzi na warunki środowiskowe [3]. Do czynników zewnętrznych i środowiskowych ryzyka rozwoju astmy można zaliczyć otyłość w trakcie ciąży u matki, narażenie na bierne palenie, poród drogą cięcia cesarskiego, poród przedwczesny, niską masę urodzeniową, antybiotykoterapię w pierwszym roku życia, infekcję o etiologii RSV we wczesnym dzieciństwie, zanieczyszczenie powietrza [4]. Choroba ta jest nierozerwalnie związana z mikroorganizmami, jako że najczęstszą przyczyną jej zaostrzenia są infekcje, głównie wirusowe [5]. Obecnie postuluje się o znacznie szerszym oddziaływaniu szeroko pojętego mikrobiomu względem etiologii i przebiegu astmy oskrzelowej [6,7].

Rozwój nauki przyczynił się do udoskonalenia metod oznaczania drobnoustrojów w organizmie ludzkim. Nadal szeroko stosowane metody klasycznej hodowli bakteriologicznej ustępują obecnie miejsca testom antygenowym oraz izolacji materiału genetycznego przy pomocy amplifikacji metodą PCR. Obecnie szeroko stosowanymi w literaturze metodami klasyfikacji bakterii jest taksonomiczna jednostka operacyjna (OTU – Operational Taxonomic Unit). Według definicji stanowi ona grupę organizmów ze zbliżoną sekwencją DNA w obrębie charakterystycznego genu markerowego w obrębie 16S rRNA [8]. Na podstawie przeprowadzonych badań przyjęto, że podobieństwo kodu rybosomalnego RNA w co najmniej 97% pozwala na przypisanie organizmów do wspólnego klasteru [9].

Powyższe udoskonalenia pozwoliły na znacznie dokładniejsze rozpoczęcie zgłębiania ludzkiego mikrobiomu. Według definicji zaproponowanej przez Lederberga, mikrobiom jest to ogół mikroorganizmów symbiotycznych, komensalnych i patogennych, zamieszkujących dane siedlisko [10]. Niektóre definicje traktują mikrobiom jako szersze pojęcie, odnoszący się do całości genomu drobnoustrojów, w tym jego metabolitów, wyróżniając dla różnicowania pojęcie mikrobioty jako zespołu organizmów [11].

Przez wiele lat dolne drogi oddechowe uważano za sterylne [12]. Pionierską pracą w zakresie oceny bakteryjnej mikrobioty dróg oddechowych została opublikowana w 2010 roku [13]. Wykazano wówczas istotne statystycznie różnice pomiędzy mikrobiotą górnego i dolnego piętra układu oddechowego u astmatyków w stosunku do osób zdrowych oraz potwierdzono obecność bakterii w znaczącym mianie w materiale z oskrzeli [13]. Od tego czasu powstało wiele prac na temat flory bakteryjnej układu oddechowego u astmatyków, jednakże nie udało się do tej pory jednoznacznie odpowiedzieć w jaki sposób te mikroorganizmy wpływają na rozwój choroby, jej fenotyp, czy też odpowiedź na leczenie. Szczególną uwagę zwraca fakt małej ilości prac na temat mikrobiomu dolnych dróg oddechowych u dzieci, ze względu na inwazyjność procedur pobierania materiału; głównymi są wymazy szczoteczkowe i popłuczyny oskrzelowo-płucne, uzyskiwane w ramach bronchoskopii [14]. Z metod nieinwazyjnych badacze wybierają indukowaną plwocinę, jednakże badania porównawcze wskazują na duże rozbieżności wyników tego materiału względem pobieranych w sposób inwazyjny w populacji pediatrycznej [15].

Kondensat powietrza wydychanego (exhaled breath condensate, EBC) stanowi alternatywę dla pozyskiwania materiału z dolnych dróg oddechowych w sposób nieinwazyjny. Według definicji European Respiratory Society i American Thoracic Society, jest to płyn lub zamrożony materiał pozyskiwany przez chłodzenie wydychanego powietrza w kontakcie z chłodną powierzchnią lub kondensatorem [16]. Kondensat składa się w zdecydowanej większości z wody, a obecnie w niej rozpuszczone związki oraz cząsteczki nierozpuszczalne są szeroko stosowane w badaniach na temat stanu zapalnego i powiązanych z nimi związków, w tym cytokin [17]. W ramach dostępnej literatury przed rozpoczęciem badania dostępne były dane odnośnie izolacji z powodzeniem materiału do oznaczeń taksonomicznych na modelu zwierzęcym [18] oraz badania odnośnie bakteryjnego mikrobiomu [19,20] i mykobiomu u osób dorosłych [21,22], co skłoniło nas do wykorzystania tej metody. Niestety nadal brakuje wytycznych odnośnie ujednolicenia procedur oznaczania DNA w tego typu próbkach.

W ramach mojej wcześniejszej działalności naukowej byłem również współautorem pracy poglądowej na temat kondensatów powietrza wydychanego, ich składu i możliwości wykorzystywania w badaniach dotyczących astmy oskrzelowej [17].

5. CELE I ZAŁOŻENIA PRACY

5.1. Praca oryginalna

Celami pracy oryginalnej były odpowiedzi na poniższe hipotezy badawcze:

- Czy istnieją różnice w składzie ilościowym oraz jakościowym bakterii dróg oddechowych u dzieci chorujących na astmę oskrzelową w porównaniu do ich zdrowych rówieśników?

- Czy istnieją istotne różnice pomiędzy bakteryjną mikroflorą górnych i dolnych dróg oddechowych w populacji dziecięcej?

- Czy bakteryjny mikrobiom dróg oddechowych populacji pediatrycznej różni się istotnie od populacji osób dorosłych (na podstawie porównania wyników badań własnych i piśmiennictwa dotyczącego populacji dorosłych)?

- Czy bakteryjny mikrobiom dróg oddechowych zmienia się w zależności od wieku pacjenta pediatrycznego?

- Czy można wyodrębnić czynniki zewnętrzne i uwarunkowania środowiskowe, które wpływają na różnice w bakteryjnym mikrobiomie dróg oddechowych w badanej populacji dziecięcej?

5.2. Praca poglądowa

Celem pracy poglądowej było :

- usystematyzowanie dotychczasowej wiedzy o mikrobiomie bakteryjnym górnych oraz dolnych dróg oddechowych w populacji pediatrycznej astmatyków

- określenie głównych różnic w zakresie taksonomicznym pomiędzy dziećmi chorującymi na astmę a zdrowymi rówieśnikami/dziećmi z innymi chorobami układu oddechowego

- podkreślenie głównych implikacji klinicznych powyższych prac, w tym próba poszukiwania poszczególnych typów/gatunków bakterii odpowiedzialnych zarówno za protekcyjny charakter względem rozwoju astmy oskrzelowej, jak i odpowiedzialnych za zaostrzenia choroby

- naświetlenie potrzeby dalszych badań odnośnie mikrobiomu bakteryjnego, szczególnie dolnych dróg oddechowych w populacji dziecięcej astmatyków z racji ograniczonej ilości danych na chwilę obecną

6. MATERIAŁY I METODY

Szczegółowy opis metodologiczny zawarty jest w pracy oryginalnej, wchodzącej w skład przewodu doktorskiego.

6.1. Materiał

Grupę badaną stanowiło 19 dzieci w wieku 6-17 lat (13 chłopców, 6 dziewczynek), z rozpoznaną w przeszłości przez alergologa astmą oskrzelową. Pacjenci rekrutowani byli w ramach przyklinicznej poradni alergologicznej dla dzieci Oddziału Alergologii Dziecięcej Uniwersyteckiego Szpitala Klinicznego we Wrocławiu. Grupę kontrolną stanowiły dzieci w tym samym przedziale wiekowym, bez chorób towarzyszących (12 dziewczynek, 7 chłopców). Kryteriami wykluczającymi udział w badaniu były: jakakolwiek infekcja i/lub antybiotykoterapia do 30 dni przed włączeniem do badania oraz jakakolwiek choroba przewlekła, poza astmą oskrzelową, alergicznym nieżytem nosa, atopowym zapaleniem skóry oraz alergicznym zapaleniem spojówek w grupie badanej. Spośród pacjentów w grupie badanej 89,47% (n=17) chorowało na astmę atopową, potwierdzoną uczuleniami wykazanymi w testach skórnych lub specyficznymi IgE dla danych alergenów w surowicy krwi. Wszyscy pacjenci charakteryzowali się dobrą kontrolą astmy w momencie rekrutacji, 68,4% (n=13) przyjmowało leki przeciwastmatyczne, z czego 53,63% (n=10) codzienne inhalacje z glikokortykosteroidów.

Badanie uzyskało zgodę Komisji Bioetycznej przy Uniwersytecie Medycznym we Wrocławiu KB-175/19 dnia 08.01.2019r. (Załącznik 11.3)

Badanie zostało częściowo sfinansowane przy pomocy stypendium Projektu dla Młodych Naukowców Uniwersytetu Medycznego we Wrocławiu, nr STM.A220.20.057.

6.2. Metody badawcze

Uczestników badania rekrutowano w okresie od kwietnia 2019r. do sierpnia 2020r. Uczestnik badania oraz jego rodzice/przedstawiciele ustawowi zostali szczegółowo poinformowani o badaniu, wyrazili świadomą, pisemną zgodę na udział w nim. Każde dziecko zostało przeze mnie szczegółowo zbadane przedmiotowo przed przystąpieniem do pobierania materiału celem wykluczenia ewentualnych cech obecnej infekcji lub zdrowotnych przeciwwskazań do uczestnictwa w niniejszym badaniu. Dodatkowo rodzic pacjenta/przedstawiciel ustawowy był proszony o wypełnienie autorskiej ankiety odnośnie

informacji o ogólnym stanie zdrowia, warunkach socjalnych i rodzinnego narażenia na choroby alergiczne (załącznik 11.3).

Pierwszym materiałem pobieranym od uczestnika badania był wymaz z jamy ustno-gardłowego za pomocą sterylnych wymazówek bez podłoża transportowego. Przed wymazem dziecko samodzielnie przepłukiwało jamę ustną butelkowaną wodą mineralną, żeby usunąć z niej naddatek bakteryjny, następnie w pozycji siedzącej pobierano wymaz z tylnej ściany gardła, ze zwróceniem szczególnej uwagi na niedotknięcie pozostałych fragmentów jamy ustno-gardłowej. Próbka była niezwłocznie transportowana do zamrażarki o temperaturze -20°C. Następnie proszono uczestnika o przepłukanie jamy ustnej i gardła 5% roztworem dwutlenku sodu celem usunięcia tła bakteryjnego w obrębie górnych dróg oddechowych jako potencjalnego zanieczyszczenia kolejnego pobieranego materiału. Celem pobrania materiału z dolnych dróg oddechowych wykorzystano kondensaty powietrza wydychanego. W ramach naszej pracy użyto jednorazowych zestawów RTube (Respiratory Research, Inc., Stany Zjednoczone), które składają się z plastikowej tuby do kolekcji materiału, ustnika z jednostronną zastawką przepuszczającą powietrze wydychane oraz zbiornikiem na ślinę oraz z powłoki chłodzącej wraz z termoizolacyjnym rękawem. Uczestnik badania był proszony o swobodne oddychanie przez usta w czasie 10 minut, w pozycji siedzącej, z klipsem na nosie; materiał chłodzący przed pobraniem osiągał temperaturę -30°C. Po pobraniu kondensat powietrza wydychanego był zamykany gumowymi zabezpieczeniami w tubie do kolekcji (w zestawie zapewnianym przez producenta) i niezwłocznie transportowany do zamrażarki o temperaturze -20°C.

Oba typy materiałów były przechowywane w niezmienionych warunkach do czasu analizy laboratoryjnej.

Celem oceny mikrobioty bakteryjnej z pobranych próbek oznaczano regiony V3-V4 16s rRNA. Ze względu na niewystarczającą ilość materiału genetycznego w niektórych próbkach, finalnie analizie poddano w grupie badanej 19 kondensatów powietrza wydychanego oraz 12 wymazów z jamy ustno-gardłowej, w grupie kontrolnej 14 kondensatów i 14 wymazów.

Całość analizy laboratoryjnej przeprowadzono w Laboratorium Genomiki i Bioinformatyki Instytutu Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk we Wrocławiu. W przypadkach obu typów próbek przeprowadzono następujące etapy : ekstrakcję bakteryjnego

DNA, przygotowanie bibliotek DNA do sekwencjonowania, amplifikację materiału genetycznego metodą PCR, sekwencjonowanie uzyskanego materiału.

Początkowy etap analizy laboratoryjnej obejmował ekstrakcję bakteryjnego DNA przy użyciu QIAamp Power Fecal Pro DNA Kit (QIAGEN, Niemcy). W przypadku wymazów z nosogardła ekstrakcja przebiegała zgodnie z zaleceniami producenta, z wyjątkiem zamiany procesu z użyciem kulek homogenizujących na odwirowanie materiału w roztworze 800µg buforu lizy (CD1) i następczej inkubacji przez 30 minut w temperaturze pokojowej. Dla kondensatów powietrza wydychanego pierwszym etapem było dodanie 600µl roztworu wysoko stężonej soli CD3 do próbki o objętości średnio 700µl. Następne kroki wykonano zgodnie z zaleceniami producenta.

Kolejnym etapem było przygotowanie bibliotek DNA do sekwencjonowania. Materiał z wymazów z jamy ustno-gardłowej został przygotowany zgodnie z zaleceniami producenta, przy użyciu QIAseq 16S/ITS Region Panel (QIAGEN, Niemcy), swoistego dla rejonu zmiennego V3V4 16S rRNA. Stężenie przygotowanych bibliotek zmierzono przy pomocy Quantus Fluometer (Promega Corporation, Stany Zjednoczone) i przetestowano przy użyciu TapeStation4200 (Agilent Technologies, Stany Zjednoczone) oraz High Sensitivity D1000ScreenTape (Agilent Technologies, Stany Zjednoczone). Znormalizowane biblioteki zostały połączone w tej samej objętości, celem wygenerowania odpowiedniej ilości surowych odczytów przy pomocy każdej z bibliotek. Ostateczny roztwór został rozcieńczony do stężenia 10pM i użyty do sekwencjonowania.

Powyższy sposób przygotowania bibliotek dla kondensatów powietrza wydychanego zawiódł, w związku z czym użyto autorskiego schematu. Do amplifikacji metodą PCR użyto zestawu KAPA HiFi HotStart ReadyMix PCR (Roche, Szwajcaria). Szczegółowy opis zastosowanej metody znajduje się w publikacji oryginalnej. Jakość przygotowanych bibliotek została sprawdzona przy pomocy TapeStation4200 (Agilent Technologies, Inc., Stany Zjednoczone) oraz High Sensivity D1000 Screen Tape (Agilent Technologies, Inc., Stany Zjednoczone). Znormalizowane biblioteki zostały umieszczone w takiej samej objętości. Ostatecznie biblioteki zostały rozcieńczone w stężeniu 6pM i użyte do sekwencjonowania.

Następnie wykonano sekwencjonowanie obu materiałów. Dla wymazów z jamy ustno-gardłowej użyto Illumina MiSeq, kartridż v3, 600 cykli, komórka przepływowa V3, zgodnie z

zaleceniami producenta. Dla kondensatów powietrza wydychanego użyto Illumina MiSeq, kartridż v2 500 cykli, komórka przepływowa v2 nano, zgodnie z zaleceniami producenta.

Analiza bioinformatyczna została wykonana przy użyciu QIIME2 2021.8 i dodatkowych wtyczek, uwzględniające inne metody analizy mikrobiomu. Wtyczka różnorodności q2 została wykorzystana do oszacowania różnorodności alfa : wskaźnika różnorodności Shannona, Observed Features, różnorodności filogenetycznej (Faith) i wskaźnika równomierności (Pielou) oraz różnorodności beta : indeksu Jaccarda, odległości Braya-Curtisa, ważonego wskaźnika UniFrac, nieważonego wskaźnika UniFrac, a także przeprowadzono analizę głównych składowych (PCoA). Test PERMANOVA przeprowadzono w celu przetestowania istotności statystycznej beta-różnorodności, test Kruskala-Wallisa pod kątem istotności statystycznej alfa-różnorodności oraz współczynnik korelacji rang Spearmana dla kolumn metadanych z prób ciągłych, wartość $p < 0,05$ uznano za statystycznie istotną. Naiwny klasyfikator bayesowski został użyty do przypisania taksonomii do wariantów sekwencji amplikonu (ASV). Do oceny różnic pomiędzy mikrobiomami poszczególnych grup uczestników użyto również metody ANCOM (analysis of composition of microbiomes). Szczegółowy opis wszystkich użytych wtyczek znajduje się w pracy oryginalnej.

7. PUBLIKACJE CYKLU DOKTORSKIEGO

7.1. Praca oryginalna

Pierwszy z artykułów jest pracą oryginalną, której materiał i metody zostały szczegółowo opisane w poprzednim rozdziale.

Mikrobiota bakteryjna wymazów jamy ustno-gardłowej (n=26) charakteryzowała się większą alfa-różnorodnością względem ilości odmiennych typów bakterii (wskaźnik Shannona $p=9{,}108x10^{-8}$), niż ta z kondensatów powietrza wydychanego (n=33). Oba materiały stanowiły również osobne nisze bakteryjne względem beta-różnorodności (Jaccard, Bray-Curtis, ważony i nieważony UniFrac, we wszystkich przypadkach p=0,001, PERMANOVA 999 permutacji).

Biorąc pod uwagę materiał uzyskany od samych astmatyków, w wymazach (n = 12) w porównaniu do EBC (n = 19) obserwowano większą obfitość bakteryjną we wskaźniku różnorodności Shannona ($p = 1{,}907 \times 10^{-4}$) i Observed Features ($p = 3{,}651 \times 10^{-6}$), a także różnorodności filogenetycznej (Faith, $p = 3{,}777^{-6}$); nie zaobserwowano takiej zależności, wykorzystując wskaźnik równomierności Pielou (p = 0,068). Ekologiczne dystanse różniły się pomiędzy wymazami z jamy-ustno gardłowej (n = 12) i EBC (n = 19) u astmatyków względem beta-różnorodności (wszystkie użyte wskaźniki p=0,001, PERMANOVA 999 permutacji). W odniesieniu do samej grupy kontrolnej, wymazy (n = 14) charakteryzowały się większą alfa-różnorodnością (wskaźnik różnorodności Shannona, $p = 1{,}648 \times 10^{-4}$, Observed Features $p = 6{,}607 \times 10^{-6}$, różnorodność filogenetyczna Faith, $p = 1{,}927 \times 10^{-5}$), w porównaniu do kondensatów (n = 14). W tym przypadku również oba materiały stanowiły również osobne nisze bakteryjne względem beta-różnorodności (Jaccard, Bray-Curtis, ważony i nieważony UniFrac, we wszystkich przypadkach p=0,001, PERMANOVA 999 permutacji).

Znaleziono statystycznie istotne różnice w alfa-różnorodności kondensatów u astmatyków, w stosunku do dzieci zdrowych. Mikrobiota dolnych dróg oddechowych astmatyków cechowała się większą obfitością bakteryjną (wskaźnik różnorodności Shannona, p=0,026) i bardziej równą dystrybucją bakterii w udziale całej niszy (wskaźnik równomierności Pielou, p=0,002). Beta-różnorodność wykazała, że obie grupy różniły się istotnie względem siebie (Jaccard, p=0,012, Bray-Curtis p=0,038, ważony UniFrac p=0,043,

PERMANOVA test, 999 permutacji). Wykazano ujemną korelację wieku z alfa-różnorodnością w EBC; im starsze dziecko, tym mniejsza obfitość i równomierność rozkładu bakterii w grupie (wskaźnik Shannona, r=0,357, p=0,041; równomierność Pielou, r=0,378, p=0,03; r - współczynnik korelacji rang Spearmana). Dodatkowo wykazano różnice w alfa- i beta-różnorodności w zależności od drogi porodu w materiale z kondensatów. Dzieci urodzone siłami natury (n=17) charakteryzowały się większą różnorodnością gatunkową (Shannon p=0,002, Faith p=0,003) i bardziej równomiernym rozkładem bakteryjnym (Pielou p=0,003), niż te urodzone poprzez cięcie cesarskie (n=16). Na zwiększenie alfa-różnorodności w próbkach z dolnych dróg oddechowych u astmatyków miały wpływ również dodatni wywiad rodzinny w kierunku chorób alergicznych (dodatni n=14, ujemny n=4), alergie wziewne (z alergiami wziewnymi n=17, bez n=2), jednak ze względu na duże dysproporcje w obrębie grup uwzględniających badane parametry należy liczyć się z możliwym zafałszowaniem wyniku. Dodatkowymi czynnikami, które różnicowały nisze mikrobioty kondensatów w beta-różnorodności, były : narażenie na bierne palenie (narażeni n=7, bez narażenia n=12), dodatni wywiad rodzinny w kierunku chorób alergicznych (dodatni n=14, ujemny n=4), przyjmowane na co dzień leki przeciwastmatyczne (tak n=14, nie n=5) i miejsce zamieszkania (miasto n=11, wieś n=8). W grupie kontrolnej dzieci urodzone siłami natury (n=4) charakteryzowały się bardziej zróżnicowaną mikrobiotą, niż urodzone drogą cięcia cesarskiego (n=10). Nie wykazano takiej zależności dla astmatyków, natomiast grupa ta charakteryzowała się odwrotnymi proporcjami (poród siłami natury n=13, drogą cięcia cesarskiego n=6).

Porównując materiał z wymazów z jamy ustno-gardłowej, nie znaleziono żadnych istotnie statystycznych różnic pomiędzy astmatykami a grupą kontrolną dzieci zdrowych.

Celem oceny dominujących klas bakteryjnych, analizowano te, których bakterie obecne były w co najmniej 50% danego typu próbki i stanowiły powyżej 1% średniej relatywnej liczebności. Dla kondensatów powietrza wydychanego były to : Bacilli, Gammaproteobacteria, Alphaproteobacteria i Actinobacteria, obecne w 100% próbek. Dla wymazów dominujące klasy przedstawiały się następująco : Bacilli, Gammaproteobacteria, Bacteroidia, Fusobacteriia, Actinobacteria, Negativicutes, Clostridia, Campylobacteria.

W odniesieniu do typów bakterii, w przypadku obu pięter układu oddechowego najbardziej liczne były kolejno Firmicutes, Proteobacteria i Actinobacteriota. Przy pomocy

metody ANCOM wykryto, że w obrębie klas stężenie Gammaproteobacteria i Bacilli było większe w materiale z EBC w grupie dzieci zdrowych, niż u astmatyków (W=1, obie klasy).

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Airway Bacterial Biodiversity in Exhaled Breath Condensates of Asthmatic Children—Does It Differ from the Healthy Ones?

Kamil Bar [1,*], Paulina Żebrowska [2], Łukasz Łaczmański [2] and Barbara Sozańska [1]

[1] 1st Department and Clinic of Paediatrics, Allergology and Cardiology, Wroclaw Medical University, 50-367 Wroclaw, Poland
[2] Laboratory of Genomics and Bioinformatics, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland
* Correspondence: kamilbarmd@gmail.com

Abstract: Asthma etiopathology is still not fully determined. One of its possible causes can be found in airway microbiome dysbiosis. The study's purpose was to determine whether there are any significant differences in the bacterial microbiome diversity of lower airways microbiota of asthmatic children, since knowledge of this topic is very scarce. To the authors' knowledge, this is the first research using exhaled breath condensates in children's lower airways for bacterial assessment. Exhaled breath condensates (EBC) and oropharyngeal swabs were obtained from pediatric asthmatic patients and a healthy group (n = 38, 19 vs. 19). The microbial assessment was conducted through genetic material PCR amplification, followed by bacterial 16S rRNA amplicon sequencing. Collected data were analyzed, in terms of taxonomy and alpha and beta diversity between assessed groups. Swab samples are characterized by higher species richness compared to exhaled breath condensates (Shannon diversity index (mean 4.11 vs. 2.867, $p = 9.108 \times 10^{-8}$), observed features (mean 77.4 vs. 17.3, $p = 5.572 \times 10^{-11}$), and Faith's phylogenetic diversity (mean 7.686 vs. 3.280 $p = 1.296 \times 10^{-10}$)). Asthmatic children had a higher abundance of bacterial species (Shannon diversity index, mean 3.029 vs. 2.642, $p = 0.026$) but more even distribution (Pielou's evenness, mean 0.742 vs. 0.648, $p = 0.002$) in EBC than healthy ones; the same results were observed within pediatric patients born naturally within EBC samples. In children with a positive family history of allergic diseases, alpha diversity of lower airway material was increased (Shannon's diversity index $p = 0.026$, Faith's phylogenetic diversity $p = 0.011$, observed features $p = 0.003$). Class Gammaproteobacteria and Bacilli were less abundant among asthmatics in the exhaled breath samples. The most dominant bacteria on a phylum level in both sample types were Firmicutes, followed by Proteobacteria and Actinobacteriota. The obtained outcome of higher bacterial diversity of lower airways among asthmatic patients indicates a further need for future studies of microbiota connection with disease pathogenesis.

Keywords: asthma; microbiome; exhaled breath condensate

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1. Introduction

Asthma is a heterogeneous disease of which etiopathology is not yet fully determined [1]. As defined by The Global Initiative for Asthma, the chronic airway inflammation causes variable expiratory airflow limitations that can provoke symptoms such as wheezes, shortness of breath, chest tightness, and cough [2]. It is the most frequent chronic lung disease among the pediatric population and its, and other allergic diseases, morbidity is still rising, especially in developing countries [3]. One of the possible causes is to be found in the biodiversity hypothesis. The lack of frequent contact with various environmental microbes, typical for modern civilization, disturb enriching the human microbiome and, by that, a proper immunological response, thus promoting inflammation and allergic reactions [4]. On the other hand, certain pathogens are known as asthma triggers, such as RSV and rhinovirus infection [5]. Latest asthma prevention interventions promote natural

colonization and advice to reduce iatrogenic factors that may lead to early life dysbiosis, underlying the impact of microbiota in asthma pathogenesis [6].

For many years, lower airways were thought to be sterile because standard cultures did not show any significant growth of pathogens in healthy people [7]. The improvement of microorganism detection through culture-free identification methods, such as high-throughput sequencing, declined that thesis, showing that the bronchial microbiome exists. Studies on adults have proven that, in asthmatic patients, there are qualitative and quantitative differences in the bacterial composition of the lower airway, especially a higher abundance of Proteobacteria, with a lower abundance of Bacteroides and Firmicutes [8].

Exhaled breath condensate (EBC) is a new potential material for the detection of bacteria in lower airways in children. It is a non-invasive method in which a sample is obtained from tidal breathing through a cooled collection device, on which surface droplets of airway water condense. Its usefulness in asthma studies has been widely proven in search of inflammation/metabolomic changes, but as for microbial studies, no specific recommendations were proposed [9].

In terms of pediatric patients, the data about upper respiratory tract microbiota and its impact on asthma is a topic of many studies, whereas knowledge of lower airways microbiome is limited, due to the invasive form of specimen collection during bronchoscopy, which, in most cases, requires anesthesia (protected brush specimen/bronchoalveolar lavage), and the noninvasive one—induced sputum—is not sensitive enough [10]. There are only a few studies to date that investigated both the upper and lower respiratory tracts in children and, due to small groups of participants, there are no strong conclusions yet to be found about their mutual relevance in children's asthma.

To further investigate the biodiversity of upper and lower airways among children with asthma, in our study, we collected exhaled breath condensates and oropharyngeal swabs for lower and upper airways bacterial abundance and composition comparison. We aimed to assess if there are any significant differences between asthmatic and non-asthmatic children and between upper and lower respiratory tracts, as well as if there are any additional factors connected with airway bacterial abundance in asthmatic and non-asthmatic pediatric patients.

2. Materials and Methods

2.1. Study Participants

Our research was planned as a case-control study. The study group comprised of patients randomly recruited in an Allergology Outpatient Clinic of 1st Department of Pediatrics, Allergology, and Cardiology at the WroclawMedical University, Poland. A total of 19 children aged 6–17 with asthma diagnosed by an allergologist were enrolled in a study group, whereas the control group consisted of 19 healthy children within the same age range (Table 1). The exclusion criteria for both groups were: any infection and antibiotic treatment 30 days prior to the enrollment and any serious chronic diseases (besides asthma, allergic rhinitis, atopic dermatitis, and allergic conjunctivitis in the study group). All patients' asthma were assessed as well controlled by the time of the study, 68.4% (n = 13) were treated with asthma medications daily, of which, inhaled corticosteroids daily intake patients were 52.63% (n = 10). A total of 89.47% (n = 17) asthmatics were characterized as atopic asthma, with sIgE/SPT proven inhalant allergy. The Human Investigation Ethics Committees at the Wroclaw Medical University approved the study. Written informed consent was obtained from all patients prior to inclusion. All participants were asked to fulfill an authorial questionnaire, regarding their general social status and health.

Table 1. Study group characteristics.

	Asthmatic Group $n = 19$	Healthy Control $n = 19$
Swab collection	12 (63.16%)	14 (73.68%)
EBC † collection	19 (100%)	14 (73.68%)
Sex (women/men)	6 (31.58%)/13 (68.42%)	12 (63.16%)/7 (36.84%)
Mean age (years)	10.3	12
Place of living (town/village)	11 (57.89%)/8 (42.11%)	10 (52.63%)/9 (47.37%)
Overweight (>85 centile of BMI)	3 (15.79%)	3 (15.79%)
Underweight (<3 centile of BMI)	1 (5.26%)	1 (5.26%)
Asthma medications daily intake/Inhaled corticosteroids (ICS) treatment on a daily basis	14 (73.68%)/10 (52.63%)	none
Natural birth/cesarean section (cc)	13 (68.42%)/6 (31.58%)	7 (36.84%)/12 (63.16%)
Inhalant allergies	17 (89.47%)	none
Family history of allergic diseases	14 (73.68%)	none
Active smokers within household	7 (36.84%)	4 (21.05%)

† EBC–exhaled breath condensate

2.2. Sample Collection

From all of the participants', oropharyngeal swabs and exhaled breath condensates were obtained. Firstly, a child was asked for a quick mouthwash with a bottled mineral water to rinse additional bacterial flora from oral cavity epithelium, then, in a sitting position, a swab was taken from a posterior wall of pharynx with sterile, non-cotton swabs without transport medium. Secondly, a participant was asked for mouthwash and to gargle with 5% sodium bicarbonate solution, as similar ones have been recommended in the previous studies [11]. Then, an exhaled breath condensate was acquired, according to European Respiratory Society (ERS)/American Thoracic Society (ATS) recommendations [9]. For our study, we used the Rtube collection system (Respiratory Research, Inc., Austin, TX, USA), which consists of the collection tube, the mouthpiece with a saliva trap and one way-valve, and a cooling sleeve with an insulating cover. A participant was asked to breathe tidally in a sitting position, with a nose clip on during the procedure, for 10 min through a mouthpiece, as such time is recommended by the producer for children's sampling. The cooling sleeve, prior to the procedure, was cooled down to −30 °C. By the end of the collection, the tube containing EBC was immediately sealed from both sides with rubber caps included by the manufacturer and subsequently stored in the freezer (−20 °C) until analysis. Due to insufficient DNA material, not all of the samples were possible to analyze; from the asthmatics group, 19 EBC and 12 swabs were included, and from the control group, 14 EBC and 14 swabs were included.

2.3. Genetic Material Isolation and Amplification

2.3.1. DNA Extraction

Bacterial DNA extraction was performed using the QIAamp PowerFecal Pro DNA Kit (QIAGEN, Hilden, Germany). For the exhaled breath condensate (EBC), standard protocol was modified as follows: the steps sample preparation, cell lysis, and inhibitor removal technology were omitted. The first step in extracting DNA from EBC samples was, therefore, adding 600 µL of high-concentration salt solution CD3 to an average of 700 µL of the sample. The manufacturer's instructions were followed from that step (bind DNA, wash, elute). Elution was performed in a volume of 50 µL of C6 solution. DNA extraction of throat swabs was performed according to the manufacturer's instructions, only no bead tubes were used, and instead of this step, the swab was vortexed in 800 µL of lysis buffer (CD1) and incubated at room temperature for 30 min.

2.3.2. Preparation of DNA Libraries for Sequencing

Oropharyngeal swab DNA libraries from DNA samples extracted from throat swabs were prepared according to the manufacturer's instructions [12], using the kit QIAseq 16S/ITS Region Panel (QIAGEN, Hilden, Germany) targeting 16S variable region V3V4. The concentration of the prepared libraries was measured using Quantus Fluorometer (Promega Corporation, Madison, WI, USA), and that was tested using TapeStation4200 (Agilent Technologies, Inc., Santa Clara, CA, USA), High Sensitivity D1000 ScreenTape (Agilent Technologies, Inc., Santa Clara, CA, USA). All normalized libraries were pooled in the same volume to generate an equivalent number of raw reads with each library. The final pool was diluted at a concentration of 10 pM and used for sequencing.

EBC Preparation of DNA libraries using the above protocol was not successful; therefore, an alternative approach was used as described below. During the following PCR amplification, KAPA HiFi HotStart ReadyMix PCR Kit (Roche, Basel, Switzerland) was used. As a first, the entire 16S rRNA gene was amplified using the following primers forward primer [5′-AGAGTTTGATCMTGGCTCA-3′] and reverse primer [5′-AAGGAGGTGWTCCARCC-3′], under the conditions: 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 65 °C for 15 s, 72 °C for 72 s, and 72 °C for 5 min, 12 °C hold. The template for this reaction was total DNA extracted from EBC samples in a volume of 10 µL. Following the manufacturer's guideline of library preparation [12] during the second PCR reaction, 16S rRNA V3 and V4 regions were amplified using primers: 16S Amplicon PCR Forward Primer [5′-TCGTCGGCAGCGTCAGATGTTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′] and 16S Amplicon PCR Reverse Primer [5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′], with the untreated products of the first PCR reaction as a template. In that step, Illumina adapter sequences were attached to amplicons (forward overhang 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]; reverse overhang 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-(locus-specific sequence)). The conditions for the reaction were: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min, 12 °C hold. The products of the second PCR reaction were purified using KAPA Pure Beads. The next step was to attach Nextera DNA CD Indexes (Illumina, Inc., San Diego, CA, USA), and NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, Inc., Ipswich, MA, USA) was used in this step, according to the standard protocol. The concentration of the purified amplicons with Illumina adapters and indices was measured using Quantus Fluorometer (Promega Corporation, Madison, WI, USA), and the quality of prepared libraries was tested using TapeStation4200 (Aligent Technologies, Inc., Santa Clara, CA, USA), High Sensitivity D1000 ScreenTape (Agilent Technologies, Inc., Santa Clara, CA, USA). Similarly, normalized libraries were pooled in the same volume. The final pool was diluted at a concentration of 6 pM and used for sequencing.

2.3.3. 16S rRNA Gene Sequencing

Oropharyngeal swab sequencing was performed using Illumina MiSeq, v3 cartridge 600 cycles, flow cell v3, according to producer protocol.

EBC Sequencing was performed using Illumina MiSeq, v2 cartridge 500 cycles, flow cell v2 nano, according to producer's protocol. PhiX input was 6 pM, 5%.

2.4. Data Analysis

Bioinformatics analysis of microbiota was performed using QIIME2 2021.8 [13] and additional plugins, including other methods of microbiome analysis. Demultiplexing was performed using a custom script that uses cutadapt [14] to cut V3V4 primer sequences. Evaluation of the quality of the reads was performed using the summary method from the demux plugin on the artifact constructed from previously demultiplexed data. Paired-end sequencing was performed, but due to insufficient read quality, which made it impossible to combine forward and reverse reads, it was decided to cut forward read to 70 nt and use trimmed single-end read for further analysis. Trimming, denoising, dereplication, and

chimera filtering was performed with the dada2 plugin for single-end reads [15]. The multiple sequences alignment was performed using mafft [16], and then the phylogenetic tree was constructed using fasttree [17]. Before the construction of the phylogenetic tree, highly variable positions were masked. All these steps are implemented as a single routine align-to-tree-mafft-fasttree from q2-phylogeny plugin. The q2 diversity plugin was used to estimate alpha (Shannon's diversity index, observed features, Faith's phylogenetic diversity [18], and Pielou's evenness [19]) and beta diversity (Jaccard distance, Bray-Curtis dissimilarity, weighted UniFrac [20], unweighted UniFrac [21], and performed principal coordinate analysis (PCoA). The samples were rarefied (subsampled without replacement) to 16,830 sequences per sample. The PERMANOVA was performed to test beta diversity group significance, Kruskal–Wallis for alpha diversity group significance, and Spearman's rank correlation coefficient for continuous sample metadata columns, p-value < 0.05 was considered statistically significant. The naïve Bayesian classifier was used to assign taxonomy to ASVs. It was trained using the method fit-classifier-naive-Bayes from feature-classifier plugin [22,23] on fragments of the 16S rRNA gene sequences derived from the SILVA 138 SSURef NR99 [24,25] database, where the sequences were aligned to the primer of the V3V4 fragment and trimmed to a length of 70 nt, as in the case of the analyzed sequences. ANCOM [26], which is a tool that identifies differentially abundant features in a group of samples, was used as implemented in the q2-composition plugin with default parameters.

3. Results

Microbiota of the lower (EBC) and upper (swab) respiratory tract varied, as statistically significant differences in alpha and beta diversity can be found between swab (n = 26) and EBC samples (n = 33).

Higher richness of microbiota is found in swab samples, compared to EBC, as measured by the Shannon diversity index ($p = 9.108 \times 10^{-8}$), observed features ($p = 5.572 \times 10^{-11}$), Faith's phylogenetic diversity ($p = 1.296 \times 10^{-10}$), swab vs. EBC, respectively, and Kruskal–Wallis test (Figure 1A,B,D, respectively). The distribution of taxa is not significantly different in swab and EBC samples, as measured by Pielou's evenness (p = 0.109, Figure 1C). Ecological distances differ between these groups of samples (Figure 2).

Similarly, in the asthma group alone, swab (n = 12) vs. EBC (n = 19) higher richness was observed in swab samples in Shannon diversity index ($p = 1.907 \times 10^{-4}$, Figure S1A) and observed features ($p = 3.651 \times 10^{-6}$, Figure S1B), as well as higher phylogenetic diversity (Faith's, $p = 3.777^{-6}$, Figure S1D); contrarily, the distribution measured by Pielou's evenness did not differ significantly between these two samples type (p = 0.068, Figure S1C).

The ecological distances differ between swab (n = 12) and EBC (n = 19) from asthmatic patients in Jaccard (p = 0.001), Bray-Curtis (p = 0.001), unweighted UniFrac (p = 0.001) and weighted UniFrac (p = 0.001) metrics, PERMANOVA test, and 999 permutations.

In terms of the alpha diversity of healthy controls alone, oropharyngeal swabs (n = 14) samples were found to have higher bacterial diversity (Shannon's diversity index $p = 1.648 \times 10^{-4}$, observed features $p = 6.607 \times 10^{-6}$, Faith's phylogenetic diversity, $p = 1.927 \times 10^{-5}$), compared to exhaled breath condensates (n = 14, Figure S2).

The ecological distances differ between swab (n = 14) and EBC (n = 14) in healthy control group in Jaccard (p = 0.001), Bray-Curtis (p = 0.001), unweighted UniFrac (p = 0.001) and weighted UniFrac (p = 0.001) metrics, PERMANOVA test, and 999 permutations.

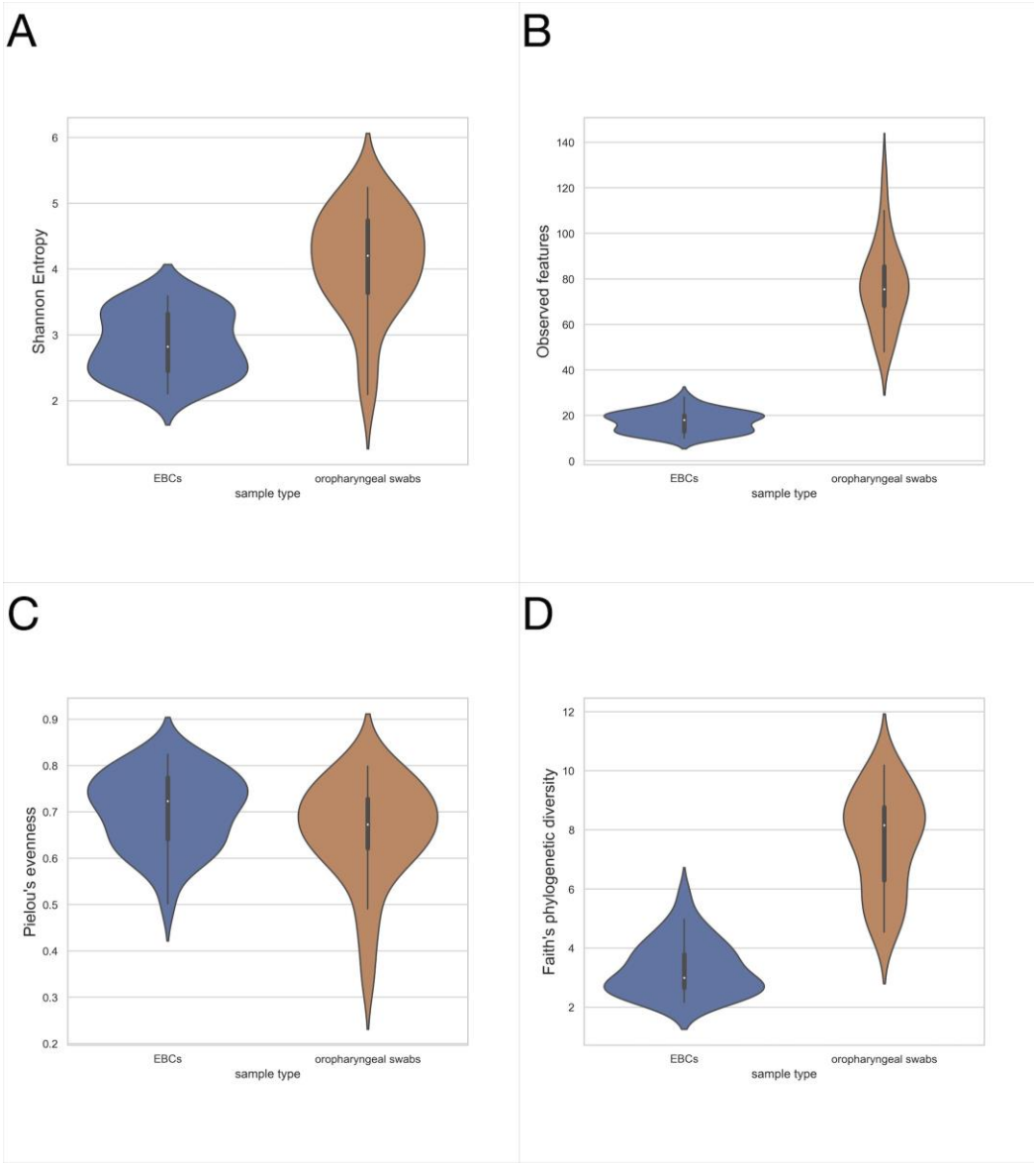

Figure 1. Alpha diversity metrics for sample type, asthmatic patients, and healthy controls taken together. (**A**) Shannon diversity index, mean 4.112 (±0.794) vs. 2.867 (±0.481), $p = 9.108 \times 10^{-8}$; (**B**) Observed Features, mean 77.4 (±18.1) vs. 17.3 (±4.4), $p = 5.572 \times 10^{-11}$; (**C**) Pielou's evenness, mean 0.657 (±0.109) vs. mean 0.703 (±0.083), $p = 0.109$; (**D**) Faith's Phylogenetic Diversity, mean 7.686 (±1.672) vs. 3.280 (±0.829), $p = 1.296 \times 10^{-10}$ swab ($n = 26$) vs. EBC ($n = 33$), respectively, Kruskal–Wallis test.

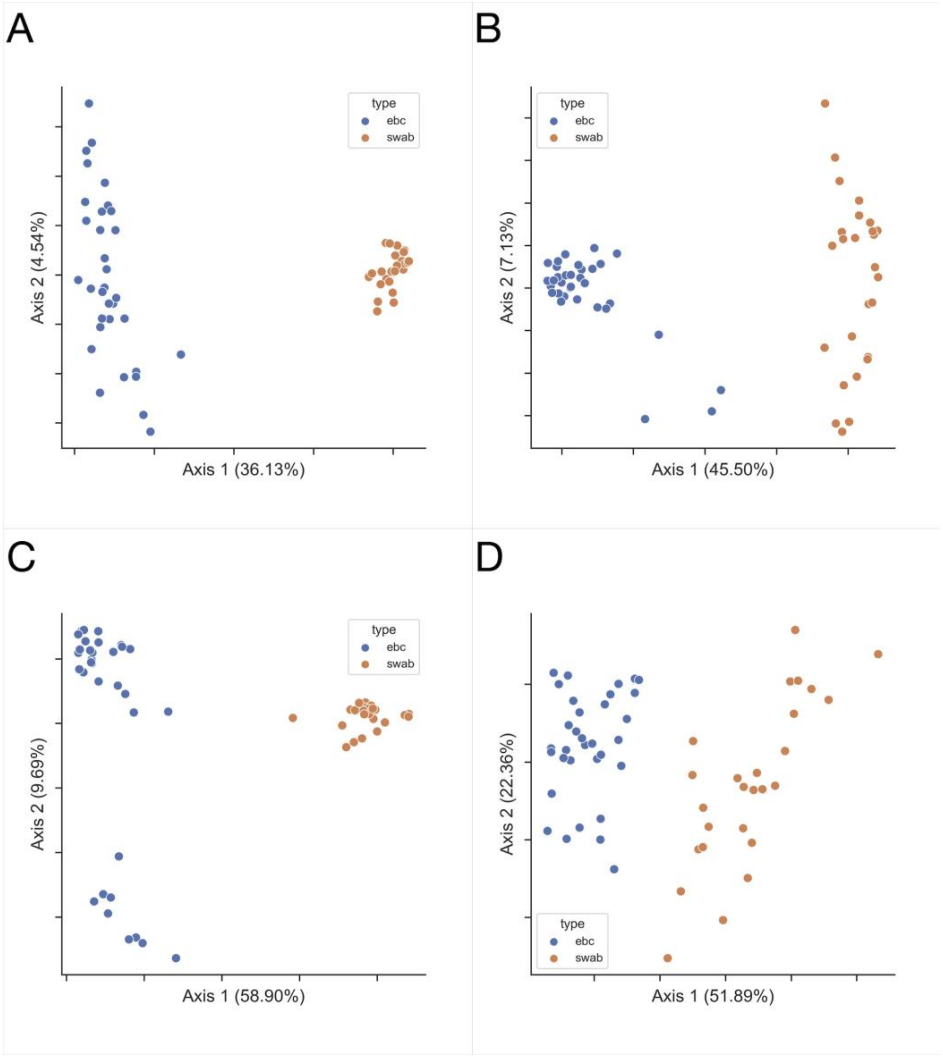

Figure 2. The microbiota of lower airways (EBC samples in blue, $n = 33$) differs from the microbiota of upper airways (swab samples, in orange, $n = 26$) asthma and control group taken together, as principal coordinate analysis (PCoA) plots capture the beta diversity for (**A**) Jaccard, (**B**) Bray-Curtis, (**C**) Unweighted UniFrac, and (**D**) Weighted UniFrac metrics, in all cases $p = 0.001$, PERMANOVA test, 999 permutations.

3.1. Exhaled Breath Condensate Samples Analysis

Statistically significant differences in alpha diversity were found between the asthmatic and control EBC samples.

Higher richness of microbiota (Shannon diversity index, $p = 0.026$) and more even bacterial distribution (Pielou's evenness, $p = 0.002$) were found in the EBC from asthmatic patients, compared to healthy controls ($n = 19$ vs. $n = 14$, Figure 3). No relevant changes were found in other alpha diversity metrics, observed features, $p = 0.854$, mean 17.4 ($\pm$4.8) vs. 17.4 ($\pm$4.4), Faith's phylogenetic diversity, $p = 0.362$, 3.370 ($\pm$0.807) vs. 3.150 ($\pm$0.839), asthmatic ($n = 19$) vs. control ($n = 14$), respectively, and Kruskal–Wallis test.

Statistically significant differences in beta diversity can be found between asthmatic ($n = 19$) and control ($n = 14$) EBC samples. Ecological distances differ between these groups of samples in Jaccard ($p = 0.012$), Bray-Curtis ($p = 0.038$), weighted UniFrac ($p = 0.043$) metrics, PERMANOVA test, and 999 permutations (Figure 4).

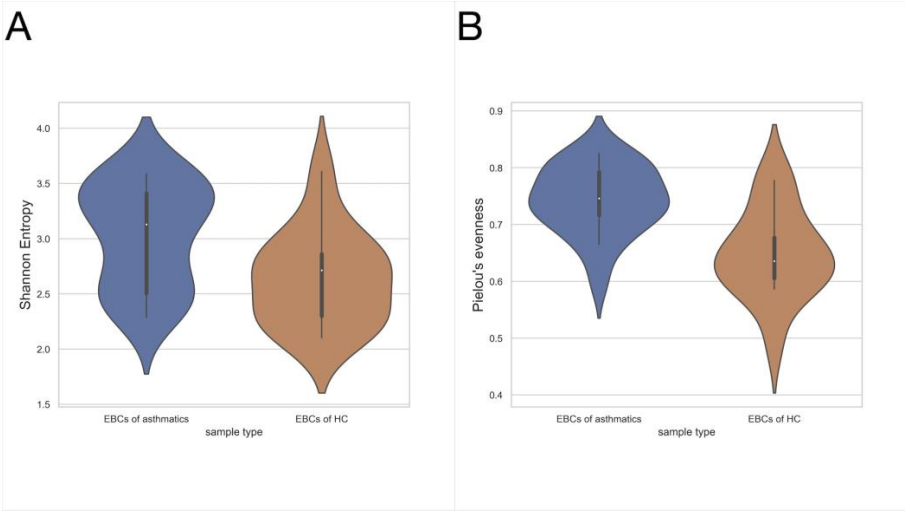

Figure 3. Alpha diversity metrics for EBC, asthmatic patients vs. healthy controls. (**A**) Shannon diversity index, mean 3.029 (±0.462) vs. 2.642 (±0.424), $p = 0.026$; (**B**) Pielou's evenness, mean 0.742 (±0.060) vs. mean 0.648 (±0.078), $p = 0.002$; asthmatics ($n = 19$) vs. control ($n = 14$), respectively, Kruskal–Wallis test.

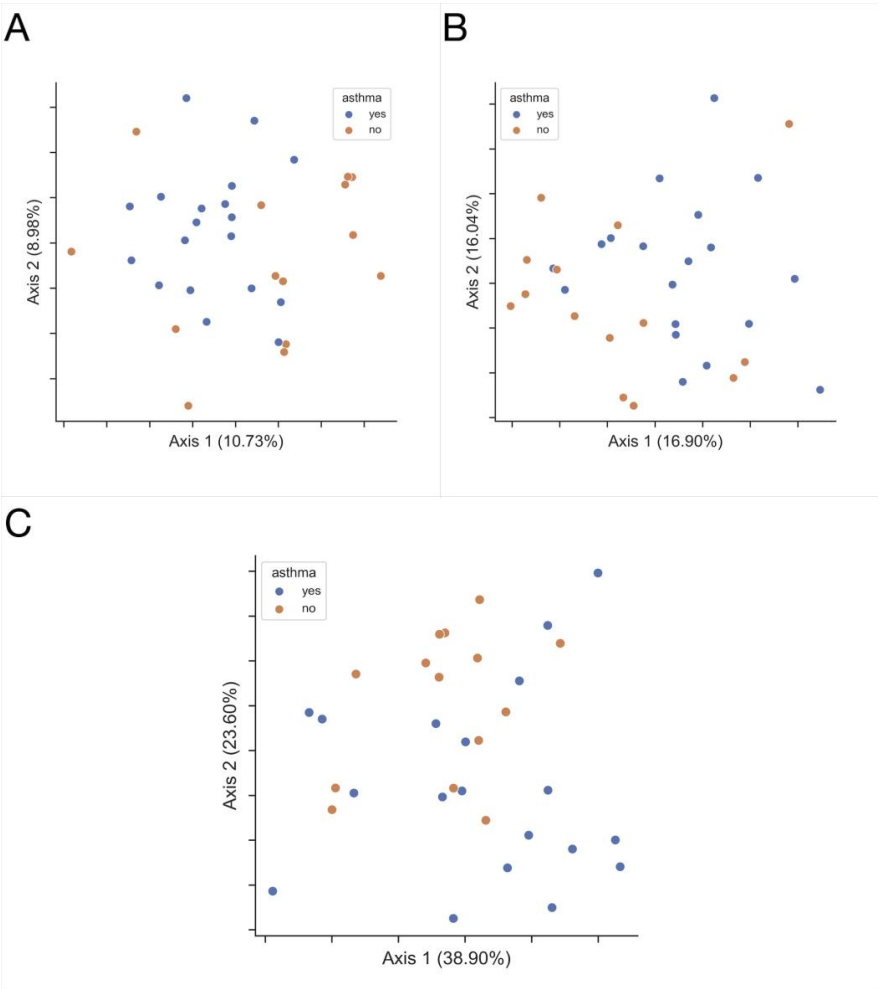

Figure 4. The microbiota of lower airways differs between asthmatics (blue) and HC (orange). Principal coordinate analysis (PCoA) plots capturing the beta diversity among EBC samples, for (**A**) Jaccard, $p = 0.018$; (**B**) Bray-Curtis, $p = 0.039$ and (**C**) Weighted UniFrac, $p = 0.035$.

Some differences were also observed within the EBC sample type, due to factors such as smoking or exposure to cigarette smoke, route of delivery, family burden of allergic diseases, medications taken, and age. The older the probant, the less diverse microbiota of lower respiratory tract (alpha diversity in the Shannon metric, r = −0.357, $p = 0.041$), and with age, it tended to lead to a less even distribution of taxa (Pielou's evenness, r = −0.378, $p = 0.030$), all EBC samples ($n = 33$), and Spearman's rank correlation coefficient. In children born with a naturally lower airway, bacterial microbiota ($n = 17$) were found to be more diverse (Shannon's diversity index $p = 0.002$, Faith's phylogenetic diversity $p = 0.003$) and more evenly distributed (Pielou's evenness, $p = 0.003$) than in children born through C-section ($n = 16$, Figure 5). They can be distinguished as separate groups in terms of the route of delivery (beta diversity, Jaccard, $p = 0.019$ and Unweighted UniFrac, $p = 0.021$; PERMANOVA, 999 permutations).

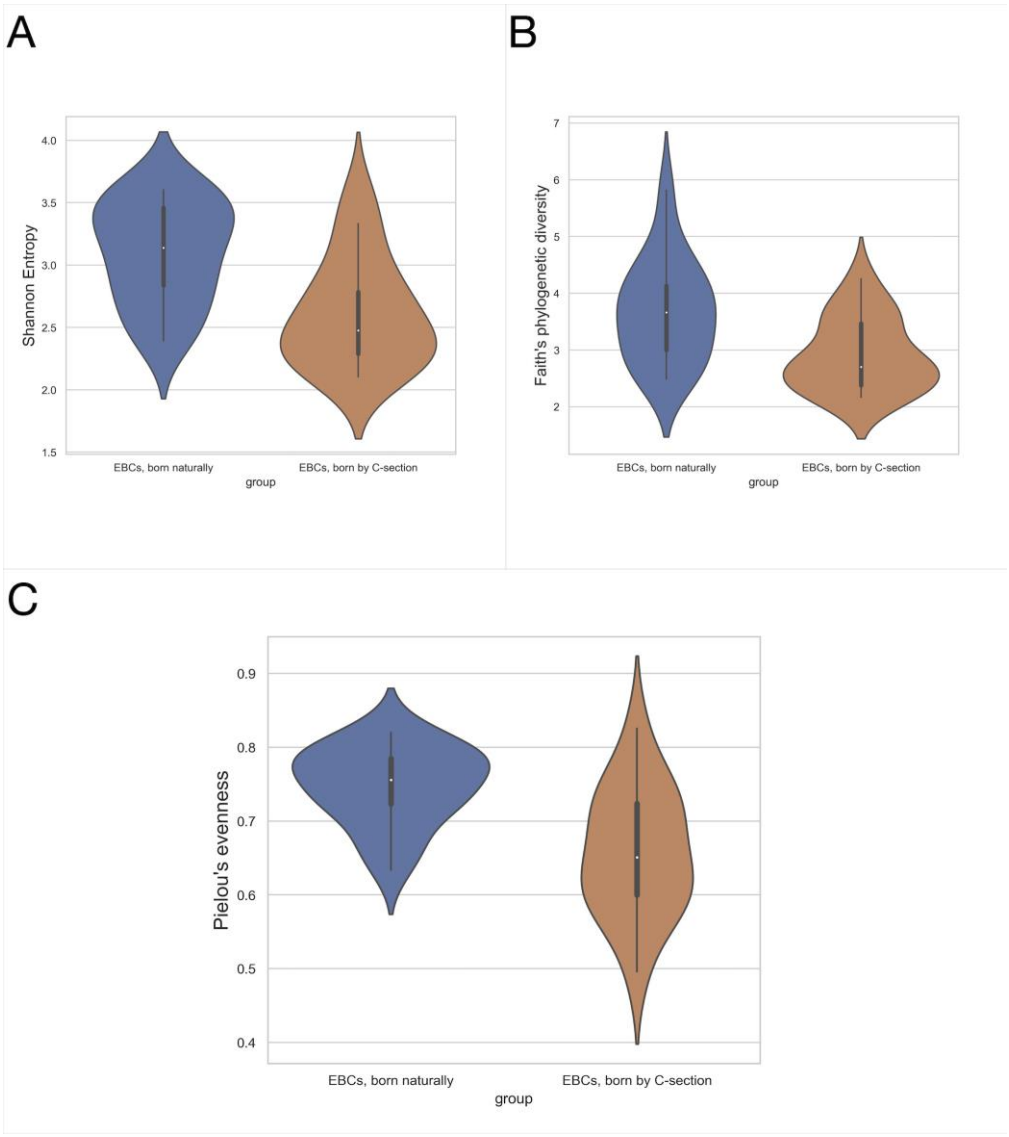

Figure 5. Alpha diversity metrics for EBC, children born naturally ($n = 17$) vs. born by C-section ($n = 16$). (**A**) Shannon diversity index, mean 3.1 (±0.409) vs. 2.615 (±0.434), $p = 0.002$; (**B**) Faith's Phylogenetic Diversity, mean 3.675 (±0.907) vs. 2.91 (±0.637), $p = 0.003$; (**C**) Pielou's evenness, mean 0.748 (±0.053) vs. 0.657 (±0.085), $p = 0.003$; Kruskal–Wallis test.

Factors found significant among all EBC samples ($n = 33$) were tested in the EBC from patients with asthma ($n = 19$) and healthy controls ($n = 14$) separately.

Microbiota of EBC from asthmatic patients with family burden of allergic diseases was more diverse than those patients without this risk factor (with family burden $n = 14$ vs. without family burden $n = 4$; Shannon's diversity index $p = 0.026$, Faith's phylogenetic diversity $p = 0.011$, observed features $p = 0.003$; Figure S3). Asthmatic patients with concurrent inhalant allergies ($n = 17$) tended to have slightly more uniformly distributed taxa (Pielou's evenness $p = 0.034$), compared to asthmatic patients without allergies ($n = 2$; Figure S4). In beta diversity, the factors allowing for distinguishing internal groups within the asthmatics were cigarette passive smoke (exposed, $n = 7$; not exposed, $n = 12$, unweighted UniFrac, $p = 0.015$), family risk (yes, $n = 14$; no risk, $n = 4$, unweighted UniFrac, $p = 0.043$), medications taken (yes, $n = 14$; no, $n = 5$, weighted UniFrac, $p = 0.017$), and the place of living (village, $n = 8$; town, $n = 11$, weighted UniFrac, $p = 0.007$; unweighted UniFrac, $p = 0.014$).

Healthy controls born naturally ($n = 4$) had more diverse microbiota found in EBC, compared to those born by C-section ($n = 10$, Shannon's diversity index $p = 0.011$; Faith's phylogenetic diversity $p = 0.034$; Figure S5). No such relationship was found in the group of asthmatics; however, the inverted proportion was observed, as the group of asthmatic patients born naturally counted13 people, and those born by cesarean section was 6.

3.2. Oropharyngeal Swabs

No significant changes in alpha and beta diversity were found between swab samples from asthmatic patients ($n = 14$) and healthy control ($n = 12$).

Shannon diversity index, mean 4.232 ($\pm$0.844) vs. 4.021 ($\pm$0.749), $p = 0.411$, observed features, mean 78.667 ($\pm$20.624) vs. 76.786 ($\pm$16.494), $p = 0.777$, Faith's phylogenetic diversity, mean 8.046 ($\pm$1.273) vs. 7.559 ($\pm$1.739), $p = 0.918$, Pielou's evenness, mean 0.673 ($\pm$0.111) vs. 0.644 ($\pm$0.107), $p = 0.440$, asthmatic vs. healthy control, respectively, Kruskal–Wallis test. Jaccard $p = 0.197$, Bray-Curtis $p = 0.733$, unweighted UniFrac $p = 0.18$ and weighted UniFrac $p = 0.913$, PERMANOVA test, and 999 permutations.

3.3. Dominant Taxa

To assess the dominant class in each sample type, we searched for bacteria that were present in at least 50% of EBCs/swabs and had a mean relative abundance >1%.

Among exhaled breath condensates, on class level complied with above (in consecutive order): Bacilli, Gammaproteobacteria, Alphaproteobacteria, and Actinobacteria, all present in 100% of EBC samples (Table 2).

Table 2. Dominant classes among EBC samples, in consecutive order.

Taxon	Sample Presence [%]	Mean Relative Abundance [%]
d__Bacteria;p__Firmicutes;c__Bacilli †,‡,§	100.00	35.44
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	100.00	20.92
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	100.00	17.86
d__Bacteria;p__Actinobacteriota;c__Actinobacteria	100.00	13.72

† d_—Domain, ‡ p_—Phylum, § c_—Class.

Within swab samples the most dominant class, in consecutive order, were: Bacilli, Gammaproteobacteria, Bacteroidia, Fusobacteriia, Actinobacteria, Negativicutes, Clostridia, Campylobacteria (Table 3).

Table 3. Dominant classes among swab samples, in consecutive order.

Taxon	Sample Presence [%]	Mean Relative Abundance [%]
d__Bacteria;p__Firmicutes;c__Bacilli †,‡,§	100.00	33.63
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	100.00	16.63
d__Bacteria;p__Bacteroidota;c__Bacteroidia	100.00	13.35
d__Bacteria;p__Fusobacteriota;c__Fusobacteriia	96.43	11.71
d__Bacteria;p__Actinobacteriota;c__Actinobacteria	100.00	10.62
d__Bacteria;p__Firmicutes;c__Negativicutes	96.43	7.54
d__Bacteria;p__Firmicutes;c__Clostridia	92.86	3.71
d__Bacteria;p__Campilobacterota;c__Campylobacteria	92.86	1.83

† d_—Domain, ‡ p_—Phylum, § c_—Class.

3.4. Taxonomic Assignment

Both exhaled breath condensates and oropharyngeal swabs were assessed, ranging from phyla to genera. The most abundant phylum in both the upper (Figure 6B) and lower (Figure 6A) respiratory tracts was Firmicutes, followed by Proteobacteria and Actinobacteriota. There were differences in the compositions of microbiota in EBC samples. Using the ANCOM method [26], it was detected that at the class levels Gammaproteobacteria (Figure 7B) and Bacilli (Figure 7A) were the more abundant among healthy controls, compared to asthmatic patients (W = 1, both). No statistically relevant differences were found between asthmatics and the control group, both comparing swabs. As the ANCOM method assumes that the groups differ by less than 25% of features (bacteria), the EBC vs. swab was not performed.

A

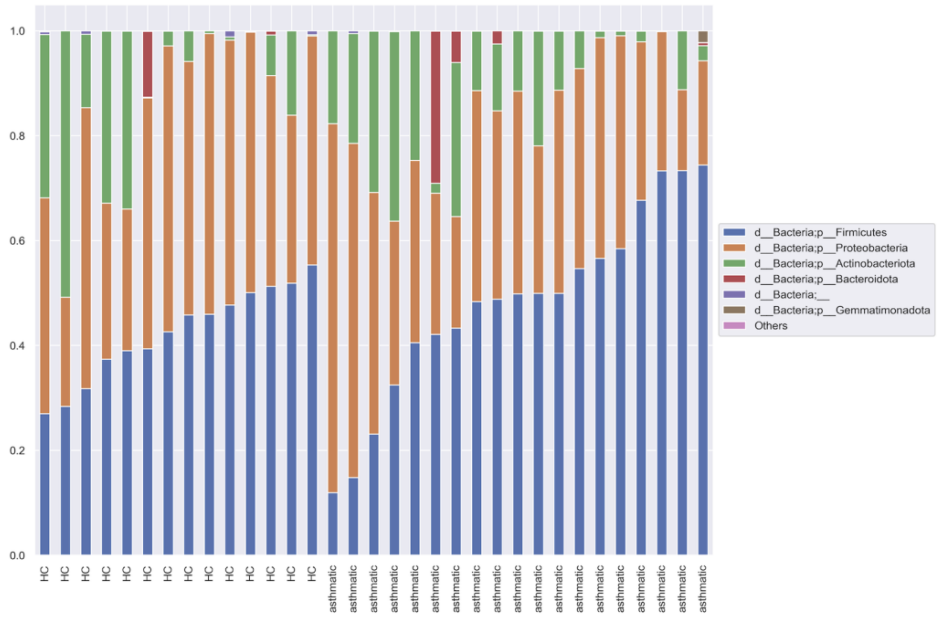

Figure 6. *Cont.*

B

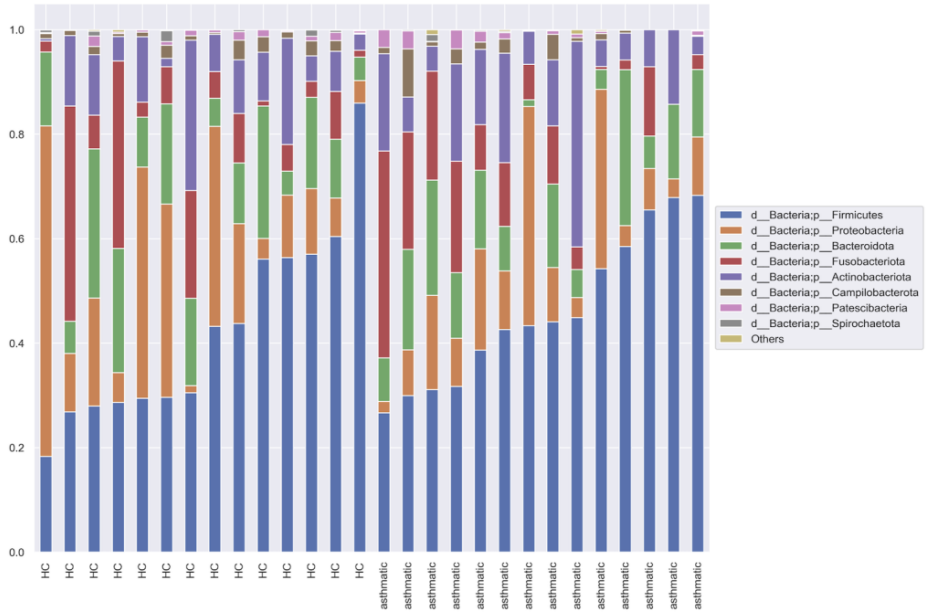

Figure 6. The relative abundance at the phylum level in lower airways—EBC samples (**A**); in upper airways—swab samples (**B**). d_—domain, p_—phylum.

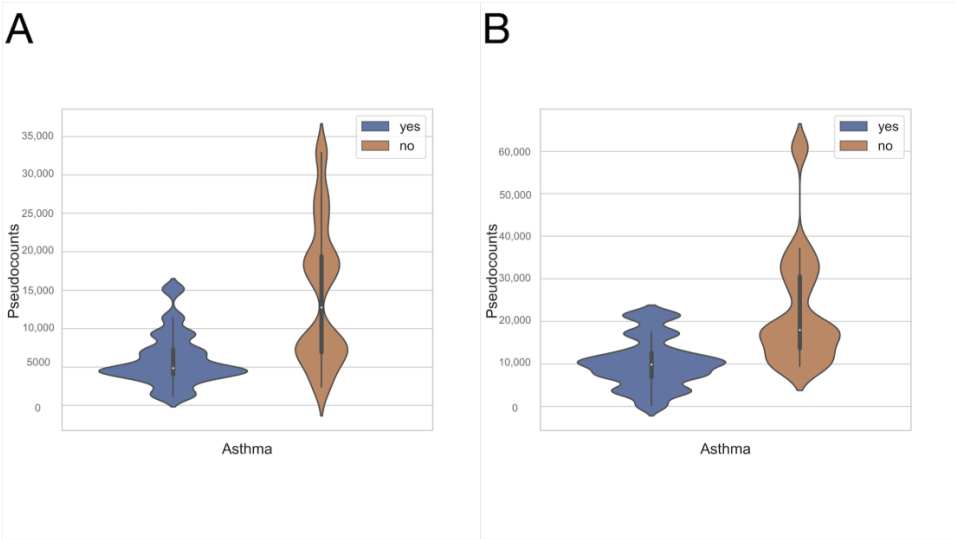

Figure 7. Relative abundance of differentially abundant taxa indicated by the ANCOM method, among EBC samples of asthmatics and healthy controls. The bacteria of class Bacilli (W = 1) (**A**) and Gammaproteobacteria (W = 1) (**B**) were more abundant in the group of asthmatics than in healthy controls.

4. Discussion

No statistically significant differences were found between asthmatics and the control group in oropharyngeal swabs, in terms of bacterial alpha and beta diversity, also the taxa of significantly different abundance could not be observed. The microbiota of the lower (tested in exhaled breath condensates) and upper (tested in swabs) respiratory tracts varied. In our study, we found that asthmatic EBC samples were characterized by higher richness (Shannon's diversity index) and more even distribution (Pielou's evenness), in terms of alpha diversity. Beta diversity also showed relevant differences in the bacterial composition of this type of sample (Jaccard, Bray-Curtis, and weighted UniFrac), compared to healthy

controls. Additionally, negative correlations of age with richness (Shannon index, $p = 0.041$) and evenness (Pielou's evenness, $p = 0.030$) were observed; however, the correlations were low. More diverse bacterial microbiota, in terms of richness (Shannon index, Faith's phylogenetic diversity) and evenness, were found in EBC samples of children born naturally. In terms of beta diversity, separate groups could also be observed (Jaccard and unweighted UniFrac). However, after the division into groups, among children from the control group, the above-mentioned relationships remained, but they could not be observed in the group of children with asthma. Notably, the asthmatics group was dominated by naturally born children, while the inverse proportion in the control group was observed as children born via cesarean section predominated in that group. The asthmatics with a family burden of allergic diseases in this study were discovered to have increased microbial richness, but this result should be treated with caution, as the number of people with asthma without a burden was very low and incomparable with the group of people with such a burden. Such disproportion can be explained by the fact of a high heritability of allergic diseases, ranging up to 95%, when assessing asthma [27]. Similarly, it appears that people with asthma and comorbid allergies had a more uniformly distributed microbiota, but also the size of the group was too small to draw such a conclusion. Factors affecting significant differences in beta diversity between EBC samples from asthmatic patients were exposure to cigarette smoke, family burden, inhalant corticosteroid intake, and place of living. The most abundant phyla in EBC samples were Firmicutes, Proteobacteria, and Actinobacteriota, and in swabs, Firmicutes, Proteobacteria, and Bacteroidetes. Among exhaled breath samples representing lower airways microbiota, the classes Gammaproteobacteria and Bacilli were found to be more abundant within the control group, compared to asthmatics. Asthma, and its connection with human microbiome dysbiosis, is still a topic of many researchers. To our present knowledge, the microbiota of the lower respiratory tract exists and differs from that of the upper respiratory tract.

The Perez-Losada et al. study assessed the nasopharyngeal microbiome of both children and adults asthmatics in a group of 40 patients, concluding that *Moraxella*, *Haemophilus*, *Staphylococcus*, *Streptococcus*, and *Fusobacterium* are the core genera of their upper respiratory tract [28]. In his another study on children exclusively, five phyla were dominant in nasal swabs: Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria, in descending order, which also comprises our EBC findings [29]. Additionally, in this particular research, including asthmatics only, varieties in genera composition among groups divided by certain phenotypes were observed, mainly in *Moraxella*, *Corynebacterium*, *Prevotella*, *Dolosigranulum*, and *Staphylococcus* abundance [29]. Depner et al.'s study, conducted in children with asthma, revealed lower alpha and beta diversity of nasal bacterial microbiota, whereas no difference was found within throat swabs, compared to healthy control [30]. Oropharyngeal swabs analyzed by Boutin et al., obtained from children with cystic fibrosis and asthmatics, revealed lower alpha diversity in the CF group, compared to asthmatics, but no difference was found comparing children with asthma to the healthy control [31]. In our study, no statistical relevance in the biodiversity of the upper respiratory tract has been observed within the compared groups.

Interestingly, studies on adults with the use of induced sputum were comparable with our findings. Marri et al.'s study showed that Firmicutes, Proteobacteria, and Actinobacteria accounted for more than 90% of the found sequences [32], which was similar to our taxonomical outcomes, despite other sample type sources. The same bacterial composition, with the addition of Bacteroidetes, accounted for 98% in the sputum samples of Pang et al.'s study; moreover, lower alpha diversity was observed within non-eosinophilic asthmatics [33]. The same sample type in the Simpson et al.'s study revealed certain microbial dysbiosis, regarding the asthma phenotype. *Haemophilus influenzae* was found to be more abundant among neutrophilic asthmatics, whereas *Tropheryma whipplei*, a member of Actinobacteria phylum, was among the eosinophilic [34].

Research including the lower airway microbiome in asthmatic children is very scarce. The study by Hilty et al. was the first based on endobronchial brushings of adults and BAL

samples in pediatric patients [35]. The adult groups (n = 24) comprised asthmatic, COPD patients, and healthy controls, whereas, among children, 13 were characterized as difficult asthma (defined as the need for at least 3 times a week rescue medications, besides high doses of inhaled and/or oral steroids), and 7 non-asthmatics underwent bronchoscopy for other indications. All of the participants had their nose and oropharyngeal swabs obtained, as well. Bronchial brushings were collected from the left upper lobe (LUL, n = 23) and right lower lobe (RLL, n = 14); not all of the patients tolerated right lobe samplings. The nasal microbiota clustered differently from other sample sites within all studied groups. Oropharyngeal microbiota of healthy controls clustered with their LUL samples and oropharyngeal swabs of asthmatics. A higher abundance of the Proteobacteria phylum and lower of Bacteroidetes were found in both the adult and pediatric asthmatics, as well as COPD adults in lower airways samples, compared to the controls. On a genus level, *Haemophilus* spp. were more abundant in asthmatics, regardless of age and COPD patients, additionally in the pediatric group higher richness of *Staphylococcus* spp. and lower of *Prevotella* spp. were found in asthmatics, compared to the controls. However, a small group of pediatric patients and concomitant diseases of "non-asthmatic" controls could have affected the outcomes. In another study by Goldman et al., bronchoalveolar lavage (BAL) of asthmatics was mainly enriched by the *Streptococcus* and *Prevotella* genera, members of Firmicutes and Bacteroidetes phyla, respectively [36]. Cui et al. assessed bronchoalveolar lavage fluid's bacterial richness in children with pneumonia. Patients with high total IgE levels (>60 UI/mL), including asthmatics and/or people suffering from allergic rhinitis, presented a higher abundance of *Bacteroides* and lower of *Streptococcus*, *Lactobacillus*, and *Anoxybacillus* [37]. The study's main limitation was antibiotic therapy prior, or ongoing, to the sample collection, which probably altered microbial homeostasis. In preschool children, asthma diagnosis remains challenging, since there is no objective test to perform as disease confirmation [2]. Researchers assess children with recurrent or persistent wheezing as an asthma equivalent. In Robinsons et al.'s study of episodic viral and multi-trigger wheezers underwent bronchoscopy [38]. In bronchoalveolar lavage samples (group of 14 children), two distinct groups were assessed with *Moraxella* predominance in one, with the mean relative abundance of 47.5% and the "mixed" group with more diverse bacterial contribution of other genera, whereas Moraxella's 1.2% was average. In episodic viral wheezers, the *Moraxella* higher abundance and greater neutrophilic BAL count was observed. Another recent study conducted by Wu et al. was a 2-year follow-up of children with persistent wheezing [39]. In BAL samples, the lower abundance of *Fusobacterium* and *Moraxella* with higher of *Elizabethkingia* and *Rothia* was observed, compared to the healthy control; the last two genera increased substantially in children with wheezing recurrence as the follow-up was performed [39]. Bisgaard et al. observed that infants' hypopharynx colonized with *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* at one month of age were more likely to be diagnosed with asthma at 5 years [40]. Although, in our study, asthmatic patients were characterized by a lower abundance of Gammaprotobacteria (including *Moraxella* and *Haemophilus* species) and Bacilli (including *Streptococcus* species), we cannot compare our findings with the ones on a phylum level.

We did not find any strong data supporting our finding that inhalant allergies affect bacterial diversity that can comply with our outcome of a more even distribution of bacteria in asthmatic patients EBC.

Inhaled corticosteroids (ICS) in asthma treatment and their role in microbiome diversity remains uncertain. Huang et al.'s study showed no significant relevance in sputum alpha diversity between asthmatics with and without ICS on the bacterial microbiome [41]. Denner et al. using endobronchial brushing (EB) in asthmatic adults have found a significant decrease in both alpha and beta bacterial diversity among patients using oral and inhaled corticosteroids, compared to milder asthma types with ICS treatment only [42].

EBC samples tend to be more diverse, in the sense of the bacterial microbiome, rather than BAL. In Zhang et al.'s study, patients suffering from severe asthma with the need for

using more than 2000 µg/d of inhaled beclomethasone presented a higher abundance of Firmicutes phyla, predominantly *Streptococcus* genera, and a minor increase in Proteobacteria, comparing to asthmatics with better disease control [43]. ICS treatment was also found to be less effective in patients with lower airways enriched with asthma-associated *Haemophilus* [44]. In the latter study, it was shown that fluticason treatment through inhalation in non-responsive patients caused a greater deviation in the bacterial flora of induced sputum and oral cavity samples [45]. In our study, the EBC of asthmatics prescribed with daily medication intake, including ICS, differed in beta diversity (weighted_unifrac), compared to asthmatics without regular basis treatment.

A cesarean section is proven to be an independent factor increasing the risk of asthma development [46,47], additionally making children prone to respiratory infections and obesity in the future [48]. Our findings indicate more diverse lower airway bacterial microbiota of children born naturally, which may support the earlier studies on microbiome diversity reduction as a risk factor for asthma development. Interestingly, in our study, children with asthma were delivered mostly by vaginal birth and healthy controls through C-section, but due to the relatively low participants group, that outcome cannot be taken as a negation of the above.

Rural areas residents are not only characterized by lower asthma prevalence [49,50], but allergic diseases in general [51]. In Depner et al.'s study, asthmatic children's nasal microbiota richness was inversely connected with *Moraxella OTU 1462*; however, no such observation regarded the ones living on farms [29]. Higher microbial richness with lower abundance of Streptococcae family in the indoor dust microbiota of farmhouses was found to have an impact on the prevalence of asthma among children [52]. In our EBC samples of the asthmatic group, two separate groups could be distinguished in beta diversity, taking the rural areas of living into account.

Passive smoking is also a factor that can alter the microbiome of the respiratory tract. In Bugova et al.'s study, children exposed to second-hand smoking were more frequently colonized by potentially pathogenic bacteria than healthy peers, when isolated from middle nasal meatus and nasopharynx with the use of standard cultivation methods [53]. In children with allergic rhinitis, exposed to passive smoking, bacterial microbiota were characterized by a lower Simpson index, compared to non-exposed patients; the two groups were also statistically different, when comparing them by beta diversity [54]. There are also some data regarding third-hand smoke, which is exposure to toxic chemicals accumulating in an indoor environment. Neonates admitted to intensive care unit gut microbiota showed a decrease in alpha diversity and a lower number of OTUs among children affected by smoke in their households [55]. Third-hand smoke can also alter the bacterial composition of an indoor environment, increasing its alpha diversity, as well as skin surfaces, such as outer ear [56]. In our study, in exhaled breath samples of asthmatic patients, passive smoking exposure was found to be a discriminant factor, in terms of beta diversity.

The study limitations were associated with the lack of standardization, in terms of the microbiological assessment of exhaled breath condensates [8]. The sample itself remains challenging; usually, the material's collected volume ranges between 0.5–1.5 mL, from which 99.99% is water [57]. Very little genetic material within each condensate puts an effort into its isolation and amplification. No salivary contamination has been assessed in the EBC samples, as reducing the amount of collected material caused a risk of decreasing the bacterial DNA within the condensate. Despite the small study group, compared to induced sputum research, the number of participants was relatively high, when compared to studies regarding BAL samples. Furthermore, the induced sputum taken from children is characterized by a lack of reliability [10]. By the time of DNA isolation, the previous recommendation regarding exhaled breath condensates failed to provide a sufficient amount of material, thereby a new method was implemented in our study, which significantly improves the methodology of the microbiome assessment in EBC, in our opinion.

5. Conclusions

This novel approach to noninvasive lower airways microbiome sample collection among children highlights the need for further DNA/RNA assessment in exhaled breath condensates, which is currently one of the promising noninvasive methods for a better understanding of airways microbiome and asthma pathology. To the authors' knowledge, this is the first study assessing lower respiratory microbiome in children with the use of exhaled breath condensates. In our research, a higher bacterial diversity in EBC from lower airways was found in asthmatic patients. Furthermore, having a family member suffering from other allergic diseases has also been found to have an impact on bacterial variety. Future studies on larger groups may determine the specific type of bacteria that can be applied as a biological factor to prevent asthma.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11226774/s1. Figure S1. Alpha-diversity metrics for sample type of asthmatic patients. Figure S2. Alpha-diversity metrics for sample type of healthy controls (HC). Figure S3. Alpha-diversity metrics for EBCs of asthmatics with family burden (n = 14) vs. without family burden of allergic diseases (n = 4). Figure S4. Alpha-diversity, Pielou's evenness, asthmatic patients with concurrent inhalant allergies (n = 17) vs. asthmatic patients without allergies (n = 2), 0.734 ($\pm$0.056) vs. 0.821 ($\pm$0.006), p = 0.034, Kruskal-Wallis test. Figure S5. Alpha-diversity metrics for EBC, healthy controls born naturally (n = 4) vs. born by C-section (n = 10).

Author Contributions: Conceptualization, K.B. and B.S.; methodology, K.B., B.S., P.Ż. and Ł.Ł.; formal analysis, P.Ż., funding acquisition K.B. and B.S., resources, K.B.; writing—original draft preparation, K.B., P.Ż. and B.S.; writing—review and editing, K.B., P.Ż., B.S. and Ł.Ł. All authors have read and agreed to the published version of the manuscript.

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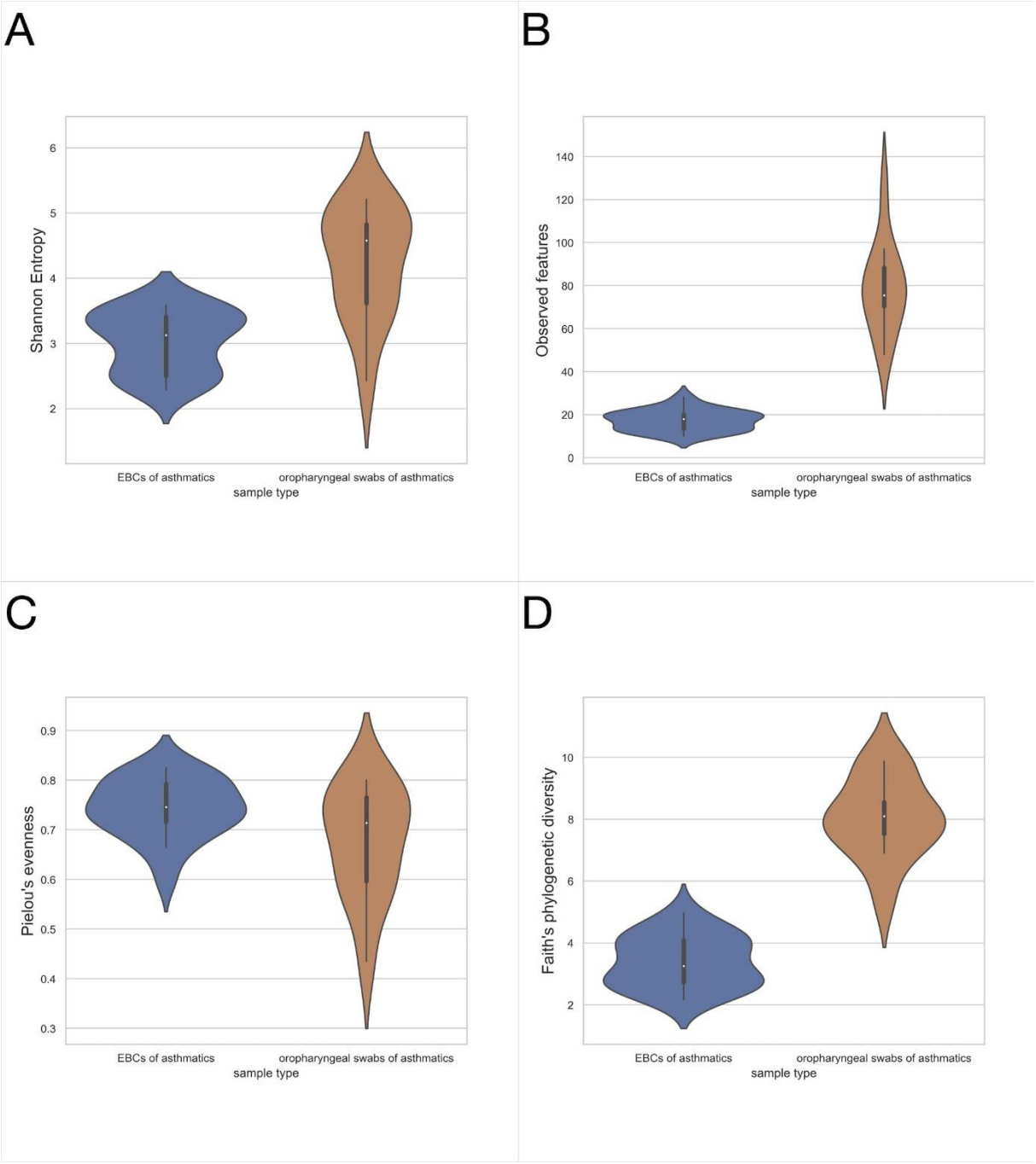
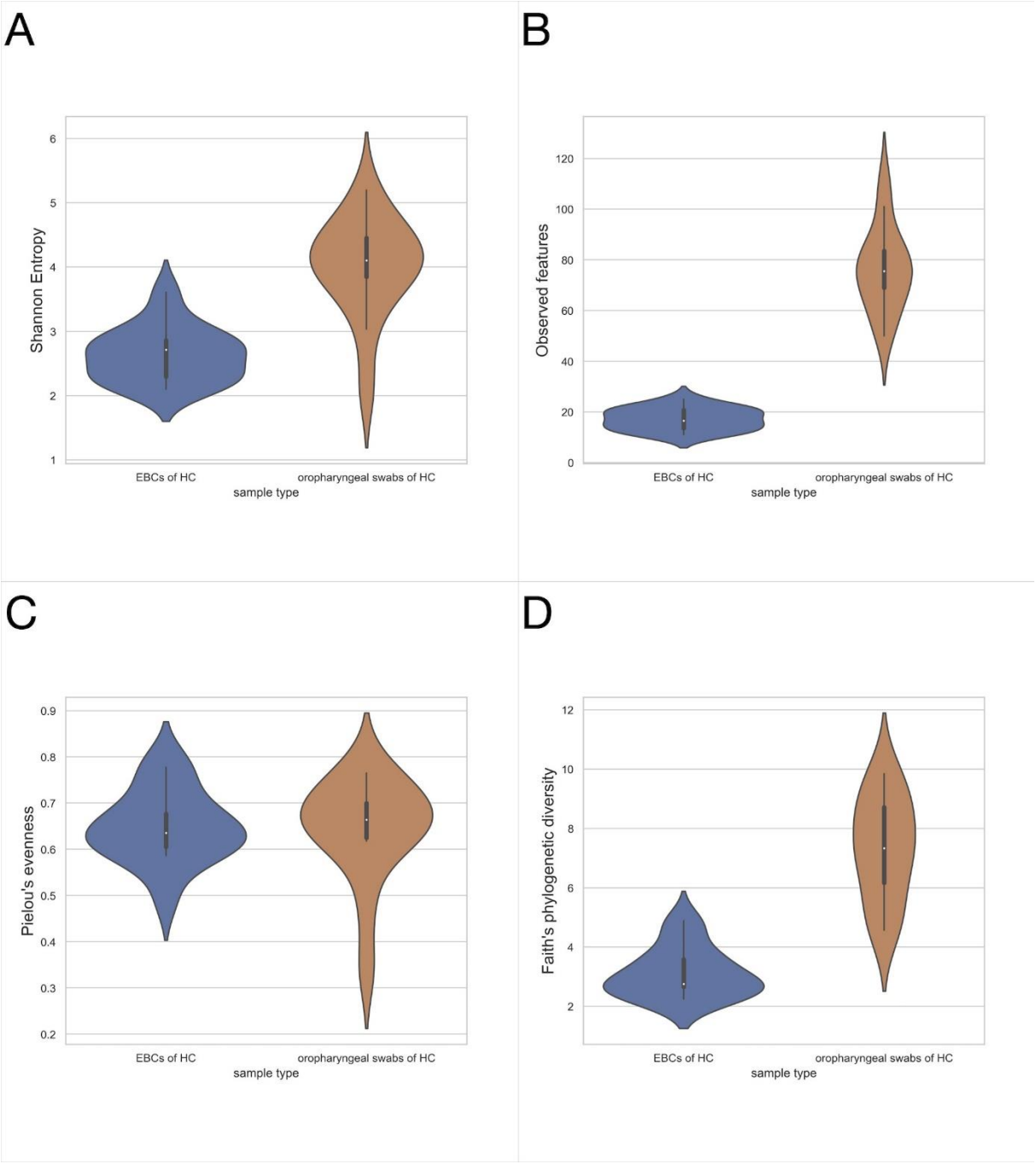
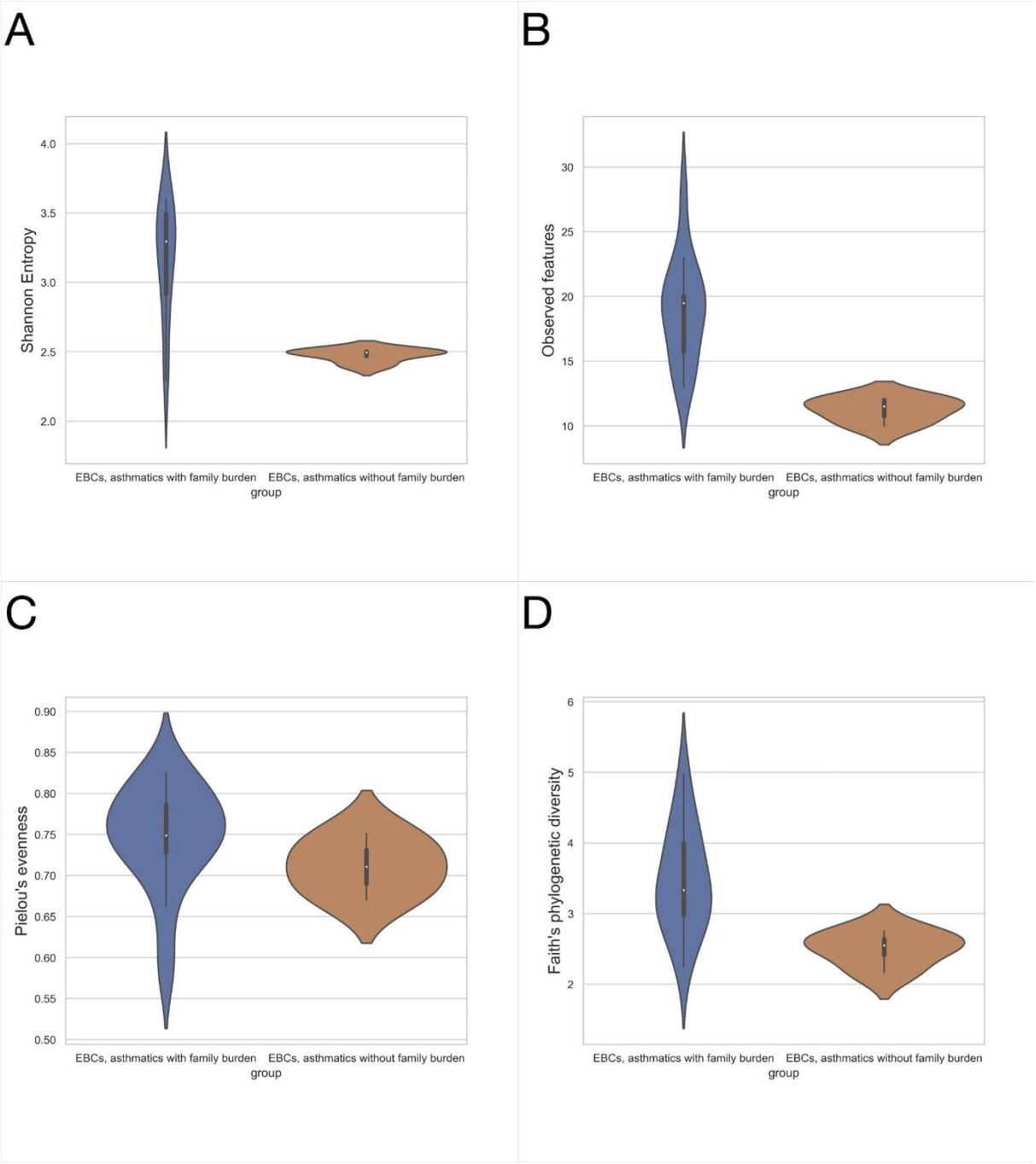


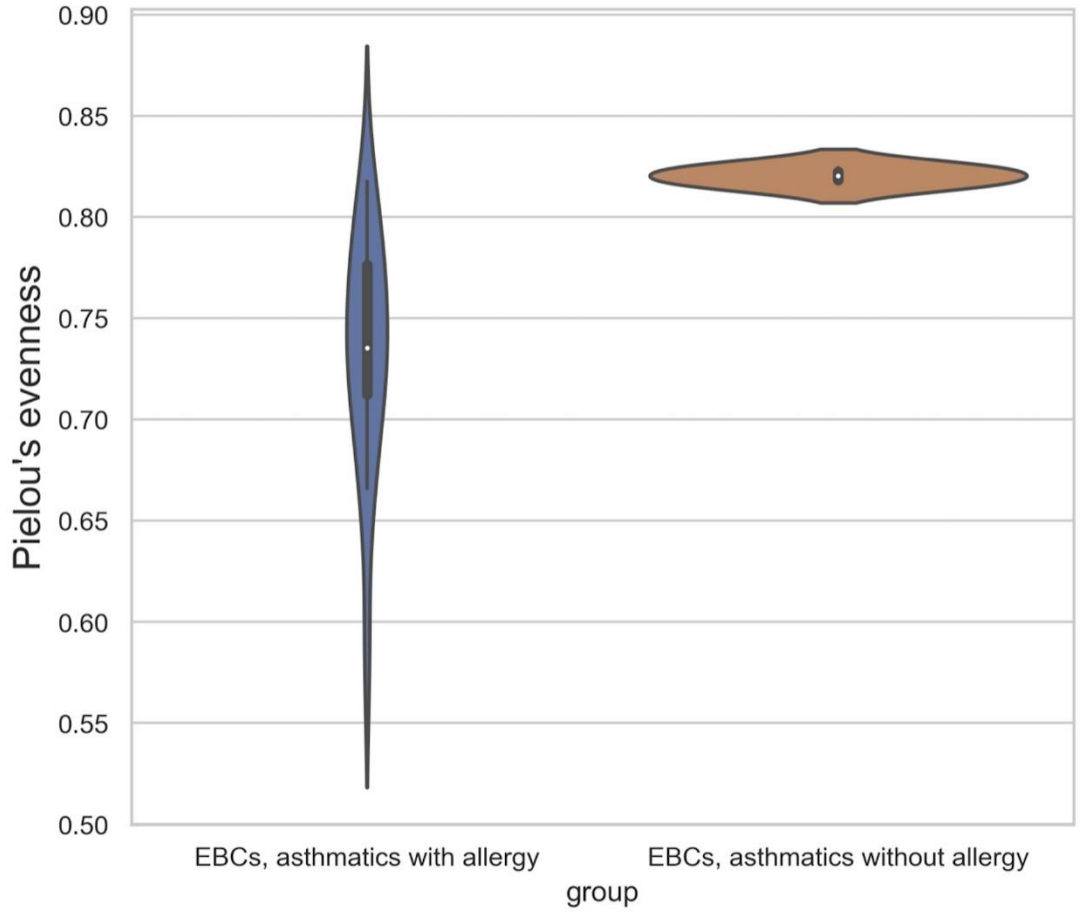
Supplementary Figure S1. Alpha-diversity metrics for sample type of asthmatic patients. (A) Shannon diversity index, mean 4.226 (±0.846) vs. 3.029 (±0.462), p= 1.907×10^{-4}; (B) Observed Features, mean 78.6 (±21.0) vs. 17.4 (±4.8), p= 3.651×10^{-6}; (C) Pielou's evenness, mean 0.672 (±0.111) vs. mean 0.742 (±0.060), p= 0.068; (D) Faith's Phylogenetic Diversity, mean 7.982 (±1.263) vs. 3.370 (±0.807), p= 3.777^{-6}, swab (n=12) vs. EBC (n=19) respectively, Kruskal-Wallis test.



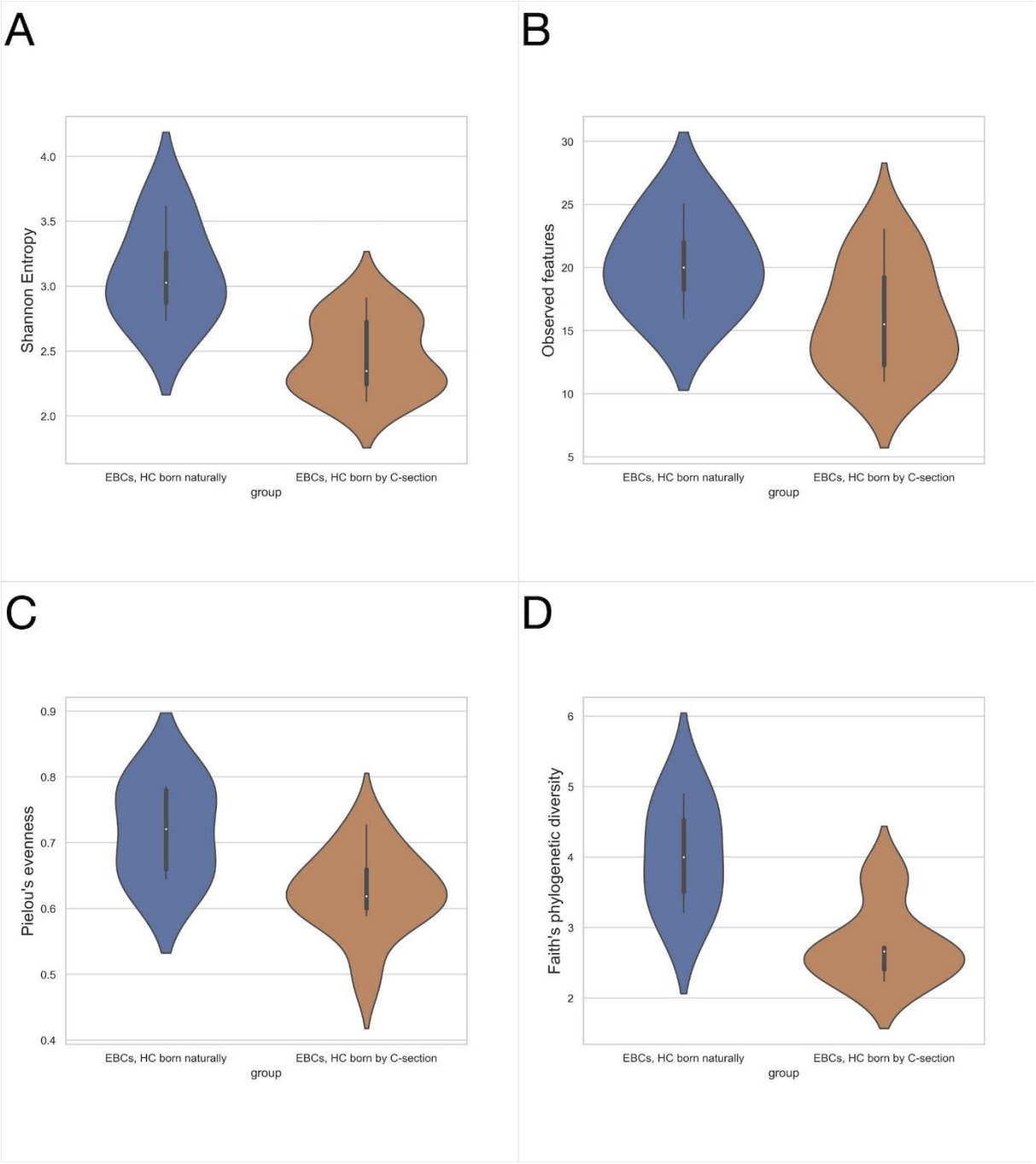
Supplementary Figure S2. Alpha-diversity metrics for sample type of healthy controls (HC). (A) Shannon diversity index, mean 4.018 (±0.763) vs. 2.642 (±0.424), $p=1.648\times10^{-4}$; (B) Observed Features, mean 76.4 (±16.6) vs. 17.4 (±4.4), $p=6.607\times10^{-6}$; (C) Pielou's evenness, mean 0.644 (±0.110) vs. mean 0.648 (±0.078), p=0.520; (D) Faith's Phylogenetic Diversity, mean 7.542 (±1.730) vs. 3.150 (±0.839), $p= 1.927\times10^{-5}$, swab (n=14) vs. EBC (n=14) respectively, Kruskal-Wallis test.



Supplementary Figure S3. Alpha-diversity metrics for EBCs of asthmatics with family burden (n=14) vs. without family burden of allergic diseases (n=4), mean respectively: (A) Shannon diversity index, 3.158 (±0.412) vs. 2.476 (±0.048), p=0.026; (B) Observed Features, 18.929 (±3.97) vs. 11.25 (±0.957), p=0.003; (C) Pielou's evenness, 0.747 (±0.063) vs. 0.711 (±0.035), p=0.243; (D) Faith's Phylogenetic Diversity, 3.47 (±0.737) vs. 2.506 (±0.249), p=0.011, Kruskal-Wallis test.



Supplementary Figure S4. Alpha-diversity, Pielou's evenness, asthmatic patients with concurrent inhalant allergies (n=17) vs. asthmatic patients without allergies (n=2), 0.734 (±0.056) vs. 0.821 (±0.006), p=0.034, Kruskal-Wallis test.



Supplementary Figure S5. Alpha-diversity metrics for EBC, healthy controls born naturally (n=4) vs. born by C-section (n=10). (A) Shannon diversity index, mean Shannon, mean 3.102 (±0.378) vs. 2.461 (±0.284), p=0.011; (B) Observed features mean 20.3 (±3.8) vs. 16.0 (±4.190), p=0.087; (C) Pielou's evenness, mean 0.718 (±0.074) vs. 0.624 (±0.062), p=0.090; (D) Faith's Phylogenetic Diversity, mean 4.027 (±0.76) vs. 2.764 (±0.537), p=0.034; Kruskal-Wallis test.

7.2. Praca poglądowa

Drugi z artykułów stanowi zbiór prac oryginalnych z ostatnich lat na temat mikrobiomu bakteryjnego dróg oddechowych u dzieci chorujących na astmę oskrzelową. Z racji trudności w rozpoznawaniu astmy wczesnodziecięcej, uwzględniliśmy również doniesienia dla grupy dzieci do lat 5 z nawracającymi świstami/obturacjami dolnych dróg oddechowych. Warunkiem włączenia pracy do przeglądu było użycie do oznaczenia bakteryjnego 16S rRNA do celów taksonomicznych oraz oceny biostatystycznej. Każda z prac została poddana analizie względem kryteriów rozpoznania astmy, materiału użytego do identyfikacji bakterii danego piętra dróg oddechowych, przedstawiono główne wnioski odnośnie wykazywanych różnic w mikrobiocie oraz transkryptomie w stosunku do grup kontrolnych.

microorganisms

MDPI

Review

Bacterial Microbiota of Asthmatic Children and Preschool Wheezers' Airways—What Do We Know?

Kamil Bar [1,*], Maja Litera-Bar [2] and Barbara Sozańska [1,*]

[1] 1st Department and Clinic of Paediatrics, Allergology and Cardiology, Wroclaw Medical University, 50-367 Wroclaw, Poland
[2] University Clinical Hospital in Wroclaw, 50-556 Wroclaw, Poland
* Correspondence: kamilbarmd@gmail.com (K.B.); barbara.sozanska@umw.edu.pl (B.S.)

Abstract: Asthma is the most chronic pulmonary disease in pediatric population, and its etiopathology still remains unclear. Both viruses and bacteria are suspected factors of disease development and are responsible for its exacerbation. Since the launch of The Human Microbiome Project, there has been an explosion of research on microbiota and its connection with various diseases. In our review, we have collected recent data about both upper- and lower-airway bacterial microbiota of asthmatic children. We have also included studies regarding preschool wheezers, since asthma diagnosis in children under 5 years of age remains challenging due to the lack of an objective tool. This paper indicates the need for further studies of microbiome and asthma, as in today's knowledge, there is no particular bacterium that discriminates the asthmatics from the healthy peers and can be used as a potential biological factor in the disease prevalence and treatment.

Keywords: asthma; children; wheezers; microbiome; wheeze; airways; bacteria

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1. Introduction

Asthma is the most chronic pulmonary disease in the pediatric population, with a rising prevalence in some countries [1]. The most common cause of the disease exacerbation is viral respiratory tract infections; bacterial impact on exacerbations remains unclear [2]. A meta-analysis of *Mycoplasma pneumoniae* infection has shown a significantly higher ratio of IgM antibodies among the asthmatics, compared to the controls, as well as in the acute asthmatics, compared to the stable asthma group [3]. Early colonization with potentially pathogenic bacteria leads to a higher risk of bronchiolitis and pneumonia in the future apart from a higher risk of asthma prevalence [4]. Patients with eosinophilic asthma are more susceptible to bacterial-pathogen respiratory infections; however, between infections, bacteria abundance is relatively low and is mostly presented by common types [5]. Nontypeable *Haemophilus influenzae* is a commensal bacterium frequently found in nasopharyngeal swabs of healthy adults; however, it is also one of the most abundant strains in the lower airways of neutrophilic asthmatics. Some evidence proves that infection in early life increases the risk of asthma in the future on an animal model [6]. Asthmatic children are more susceptible to invasive pneumococcal disease based on the current meta-analysis [7], with a higher probability of developing pneumonia, compared to peers without any risk factors [8].

Since the launch of the Human Microbiome Project, our knowledge about the connection between our body systems and microorganisms has begun to increase vastly, allowing for the isolation of certain dysbiosis patterns within diseases [9]. Human airways are inhabited by many different bacteria. The upper- and lower-airway bacterial composition remains very similar and differs in terms of concentrations, in advance of the upper respiratory tract; it can be justified by the hypothesis of the lower airway colonization through droplets aspiration [10]. Various samples collected during bronchoscopy proved that the lower-airway bacterial microbiota in adults is a sum of the mouth and nasopharyngeal

composition, acquired by subclinical aspiration [11]. The lower the tract of the airways, the lower the biomass of detected bacteria, whose composition is similar in each site among individuals [12]. Airway colonization starts from the first day of life and modulates the immune system [13]. Various environmental factors alter the composition of microbiota towards homeostasis or dysbiosis, which potentially leads to chronic illnesses [11].

The aim of this review was to highlight the current knowledge about respiratory tract bacteria among asthmatic children. We have also included preschool wheezers, since asthma diagnosis in children below 5 years of age remains challenging due to the lack of sufficient tools for its confirmation [14].

2. Upper-Airway Microbiome

As for the asthmatics, the most common sample types were nasal/nasopharyngeal swabs, followed by throat swabs, nasal washes, brushings, induced sputum, nasal blow and saliva (Table 1).

Table 1. Asthmatics' upper respiratory tract bacterial microbiome assessment. In all of the presented studies, bacterial analysis was conducted using Operational Taxonomic Units (OTUs). After isolating DNA material, bacterial 16sRNA amplification was conducted for further analysis using sequencing methods. In most cases, alpha and beta diversity and relative abundance of bacterial phyla and genera were assessed in terms of bacterial microbiota.

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Castro-Nallar 2015 [15]	8 asthmatic children and 6 healthy controls aged 6–20 years	Nasal epithelial cells	Ambiguous results in terms of biodiversity among asthmatics' samples (more species but samples dominated by fewer), mostly dominated by *Moraxella catarrhalis* species; *Escherichia* and *Psychobacter* more abundant in asthmatics' samples
Depner 2016 [16]	327 throat swabs (16.2% asthmatics) and 68 nasal samples (17.4% asthmatics) of children aged 6–12 years	Throat swabs and nasal samples	Lower alpha and beta diversity of nasal microbiota among asthmatics, higher abundance of *Moraxella* genus
Perez-Losada 2016 [17]	30 asthmatic children aged 6–18 years	Nasal washes and nasal brushes	Nasal brushes are characterized by higher alpha and beta diversity and a more abundant bacterial microbiome
Perez-Losada 2017 [18]	40 children aged 6–18 years	2 nasal washes collected within 5.5–6.5 months apart	*Moraxella*, *Staphylococcus*, *Dolosigranulum*, *Corynebacterium*, *Prevotella*, *Steptococcus*, *Haemophilus*, *Fusobacterium*, *Neisseriaceae* were the most abundant genera in consecutive order
Boutin 2017 [19]	27 asthmatic children, 57 with diagnosed cystic fibrosis, 60 healthy children aged 6–12 years	Throat swabs	Higher *Haemophilus* abundance in asthmatic children, compared to other groups, children with CF have less diverse bacterial microbiome than that of asthmatics
Birzele 2017 [20]	84 children including asthmatics aged 6–12 years	Mattress dust samples and nasal swabs	Asthma inversely associated with genus richness both in mattress dust and nasal swabs; inverse relative abundance of *Prevotella* genus in asthmatics nasal swabs
Kim BS 2018 [21]	31 children with asthma, 30 children with asthma in remission, 31 healthy controls; children aged 6–10 years	Nasopharyngeal swabs	The most dominant abundance of Proteobacteria in the control group, asthmatics with higher proportion of Fimicutes and Fusobacteria within the remission group; *Staphylococcus* being most dominant in asthma group; *Streptococcus*, *Dolosigranulum* and *Corynebacterium* more abundant in asthmatics and remission
Perez-Losada 2018 [22]	163 asthmatic children aged 6–18	Nasal washes	4 main genera detected were *Moraxella*, *Staphylococcus*, *Streptococcus and Haemophilus*
An 2018 [23]	7 asthmatics and 13 children without asthma, aged 5–16 years	Mouth swab, nose swab, throat swab, induced sputum, bronchial fluid	Actinobacteria were the most dominant in the nose and mouth swabs, Fusobacteria in throat swabs and induced sputum
Zhou 2019 [24]	214 asthmatic children aged 5–11 years	Nasal blow samples were taken at randomization point (214) and on the early loss of disease control (105)	*Corynebacterium* + *Dolosigranulum* cluster dominance was associated with a lower risk of asthma exacerbation, switching to *Moraxella*-dominant cluster involved the highest risk of exacerbation
Espuela-Ortiz 2019 [25]	57 asthmatic children aged 15.6 ± 3.3 years and 57 healthy controls aged 15.0 ± 3.9 years	Saliva samples	Higher alpha diversity among asthmatics and higher abundance of *Streptococcus* and *Veilonella* genus among the group

Table 1. *Cont.*

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Chiu 2020 [26]	60 participants, 20 with allergic rhinitis and 18 with asthma (both groups mite-sensitized, aged 4.37 ± 0.45 years), 22 healthy children aged 4.59 ± 0.36 years	Throat swab and stool sample	Lower richness and diversity of airway samples, compared to stool samples; significantly increased *Leptotrichia* and *Selenomonas* genera in asthmatics' airway samples
Chun 2021 [27]	132 asthmatic children aged 12.5 ± 3.6 years (±1 SD)	Nasal swab of 1 nare and nasal brushing of contralateral	Cat sensitization is associated with lower bacterial diversity; *Corynebacterium* and *S. epidermidis* were associated with the absence of sensitization to cat allergens
Majak 2021 [28]	133 children with chronic rhinosinusitis, including 82 asthmatics, aged 4–8 years	Nasal epithelium samples	Reduced abundance of Patescibacteria with an increase of Actinobacteria and *Staphylococcus* strains in asthmatics' samples; reduced alpha diversity and more frequent sweets consumption, compared to non-asthmatics
Aydin 2021 [29]	46 asthmatics aged 6–17 years, 61 wheezers <6 years, 39 healthy controls	Nasopharyngeal swabs	Higher Firmicutes abundance among asthmatics, atopic asthmatics were more colonized with *Streptococcus* and *Staphylococcus*
Hou 2022 [30]	33 asthmatics aged 6–17 years, 22 non-asthmatics	Flocked nasopharyngeal swabs	Group characteristic of *Moraxella* microbiome profile for longitudinal asthmatic samples, *Corynebacterium* dominated in stable asthma
Liu 2022 [31]	56 asthmatics aged 3–17 years with recurrent wheeze	Nasopharyngeal swabs, throat swabs, stool samples	In the recovery phase, there has been an increase in *Staphylococcus* and decrease in *Moraxella* abundance

Nasal brushes were characterized by higher alpha and beta diversity and more abundant bacterial microbiome than nasal washes [17]. One study used saliva as examined material [25]. In Espuela-Ortiz et al.'s study, 114 participants aged 8–21 years were equally divided into the asthmatic and control groups [25]. The whole study population was African American with the inclusion criteria of all four grandparents being African American. Asthma was defined as physician-diagnosed with at least one of active disease symptoms 2 years prior to the study. All samples in 98% of reads were dominated by the phyla Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria. When comparing the groups, alpha diversity in terms of species richness was higher among the asthmatics. The study group had a higher abundance of *Veilonella* and lower *Streptococcus* than that in healthy children. No relevant differences were found in the other most abundant genera found in the samples: *Haemophilus* and *Prevotella*. An et al. compared various upper and lower-airway samples (mouth/nose/throat swabs, induced sputum, bronchial fluid) [23]. Twenty children aged 5–16 years, of which seven were asthmatics with no specific criteria of disease diagnosis mentioned, underwent tonsillectomy. Before the procedure, induced sputum was obtained, and then after intubation, nasal secretions, mouth and pharyngeal swabs were collected. Lastly, a bronchial cytology brush was inserted through endotracheal tube for lower airway samples. From all sites, overall bacterial profiles of nasal and bronchial samples were distinct from the mouth, throat and induced sputum, which clustered together. The most dominant phyla in all sample types were Actinobacteria (most dominant in the nasal and mouth swabs), Firmicutes, Fusobacteria (most dominant in the throat samples) and Proteobacteria. Bacterial compositions did not differ statistically in each sample sites between asthmatics and non-asthmatics.

Two studies assessed nasal epithelial cells. A study on 14 children showed greater species richness and less evenness in terms of alpha diversity; asthmatics' samples were dominated by *Moraxella* species [15]. Moreover, in this study, host gene expression was also assessed with the use of libraries from the Human Microbiome Project database. The *Moraxella*-dominant samples were associated with the response of 32 genes previously connected with the immune response to these bacteria, whereas the control group showed no response. Another study was conducted on 134 patients aged 4–8 years with chronic rhinosinusitis, with asthma being a differential factor among the groups [28]. Nasopharyngeal swabs were taken for microbial assessment, and nasal epithelium was also obtained for host gene expression, as in the previously mentioned research. The asthmatic group was characterized by reduced alpha diversity (Shannon index) and an increased abundance of Actinobacteria and *Staphylococcus*. Moreover, asthmatic children were found to

consume more sweets. Nasal washes were used in three consecutive studies conducted by Perez-Losada et al. [17,18,22]. In all of the following papers regarding this researcher, the study groups were asthmatic children of the AsthMaP-2 study. All children, aged 6–18, had been diagnosed with the disease at least one year prior to enrollment, most of the group's ethnicity was African American. The exclusion criteria were a chronic or complex cardiorespiratory disease. In the first study, nasal washes of 40 asthmatic children were for bacterial composition taken during the first visit and another wash about 6 months later (±0.5 month) [18]. No relevant changes were observed in terms of alpha and beta diversity; however, the proportions of *Haemophilus*, *Moraxella*, *Staphylococcus* and *Corynebacterium* genera varied between sample collection times. The *Moraxella* genus accounted for more than 1/3 of reads (35.3%). An interesting finding in this particular study was that from all of the most abundant genera mentioned above, only *Haemophilus* had a seasonal difference in a relative proportion, with a higher abundance in the summer, compared to fall. The latter study conducted by the same head researcher with the same sampling method enrolled 163 children aged 6–18 years, of whom 42 attended a follow-up procedure after about 6 months (±0.5 month) and had another sample taken [22]. Within the study, three main asthma phenotypic clusters of patients were established based on various data acquired from the participants. The first cluster, APC 1, was female-dominant, with a lower asthma-control test score. The APC 2 cluster was characterized by the highest positive allergen-test ratio with the highest blood eosinophil rate and serum IgE value. Lastly, the APC 3 patients had the highest mean asthma-control test score and best outcomes of post-bronchodilator pulmonary test functions. When it comes to the most dominant phyla within the samples, the most abundant were Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria. The core microbiome genera presented in at least 95% of samples were *Moraxella*, *Streptococcus*, *Staphylococcus* and *Haemophilus*. Each mentioned cluster had its own significant variations in nasopharyngeal microbiome. APC1 was presented by the highest abundance of Actinobacteria and Bacteroidetes, with the dominant genera of *Corynebacterium* and *Prevotella*. APC2 participants had the highest abundance of Proteobacteria and Moraxella. The APC3 cluster was characterized by a higher abundance of Firmicutes and Fusobacteria, whereas at the genus level, no statistical differences were found, compared to other groups. Perez-Losada also compared nasal washes with nasal brushes of 30 asthmatic children [17]. The most abundant genera in nasal washes were *Moraxella* and *Staphylococcus*, whereas the most abundant in nasal brushes were *Moraxella* and *Corynebacterium*. Both sample types were different in terms of beta biodiversity and alpha biodiversity, with nasal brushes being enriched with more different bacteria. Moreover, in terms of core microbiome analysis (OTUs detected in at least 95% of samples), the main genera in nasal washes/washes OTUs were Moraxella, Pseudomonas, Enterococcus and Bacteroidetes and in nasal brushes/brushes OTUs of the genera Moraxella, Staphylococcus, Haemophilus, Streptococcus and Enterobacter. Another prospective study was meant to answer if there are any bacterial changes in the early loss of disease control [24]. A total of 254 children aged 5–11 years with doctor-diagnosed asthma of mild to moderate persistence and at least one exacerbation in the previous year were treated with a low dose of fluticasone propionate (twice 88 ug daily), with a randomization of taking either the same or a five times higher dose on the early signs of loss of asthma control ("yellow zone" defined as the use of certain amounts of rescue albuterol in a day and any night awakening due to asthma with the need of this medication); they were originally a part of another study. Thirty-eight nasal blows were taken by a researcher during the randomization visit, followed by the second blow taken at home during the early stage of disease-control loss by parents who were previously thought of the sampling technique. Samples of younger children aged 5–7 years during the randomization visit had a higher abundance of *Moraxella* and *Streptococcus*; they were more at a higher risk of developing symptoms of early asthma-control loss than older participants. Older children had a higher abundance of *Staphylococcus*. Taken the fact that viral infections are associated with asthma exacerbations, the researchers also assessed viral genetic material within the samples, of

which mostly rhinoviruses were detected. Viral-positive samples (33% of randomization samples) were associated with a higher abundance of *Moraxella* and a lower abundance of *Bacillus* and *Staphyloccoccus*. Using clustering analysis, the dominant genera were obtained, of which *Corynebacterium* + *Dolosigranulum* lowered the risk of the annual rate of "the yellow zone" symptoms and elongated the time between episodes in children, who reported at least two of them in one year. During the yellow zone sampling, more than half of the patients switched their dominant cluster to the *Streptococcus* cluster, which became most dominant at this point of the study. Moreover, total bacterial load and species richness were also higher at the early disease-control loss stage. Switching from the *Corynebacterium* + *Dolosigranulum* cluster at randomization to the *Moraxella* cluster at early symptoms of losing control was associated with the highest risk of progressing to asthma exacerbation. In Kim et al.'s study, nasopharyngeal swabs were taken from the children of three groups: asthma (n = 31), asthma in remission (n = 30) and healthy controls (n = 31) [21]. Children who participated were a part of KOREA study, the specimens taken in this particular study were from children aged 6–10 years. Asthma was diagnosed by pediatric allergologist on both clinical symptoms and either with positive methacholine challenge test (MCT) or at least 12% of forced expiratory volume improvement after albuterol administration, according to the American Thoracic Society guidelines. Asthma remission was defined as no symptoms and no need of asthma medication for at least 2 years prior to enrollment, with additional normal MCT results. All children reported no antibiotic therapy 3 months prior to sampling and at least 2 months of neither intranasal nor inhaled corticosteroids. At the phylum level, Proteobacteria, Firmicutes and Fusobacteria were most dominant, with a statistically higher proportion of Proteobacteria among the control group, Firmicutes among the asthmatics and Fusobacteria in remission. at the genus level, *Staphylococcus* was most abundant in the asthma group, whereas *Haemophilus* and *Moraxella* were more abundant in the control group. Both asthmatic groups active and in remission had a higher abundance of *Streptococcus*, *Dolosigranulum* and *Corynebacterium* than that in the healthy group. Additionally, in the remission group, the abundance of Fusobacterium, *Prevotella* and *Parvimonas* was the highest. Firmicutes at the phylum level and *Staphylococcus* at the genus level were inversely associated with the concentration of methacholine in the MCT test. Moreover, the Streptococcus genus abundance was negatively associated with the predicted FEV1. Within the study, functional genes were also obtained from the samples. When comparing taxonomical findings, Streptococcus pneumoniae in the asthmatics were associated with arachidonic acid metabolism genes, which were more abundant in both asthmatic groups. Genes associated with lysin degradation were less abundant in the asthma group than in remission group. The *Haemophilus influenzae* species was associated with these lysin degradation genes in all groups, whereas for the asthmatic group, only specific were *Neisseria lactamica*, *Neisseria meningitides*, *Parvimonas micra* and *Treponema medium*.

In Hou et al.'s study, the change in the asthmatics' bacterial microbiota during the disease exacerbation was assessed [30]. Thirty-three asthmatics aged 6–17 with physician-diagnosed asthma and at least one asthma exacerbation within the past 12 months were enrolled, as well as 20 non-asthmatic children as the control group. Asthma exacerbation was defined as either more than three uses of a beta agonist in at least 2 consecutive days, use of oral prednisolone or an unscheduled physician visit or hospitalization regarding asthma. Flocked nasopharyngeal swabs were obtained from both groups of participants, as well as five more during follow-up home visits at 2–4-week intervals. Similar to other studies, the most abundant phylum was Fimicutes, followed by Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria. Researchers distinguished six nasopharyngeal microbiomes by most abundant genera within *Moraxella*, *Corynebacterium 1*, *Dolosigranulum*, *Staphylococcus*, *Streptococcus* and *Anoxybacillus*. A higher proportion of the *Corynebacterium-1*-oriented cluster was observed in the stable asthma group, and none was detected in the asthma exacerbation group. During asthma exacerbation, the alpha diversity in the samples lowered substantially. Interestingly, the *Moraxella* abundance almost doubled

at the exacerbation point, whereas *Corynebacterium 1* and *Dolosigranulum* showed a significant decrease. Moreover, a *Moraxella* increase during the exacerbation contributed to nicotinate and nicotinamide metabolism. Liu et al. also tried to assess microbial changes during exacerbation [31]. Fifty-six asthmatic children aged 3–17 years with no control group were recruited during asthma exacerbation defined as a progressive worsening of symptoms and the need of reliever medications. The endpoint group were patients in a "recovery phase" approximately 2 weeks after exacerbation. The exclusion criteria were fever during a worsening, antibiotic therapy, an oral or parenteral steroid course for over 15 days, use of high budesonide doses in inhalation and need for at least four doses of β2-agonist a day. Patients had their nasopharyngeal swabs, throat swabs as well as stool samples collected. During the recovery phase, eight microbial clusters were distinguished, with *Corynebacterium* + *Dolosigranulum* and *Staphylococcus* being the most frequently found in the nasopharyngeal samples. Children of 3–5-year-old samples showed a statistically relevant change between the two time points. Moreover, moving towards the recovery phase, the *Staphylococcus* abundance increased while that of *Moraxella* and *Acinetobacter* decreased. The *Corynebacterium* + *Dolosigranulum* cluster was also positively associated with IgE levels in serum. Throat samples did not alter during the researched time phases, indicating no association with asthma. Researchers also compared bacterial composition of the asthmatics to other respiratory diseases. Boutin et al. compared throat swab samples of patients with cystic fibrosis (CF), asthmatics and healthy peers; all of participants were aged 6–12 years [19]. CF patients (n = 57) were diagnosed by specialists. Data on the asthmatic children (n = 27) were obtained from GABRIELA study, and asthma was defined using one of the three criteria: a parent reporting a wheeze at least two times in 12 months, a positive answer to the question about the "asthma spray" use or doctor-diagnosed asthma or a least two or more instances of obstructive bronchitis. The control group consisted of 62 healthy children. Pediatric cystic fibrosis patients had a lower *Haemophilus* abundance and less diverse bacterial microbiota than the asthmatic children. In the asthmatic group, the absence of one OTU belonging to *Aggregatibacter* was found and compared to other cohorts. When comparing the swab samples of the asthmatics and healthy controls, no difference in alpha diversity was found. Aydin et al. connected the rhinobiome of asthmatics with recurrent wheezers and healthy controls [29]. The alpha diversity in the nasopharyngeal swabs of atopic asthmatics increased, compared to a decrease in the atopic wheezers. Proteobacteria were more abundant in the wheezers group, while Actinobacteria were more abundant in the healthy controls and Firmicutes in the asthmatics. *Moraxella* was found to be the most abundant within the wheezers group, as well as the *Haemophilus* genus. The nasal cavity samples of atopic asthmatics had a higher abundance of Streptococcus and Staphylococcus. Interestingly, in this study, nasal epithelial spheroid cultures of healthy controls were infected with the *Moraxella catarrhalis* strains of the asthmatics and wheezers groups, leading to disruption of epithelial integrity after 24 h of incubation.

Environmental factors also seem to have an effect on asthma microbiota. Birzele et al. compared mattress dust samples and nasal swabs of children living on farms with non-farm residents, as a part of GABRIELA study [20]. One hundred and two children aged 6–12 years were enrolled. Asthma definition was the same as mentioned above regarding GABRIELA project. Non-asthmatics' nasal and mattress samples were characterized by a higher richness of bacteria than in the asthmatics. Mattress-dust bacterial richness was found to be a protective factor in asthma prevalence. Alpha diversity of mattress-dust bacterial microbiota was higher among the farm children, whereas the nasal samples' higher diversity was associated with the exposure to cow and straw. The *Prevotella* abundance in the nasal swabs was inversely associated with asthma. Interestingly, when comparing all nasal samples regardless of the group, farm exposure did not alter biodiversity significantly. Another related study by the GABRIELA working group included 327 throat samples and 68 nasal swabs from children aged 6–12 years with asthma definition as mentioned previously [16]. In both sample types, the asthma status nor farm exposure did not alter the bacterial load. In beta diversity of only nasal samples, the greater phylogenetic similarity

was found among the asthmatics. Alpha diversity in the nasal simples of the asthmatic children was significantly lower than that in the non-asthmatic peers, with no change, compared to the farm-exposure group. Given the taxonomics, the bacterial relative abundance in the nasal samples of the asthmatic children showed a higher Proteobacteria load at the phylum level and *Moraxella*, a member of the phylum, at the genus level. The specific abundance of *Motraxella OTU 1462* was inversely correlated with the nasal bacteria richness, but only for non-farm children. Besides rural animal exposure, another research study investigated house pet exposure, sensitization and microbiota [27]. The study enrolled 132 children with persistent asthma, with a mean age of 12 years, with asthma diagnosed using the National Heart, Lung and Blood Institute Expert Panel Report 3 Guidelines, taking various asthma medications on a daily basis and with SABA predominance (92%). Specific IgE higher than 0.1 kU/l was treated as sensitization to cat (68.9% participants) and dog (72.7%) allergens. Nasal swabs and brushing of participants revealed significantly lower alpha and beta diversity among the asthmatics sensitized to cat allergens, whereas there was no statistical difference in terms of dog sensitization. The *Corynebacterium* spp. And the *Staphylococcus epidermidis* abundance was associated with the absence of sensitization to cat or/and dog allergens. Additionally, this association was related to reduced 7-gene expressions related to IgE-mediated hypersensitivity and mast cell function. Other studies investigated if airway allergies have an impact on the microbiome. In Chiu et al.'s study, 60 children aged 4–5 years were enrolled, of which 38 were mite-sensitized and 22 were healthy controls [26]. From the study group, 18 children suffered from asthma and the other children from allergic rhinitis, both diagnoses were physician-diagnosed by a pediatric pulmonologist. All patients had not received antibiotic therapy 4 weeks prior to enrollment. Sensitization to mite dust was assessed by specific IgE levels of at least 17.5 kU/L (class 4 and above). From all participants, throat swabs and stool samples were obtained. The most abundant phylum in throat samples was Firmicutes, followed by Bacteroidetes, Proteobacteria and Fusobacteria. At the genus level, the most common were *Streptococcus*, *Prevotella* and *Fusobacterium*. As expected, lower diversity in terms of richness was observed in the airway samples, compared to the stool samples. Moreover, the alpha diversity richness in the throat samples was lower among children with mite-sensitization, but the main significance was found in children with rhinitis and not asthma, compared to the healthy group. The asthmatic children had a significantly higher abundance of *Leptotrichia* and *Selomonas* in the airway samples than patients with rhinitis. An interesting finding of the study is that the airway microbiota correlated mostly with total fecal IgE and not serum levels. Total fecal IgE levels were positively correlated with specific IgE of *Dermatophagoides pteronysinnus* and *Dermatophagoides farinae*, as well as the *Haemophilus* abundance in the airways. Contrary, a negative correlation of bacterial abundance to stool IgE was found for *Atopobium*, *Bulleidia*, *Moryella* and *Dialister*.

As for recurrent wheezers solely, the presented studies' samples were nasal and oropharyngeal swabs. All of the papers described prospective studies (Table 2).

Table 2. Wheezers' upper respiratory tract bacterial assessment. In all of the presented studies, bacterial analysis was conducted using Operational Taxonomic Units (OTUs). After isolating DNA material, bacterial 16sRNA amplification was conducted for further analysis using sequencing methods. In most cases, alpha and beta diversity and relative abundance of bacteria phyla and genera were assessed in terms of bacterial microbiota.

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Powell 2019 [32]	293 newborns enrolled in the study, 98 with complete 24-month follow-up and sequencing data	Oropharyngeal swabs	Colonization with *Neisseria* before age of 1 year is positively associated with a risk of wheezing by the age of 2 years; *Granulicatella* species are negatively associated
Cuthbertson 2019 [33]	109 preschool children with acute wheezing (median age 3.83 years) and 75 non-wheezing controls (median age 3.16 years); children aged 0–16 years	Oropharyngeal swabs	Significant beta diversity change between acute wheezing and 9-month follow-up sample, bronchiolitis diagnosis decreased alpha diversity among acute wheezers

Table 2. *Cont.*

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Dumas 2019 [34]	921 children aged <1 year hospitalized with bronchiolitis, followed up to age of 3 years	Nasopharyngeal swabs	Children with *Moraxella* dominant cluster were characterized by higher rate of rhinovirus-induced bronchiolitis, eczema and breathing problems among other groups, lower *Haemophilus* abundance in group with highest rate of RSV-induced bronchiolitis
Mansbach2020 [35]	842 infants hospitalized because of bronchiolitis, followed up to 3 years of age; median age of enrollment 3 months	Nasal swabs	Airway enrichment of *Moraxella* or *Streptococcus* after severe viral infection was associated with a higher risk of developing recurrent wheezes by the age of 3 years and wheezes accompanied by asthma at the age of 4 years
Tang 2021 [36]	289 infants followed up from 2 months to 24 months	Nasopharyngeal mucus sample	*Staphylococcus*-dominant microbiome in the first 6 months of life associated with a higher risk of recurrent wheezing at the age of 3 years, *Moraxella* dominance during wheezing illnesses associated with asthma persisting through later childhood
Aydin 2021 [29]	46 asthmatics aged 6–17 years, 6 wheezers < 6 years, 39 healthy controls	Nasopharyngeal swabs	Higher abundance of Proteobacteria in wheezers, more frequent colonization of Moraxella and Haemophilus, compared to other groups
Song 2022 [37]	Children aged 2–5 years divided into three groups: 16 recurrent wheezers with positive API score, 18 children with upper respiratory tract infection, 36 children in control group without infection	Nasopharyngeal swabs	Recurrent wheezers with dominant Proteobacteria phylum, lower alpha diversity than the healthy control group, *Moraxella catarrhalis* and *Dolosigranulum pigrum* were the most abundant species in all samples

Powell et al.'s prospective research study enrolled healthy term newborns, observed up to 24 months of age [32]. Oropharyngeal swabs from the posterior wall of the pharynx were obtained during home visits at 6 weeks, 6–9–12–18 and 24 months of age. After 2 years of visits, medical history of patients was obtained from their general practitioners to search for wheezing episodes. All necessary data and samples were obtained for 98 patients. Doctor-diagnosed wheeze was defined as recorded in medical notes, auscultation-confirmed or with a prescribed bronchodilator. Recurrent wheeze was defined as at least two wheeze episodes. Twenty-six children were diagnosed with wheeze, of whom eleven had recurrent wheezing. The bacterial density of children observed from birth to 24 months increased significantly at up to 9 months of age. Alpha and beta diversity of microbiota were correlated positively with age, with the highest change between samples taken at 6 weeks of age and any other assessment. Children with a wheeze diagnosis had a higher abundance of the *Neisseria* OTU, whether non-wheezers samples were enriched more with the *Granulicatella* and *Prevotella* OTUs, with *Neisseria* starting to differ after 9 to 24 months of age. Interestingly, at 6 weeks of age, all of the three OTUs were equally abundant. Another study conducted by Cuthberston et al. compared bacterial microbiota of children 0–16 years of age with acute wheeze and their healthy peers [33]. Oropharyngeal swabs of 109 acute wheezers (median age 3.8 years) and 75 non-wheezing children (median age 3.1 years) were obtained; in addition, follow-up samples were collected from 17 children from the wheezing group. Alpha diversity between the groups showed no statistically relevant differences. Diagnosis of bronchiolitis was a factor decreasing alpha diversity. Assessing only the acute wheeze group, the researchers also found that kindergarten attendance increased bacterial richness of oropharyngeal samples. The follow-up samples did not differ in alpha diversity either. However, beta diversity indicated the presence of distinct communities.

In Mansbach et al.'s study, 842 infants (median age 3 months) hospitalized with the first severe bronchiolitis were followed up to the age of 3 years for recurrent wheezing [35]. Each child had their nasal swab taken by a specialist in a hospital, and then parents after proper instruction collected another sample 3 weeks after hospitalization, in the summer while the children were healthy and 1 year post-hospitalization. In this multicenter study (35th Multicenter Airway Research collaboration, MARC-35), attending pediatricians diagnosed bronchiolitis based on the American Academy of Pediatrics' definition. Recurrent wheezing was defined by the 2007 National Institutes of Health Expert Panel Report 3—at least two corticosteroid-treated exacerbations in 6 months or at least four wheezing episodes in a year that lasted 1 week and affected sleep. Additionally, they extended the endpoint to

the age of 4 years and asthma diagnosis, which was defined as physician-diagnosed with either asthma medication usage or specific for its symptoms. The collected data identified that an increased abundance of *Moraxella* and *Streptococcus* 3 weeks after hospitalization or of *Streptococcus* in the summer after hospitalization was associated with a higher risk of recurrent wheezes by the age of 3 years, highlighting the possibility of post-illness colonization intervention to prevent the disease. Moreover, the same results in terms of developing asthma at the age of 4 years were obtained, with the exclusion of a higher abundance of *Streptococcus* in the summer samples, which did not increase the risk. The same cohort of MARC-35 was used in a study conducted by Dumas et al. [34]. The difference of this prospective study was that the only samples were taken during hospitalization, and the follow-up was the telephone interviews with parents of the children 1 week after hospitalization, 3 weeks after and then every 6 months from the age of 6 months onwards. Nine hundred and twenty-one children were clustered into three profiles (from A to C) depending on the latent class analysis. Profile A had the highest proportion of children with *Moraxella*-dominant profile and had a higher proportion of *Haemophilus* profile as well, compared to profile B. These children had a higher rate of rhinovirus infection and a higher proportion of eczema and breathing problems. Profile C was characterized as well by more children with *Haemophilus*-dominant profile than B, but the lowest proportion of *Moraxella*-dominant among all groups. The C group was mostly infected with RSV during enrollment hospitalization and presented more severe respiratory distress symptoms, as well as 21% of them being hospitalized for more than a week, in contrast with 0 and 1% in the other groups. In another study by Tang et al., a cohort of 289 newborns were enrolled for a 3-year follow-up [36]. All children were in risk of atopic diseases having at least one parent with asthma or other allergic diseases. Nasopharyngeal samples were obtained at 2, 4, 6, 9, 12, 18 and 24 months of age. Additional samples were taken during upper respiratory tract disease of at least moderate severity and any lower-airway infection up to 3 years of age. Wheezing respiratory illness was defined in the previous study with the same cohort as physician-diagnosed wheezing, illness with prescribed beta agonist and/or long-term controller medication or illness with diagnoses of bronchiolitis, wheezing illness, reactive airway disease, asthma or asthma exacerbation. All participants were divided into four trajectory groups of microbiome composition with each being represented by a distinct bacterial taxon in the first 4–6 months of life. The trajectory dominated by *Staphylococcus* was associated with a higher prevalence of wheezing illnesses of the second and third year of life. The acquired data allowed the researchers to extract four clusters associated with acute respiratory illnesses. Bacteria of discriminant clusters were the OTUs of *M. catarrhalis*, *S. pneumoniae* and two of *H. influenzae*. Interestingly, in this study, viruses were also taken into account. During illnesses of the respiratory tract, the most dominant correlation was in the detection of both viral and above-mentioned pathogens (66% of cases) than each individually. Moreover, the *Moraxella* dominance during acute wheezing was associated with asthma persisting through later childhood. The aspect of recurrent wheezing and infections was also assessed in the study conducted by Song et al. by the use of nasopharyngeal swabs [37]. Children aged 2–5 years were divided into the recurrent wheezing group (n = 16), who were hospitalized because of this diagnosis and had a positive stringent-asthma predictive index, the inpatient control group (n = 18) of children with no history of asthma or wheezing but having symptoms of upper respiratory tract infection by the enrollment time and the community control group (n = 36) with no allergic diseases, free of respiratory tract infection symptoms for at least 4 weeks prior to sampling. The additional exclusion criterion was antibiotic treatment in 4 weeks before the study. All samples were checked for the human rhinovirus presence. In all of the recurrent wheezers, the genetic material was found, whereas in the other groups, less than a third of participants were positive for that virus. *Moraxella catarrhalis* and *Dolosigranulum pigrum* were the most abundant species in all samples. The only phylum discriminant for recurrent wheezers was Proteobacteria with no particular genus of species of statistically higher dominance. A higher abundance of *Dolosigranulum pigrum* was found in the community

control group, when it was adjusted for the human rhinovirus status. Interestingly, the *Haemophilus influenzae* abundance was higher in inpatient controls with respiratory tract infection, and it was primarily discriminant in samples positive for the rhinovirus within the group.

Bacterial upper-airway microbiota seems to be consistent at the phylum level regardless of asthma and wheezing, with Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria being most abundant. At the genus level, the main differences concern pathogens such as *Moraxella*, *Haemophilus*, *Staphylococcus* and *Streptococcus*, which dominate in specific clusters and affect the niche in terms of biodiversity and transcriptome, which potentially may be a key to a better understanding of improper immunological responses in asthma and other allergic diseases.

3. Lower-Airway Microbiome

Data about bacterial microbiota of the lower airways are much more limited than those of the upper part, and the study participants were much fewer (see Table 3 for asthmatics and Table 4 for wheezers). Besides induced sputum, the only noninvasive method within the assessed papers, other sample types were bronchoalveolar lavage (BAL) and bronchial brushing. Additionally, in one study, sputum was collected from the trachea by a soft catheter [38]. Induced sputum however involves a risk of contamination from the upper respiratory tract and is not recommended by some researchers in children because of insufficient susceptibility, compared to other methods [23].

Table 3. Asthmatics' lower respiratory tract bacterial microbiome assessment. In all of the presented studies, bacterial analysis was conducted using Operational Taxonomic Units (OTUs). After isolating DNA material, bacterial 16sRNA amplification was conducted for further analysis using sequencing methods. In most cases, alpha and beta diversity and relative abundance of bacteria phyla and genera were assessed in terms of bacterial microbiota.

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Hilty 2010 [39]	13 asthmatics with severe asthma aged 7–15 years, 7 children as a control group (non-asthmatics)	Bronchoalveolar lavage (BAL)	Higher Proteobacteria and lower Bacteroidetes abundance in the asthmatic group, higher abundance of *Haemophilus* spp. and *Staphylococcus* spp., lower abundance of *Prevotella* spp.
An 2018 [23]	7 asthmatics and 13 children without asthma, aged 5–16 years	Mouth swab, nose swab, throat swab, induced sputum, bronchial fluid	Proteobacteria dominant in bronchial samples, compared to nose and mouth; asthmatics and non-asthmatics did not present any statistically significant differences in phylum abundance
Kloepfer 2018 [40]	36 participants, including 22 asthmatics, median age 3.3 years, interquartile range 3 months–18 years	Nasopharyngeal swabs and bronchoalveolar lavage (BAL)	BALF samples are richer and more diverse in terms of bacterial flora; Streptococcus was the most abundant genus in both sample types; *Prevotella* was more abundant in BALF
Goldman 2018 [41]	31 participants, 15 children with severe asthma (11 years ±4.5), 5 with cystic fibrosis (14.4 years ±2.7), 11 non-asthmatics (5.2 years ±4.1)	Bronchoalveolar lavage (BAL)	13 bacterial genera more abundant in asthmatic patients, compared to non-asthmatics, including *Bacteroides*, *Faecalibacterium* and *Roseburia*
Chun 2020 [42]	27 children with severe persistent asthma (aged 12.6 years ±4.4) and 27 controls (aged 12.6 years ±3.8)	Nasal and bronchial brushing for transcriptome profiling, nasal swabs and BAL for microbiome assessment	*Moraxella* and *Alloiococcus* were hub genera for nasal samples, but not for bronchial; *Corynebacterium* in upper airways and *Actinomyces* in lower airways have a negative correlation with an inflammatory response
Kim YH 2021 [43]	95 children, 67 with stable asthma, 22 with asthma exacerbation, 6 controls, aged 6–15 years	Induced sputum	Proteobacteria more abundant and Actinobacteria less abundant within exacerbations; beta but not alpha diversity changed between exacerbation and stable asthma; *Capnocytophaga* significantly more abundant among the exacerbation group
Bar 2022 [44]	38 children aged 6–18 years, 19 asthmatics, 19 healthy controls	Exhaled breath condensates and nasopharyngeal swabs	Class Gammaproteobacteria and Bacilli were less abundant among asthmatics in breath condensates

Table 4. Wheezers' lower respiratory tract bacterial assessment. in all of the presented studies, bacterial analysis was conducted with using Operational Taxonomic Units (OTUs). After isolating DNA material, bacterial 16sRNA amplification was conducted for further analysis using sequencing methods. In most cases, alpha and beta diversity and relative abundance of bacteria phyla and genera were assessed in terms of bacterial microbiota.

Reference	Subjects	Specimen	Bacterial Microbiota Findings
Robinson 2019 [45]	Children aged 1–6 years, grouped into episodic wheezers (n = 14) and multiple-trigger wheezers (n = 21)	Bronchoalveolar lavage (BAL)	Higher abundance of *Moraxella* and lower bacterial diversity are associated with lower-airway neutrophilia
Zhang X. 2020 [38]	74 infants <6 months of age with first in their life severe RSV bronchiolitis, follow up until 3 years	Sputum samples collected from trachea by a soft suction catheter	Higher Proteobacteria abundance among children who developed recurrent wheezing; at the genus level, higher abundance of *Haemophilus*, *Moraxella* and *Klebsiella* among the mentioned groups
Wu 2021 [46]	Children up to 24 months old, 35 persistent wheezers and 28 of control group	Bronchoalveolar lavage (BAL)	Higher abundance of *Elizabethkingia* and *Rothia* among wheezers
Zhang L. 2022 [47]	32 children aged 1–3 years with wheezing symptoms, 23 non-wheezers with an aspiration of foreign body	Bronchoalveolar lavage (BAL)	Both groups differed in beta but not alpha diversity; higher Proteobacteria abundance among wheezers
Yao 2022 [48]	Children aged 6–36 months divided into multiple wheezing group n = 13, persistent wheezing n = 16 and foreign-body aspiration control group n = 19	Bronchoalveolar lavage (BAL)	Both wheezing groups' bacterial diversity was lower, compared to controls; higher abundance of *Phyllobacterium* and lower abundance of *Prevotella*, *Neisseria* and *Haemophilus* in wheezers

One of the first studies regarding children's lung microbiota was conducted by Hilty et al. [39]. Bronchoalveolar lavage fluid was collected from a small group of 13 asthmatic children with difficult asthma, characterized by the need of treatment with a high dose of inhaled corticosteroids and long-acting β2-agonists and/or oral prednisolone, as well as using rescue bronchodilator at least three times a week; seven controls were also included for comparison. The study revealed a higher abundance of Proteobacteria at the phylum level. At the genus level, a higher abundance of *Haemophilus* and *Staphylococcus* was observed, compared to the non-asthmatic control group. Accordingly, non-asthmatics were characterized by a higher abundance of Bacteroidetes and *Prevotella*. The same study assessed the adults' lower airways with bronchial brushing of the left upper lobe, and the bacterial outcomes of both studied groups regardless of age were similar [39]. Limitations of the study were a small group of pediatric patients and a lack of a healthy control group; non-asthmatic children that underwent bronchoscopy had other comorbid diseases. Goldman et al. also assessed the lower airways' microbiota with bronchoalveolar lavage [41]. Thirty-one study participants were divided into severe asthmatics (n = 15, mean age 11 years), cystic fibrosis patients (n = 5, mean age 14 years) and the non-asthmatic group (n = 11, mean age 5 years). Children with severe asthma were defined as treated with high-dose inhaled or oral corticosteroids for at least half a year, with at least two minor additional criteria present. All were treated with a combination of high-dose inhaled corticosteroids and long-acting beta agonists, with or without other asthma medications. Six children also were sensitized to fungi. The non-asthmatic group included children with congenital respiratory malformations, e.g., tracheal diverticulum, tracheomalacia, etc. At the phylum level, the most dominant taxa were Firmicutes, Proteobacteria and Bacteroidetes in all groups. In all of the samples, the most dominant genera were *Streptococcus* and *Prevotella*. Regarding the asthmatic patients, 10 genera were discriminant, compared to the non-asthmatics, with a higher abundance of *Bacteroides*, *Faecalibacterium* and *Roseburia*. Moreover, a higher abundance of *Proteus* and *Capnocytophaga* was found among the non-asthmatics, compared to the asthma patients. The authors also indicated that the non-asthmatic group was younger than the others and had evidence of inflammation in the samples, which could influence the results. Some researchers tried to compare lower microbiota findings with those for the upper airways. Kloepfer et al. compared bronchoalveolar lavage fluid (BALF) samples with nasopharyngeal swabs [40]. Thirty-six children aged 3 months–18 years that underwent bronchoscopy had various diagnoses, in which asthma affected 61% of patients and recurrent bronchitis/pneumonia 67%. There

were no specific criteria mentioned in the text for defining diseases that were the reasons for the procedure. Four patients received antibiotic therapy during enrollment, whereas others claimed to be withdrawn from any at least 2 weeks prior to sampling. Twenty-four bronchial fluid samples (66.7%) were obtained from at least two or three lobes. The BALF samples were found to be more diverse and richer in bacteria than swabs with the use of alpha and beta diversity. Both sample types were dominated by Firmicutes and Proteobacteria in relative abundance. Nasopharyngeal swabs had a higher abundance of Actinobacteria, whereas BALF was characterized by a higher abundance of Bacteroidetes, compared to each other. At the genus level, relevant differences in advance of nasopharyngeal swabs regarded *Corynebacterium*, *Staphylococcus*, *Moraxella* and *Haemophilus*, whereas the lower airways had a higher abundance of three *Prevotella*-family genera. The *Streptococcus* genus in both sample types was the most dominant. BALF samples of conventional bacterial cultures were also obtained; in 22 of 34 samples, at least one or more bacteria were culture-positive. When comparing to the assessed 16S rRNA, all culture-positive bacteria were also present in the molecular outcomes, with no correlation between the OTU abundance and its presence in the standard culture. Chun et al. also compared nasal swabs with BAL fluid samples of the asthmatic children and the control group to assess bacterial microbiome and transcriptome [42]. The groups consisted of 27 children with severe persistent asthma and the same amount of healthy controls with a mean age of 12 years (~4 years SD). There was no clear definition of severe persistent asthma in the study. Nasal swabs and BAL fluid samples were obtained for a microbiome assessment, and nasal and bronchial brushings for a transcriptome. However, due to invasive procedure, in this particular study, only asthmatic patients underwent bronchoscopy and had their lower airways' samples collected. In the asthmatics group, nasal and BAL fluid samples varied in terms of both alpha and beta diversity, with bronchial samples having higher species richness. *Corynebacterium*, *Staphylococcus* and *Moraxella* were more abundant in nasal samples, while *Veilonella*, *Prevotella*, *Streptococcus* and *Neisseria* dominated in bronchial samples. Researchers also assessed the network of the associations between each genus. In nasal microbiome of the asthmatics, *Moraxella* and *Alloiococcus* were hub genera, with none of them in the BAL fluid samples. Taking into account the networks between the nasal transcriptome and microbiome, the study showed that *Corynebacterium* represented 30% of the associations, and all of them were negative, leading to lower gene expression with its higher abundance. However, that connection did not lead to any enrichment of biological processes. Interestingly, the same hub genera were found among the healthy peers, where *Corynebacterium* was negatively correlated with inflammation-promoting genes, and suggest their potential protective role in the non-asthmatics. Moreover, *Actinomyces* in the lower airways were negatively correlated with inflammation genes and can potentially be a protective factor. Kim et al. compared the induced-sputum microbiota of asthmatic children during exacerbation, with that of the stable asthma group and healthy peers, assessing the bacterial composition and its connection with inflammation cytokines [43]. Ninety-five children aged 6–15 were enrolled in the study. Asthma was diagnosed based on the current respiratory symptoms and confirmed in spirometric tests according to the ATS guidelines. Stable asthma was defined as no exacerbation in the last 4 weeks with the need of a systemic corticosteroid or an increased amount of inhaled corticosteroids, use of rescue medication less than 3 times a week and no need for a medication change. Asthma exacerbation was a disease worsening that required systemic steroid usage or hospitalization. Among 22 children in the exacerbation group, 11 were diagnosed with RSV concomitant infection, and one had a positive result for an influenza virus in the PCR analysis of nasopharyngeal swabs. No relevant differences in alpha diversity were found among the stable asthma and exacerbation groups; however, beta diversity showed statistical significance in terms of the dissimilarity of the two communities. Proteobacteria were more abundant, and Actinobacteria were less abundant in the asthma exacerbation group. At the genus level, the abundance of *Haemophilus*, *Campylobacter*, *Neisseria*, *Veilonella* was higher in the same group of patients. Moreover, in the exacerbation group, a microbiota

network was generated to assess their shared correlation, and the mostly connected genera were *Campylobacter*, *Haemophilus*, *Neisseria*, *Fusobacterium*, *Streptococcus*, *Peptostreptococcus* and *Granulicatella*. In the same study, inflammation cytokines were also assessed from the sputum. *Campylobacter* was found to be positively correlated with several inflammatory cytokines, such as granzyme B, macrophage inflammatory protein 1β (MIP-1β) and programmed death-ligand 1 (PD-L1). Additionally, *Haemophilus* was positively correlated with PD-L1, while *Porphyromonas* and *Peptostreptococcus* had negative correlation. The researchers also conducted the original research of the lower-airway microbiota using the exhaled breath condensates, which to our knowledge was the first study in pediatric asthmatics [44]. Exhaled breath condensates (EBCs) and oropharyngeal swabs were obtained from 38 children (19 asthmatics vs. 19 healthy participants). The exclusion criteria consisted in no antibiotic treatment and any respiratory illnesses at least 30 days prior to sampling. Asthma was defined as being diagnosed in the past by an allergologist. The most abundant phyla of both the lower and upper airways were Firmicutes, Proteobacteria and Actinobacteria. Between the asthmatics and the control group's samples of the exhaled breath condensates, the class Gammaproteobacteria and genus Bacilli were more abundant in the asthmatic patients. In the comparison of the samples, the swabs were characterized by a higher abundance of bacterial species than the EBCs. In terms of biodiversity, the asthmatics' lower-airway samples had higher bacterial species diversity (Shannon diversity index) and more even distribution (Pielou's evenness), than those in the healthy children.

As for wheezers, the lower-airway bacterial findings were similar to those of the asthmatics.

Robinson et al.'s study concerned episodic viral and multiple-trigger wheezers [45]. Thirty-five children aged 1–6 years with severe recurrent wheeze underwent bronchoscopy during infection with BAL fluid sampling. Wheeze was doctor-diagnosed and confirmed using a video questionnaire. The study groups were divided via a parental questionnaire into episodic wheezers during viral upper-respiratory-tract infections and multi-trigger wheezers with additional wheezes between infections. Based on the standard bacterial culture and viral detection tests, 60% of children had a positive outcome on either test. The most common bacterial results were *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*. A higher neutrophil count of BAL fluid was found in multi-trigger wheezers with positive bacterial cultures. Due to other clinically indicated tests, BAL samples from only 26 patients could be assessed for the microbiome analysis, and hence, some of the samples collected were insufficient in the latter aspect of 16S amplification and sequencing; bacterial OTUs were acquired from only 14 patients. Although no significant differences between two study groups have been found in terms of microbiota, taking all participants (into account) two other groups have been observed, with Moraxella genus being discriminant. The *Moraxella* species profile was associated with higher neutrophil counts in BAL fluid samples, while the other group of a mixed-microbiota cluster was characterized by a higher concentration of BAL macrophages and lymphocytes. All wheezers with this particular profile had Moraxella-catarrhalis-positive fluid culture outcome. The other group called "mixed" was in contrast characterized by higher bacterial diversity. Another study conducted by Wu et al., compared BAL samples of 35 infantile persistent wheezers up to 2 years old and a control group of 28 peers with foreign-body aspiration that underwent bronchoscopy [46]. Persistent wheezing was assessed according to the ATS recommendations, as the duration of a wheezing episode of more than one month despite proper treatment was an indication for further diagnostics with the use of bronchoscopy. All wheezers were followed up 24 months after enrollment to assess the recurrence of symptoms; more than half (n = 20, 57.1%) had at least one episode of wheezing, and they were further described as the "recurrence" group. No differences were measured using alpha diversity between the groups, although wheezers' samples for relative abundance were more enriched with *Elizabethkingia* and *Rothia* and had lower concentrations of *Moraxella* and *Fusobacterium* at the genus level. The most dominant phyla detected in both groups were Proteobacteria, Firmicutes, Bacteroidetes and

Actinobacteria. In the wheezer group solely, boys had a lower abundance of *Haemophilus*, *Prevotella* and *Porphyromonas*. Moreover, the route of delivery was a discriminating factor in the cesarean-section group with a lower abundance of *Streptococcus*, *Alloprevotella* and *Prevotella* and a higher abundance of *Sphingomonas*, *Elizabethkingia* and *Phenylobacterium*. Children of the "recurrence" group at the genus level had also a higher abundance of *Elizabethkingia* and *Rothia,* compared to the wheezers with no wheezing episodes in a 2-year follow-up. Using sputum samples taken from the trachea, Zhang X. et al. conducted a follow-up study of infants with the first severe RSV bronchiolitis, assessing microbiota and whether they develop recurrent wheezing by the age of 3 years [38]. The initial age of the children was less than 6 months; 74 patients were included. The exclusion criteria were any chronic illness, concomitant infection caused by other pathogens, any wheezing in the past, use of anti-gastroesophageal reflux medication and prematurity. Wheezing in a follow-up was diagnosed as a yes answer to the question about a wheezing episode requiring corticosteroid inhalation. Similar to other studies, the most dominant phyla were Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria. At the genus level, *Streptococcus* was highly dominant above all with an 80% relative abundance, with *Haemophilus* and *Moraxella* as the top three most frequently isolated genera. Infants who were later on diagnosed with recurrent wheezing (defined as three or more wheezing episodes, n = 26; 35.1%) were characterized by a higher abundance of *Moraxella*, *Haemophilus*, and, which was unique for the study, *Klebsiella*. However, the authors suggest that *Klebsiella* among Chinese children is the second most significant bacteria responsible for community-acquired pneumonia. Zhang L. et al. compared microbiota of the wheezers with that of the control group of children with foreign-body aspiration [47]. Thirty-two children aged 1–3 years with wheezing symptoms, having wheezes at least two times in the past or persistent wheezing for more than one month diagnosed by pediatric pulmonologist, and twenty-three children in the control group had their BAL fluid sample collected during bronchoscopy. In terms of biodiversity, the samples differed only in beta diversity. The most abundant phyla in both groups were Proteobacteria, Firmicutes and Bacteroidetes. The abundance of Proteobacteria phylum was statistically higher in the wheezers group. At the genus level, a higher proportion of *Stenotrophomonas*, *Sphingomonas* and a lower proportion of *Prevotella*, *Neisseria* and *Haemophilus* were obtained in the study group. Yao et al. also assessed the microbiota of wheezers divided in two groups [48]. The criteria for the enrollment in the wheezing groups were the age of 6–36 months, previous wheeze at least three times divided into duration of wheeze to less and more than a month, no history of antibiotic use for at least one week prior to the study and no history of any cardiac and pulmonary diseases. All participants were divided into three groups. The first group included participants with multiple wheezing (n = 13), defined by repeated wheezing episodes in a short period of time, the second group included participants with persistent wheezing (n = 16), defined as wheezing for more than one month without such symptoms in the past, and the control group (n = 19) included children with foreign-body aspiration. The samples of the children's BAL were obtained during bronchoscopy. As for the previous studies, the most abundant at the phylum level were Proteobacteria, Firmicutes, Bacteroidota and Fucobacteriota. At the genus level, *Phyllobacterium* was the most abundant in both wheezing groups, compared to the controls, and *Sphingomonas* additionally in the multiple wheezing group, compared to the control group. Furthermore, *Neisseria*, *Haemophilus* and *Prevotella* were less abundant in both wheezing groups.

4. Summary

The core phyla of the airway bacterial microbiota remain the same between the asthmatics and their healthy peers, and the qualitative differences in phyla seem to be provoked by the changes in the abundance of potentially pathogenic bacteria. In most cases, the asthmatics' samples from the upper respiratory tract had lower alpha diversity, compared to the healthy groups [20,28]. At the same time, higher richness of bacterial microbiota was observed in some studies [15,25]. Moreover, during the exacerbation of the disease, lower

alpha diversity was observed [16]. When comparing various sample types, BAL samples were characterized by higher richness than that of the nasal samples [40,42]. The nasal brushes also had greater bacterial richness than that of the washes [17]. The exhaled breath condensates had lower alpha diversity than that of the oropharyngeal samples [44]. Because of the sampling procedure, the EBC and induced-sputum samples could be contaminated with upper respiratory bacteria, of which researchers should be aware while assessing the outcomes of studies with those sampling methods. No discriminant bacteria were found between the upper and lower tracts of the asthmatic airways, which corresponds to the similarity of the respiratory tract microbiota in the non-asthmatics and healthy population.

No significant differences were found in the asthmatic children's microbiota that could discriminate it for the outcomes in the adult population. Moreover, the presented studies do not provide sufficient information about the influence of asthma treatment on bacterial microbiome. In one study, the asthma medication intake in the lower-airway samples of the asthmatics was statistically relevant in terms of beta diversity (n = 14 vs. 5, p = 0.014) [44]. This topic needs further investigation.

The nasal samples with high concentration of Moraxella collected from wheezers either during enrollment or during respiratory infection were found in children with a higher risk of recurrent wheezing and developing asthma in the future [34–36]. Interestingly, in some studies, clusters of the *Corynebacterium* genus in the nasal samples were associated with a lower asthma exacerbation/disease-control loss ratio [24,30]. Moreover, the Prevotella genus was inversely associated with asthma [20], and its lower abundance was found in the lower-airway samples from wheezers [47,48].

The mentioned bacteria tend to modify the immunological reaction through the promotion of specific genes in our immune system. The Moraxella-dominant samples were associated with the expression of 32 epithelial genes, mostly mediators of inflammation and apoptosis, whereas in the control group no expression was found [15]. *Streptococcus pneumoniae* in the asthmatics were associated with the arachidonic acid metabolism genes [21]. In Chun et al.'s study, *Corynebacterium* in the nasal samples was negatively correlated with inflammation-promoting genes, and *Actinomyces* in the lower-airway samples were negatively correlated with inflammation genes [42]. *Campylobacter* was found to be positively correlated with several inflammatory cytokines, such as granzyme B, MIP-1β and PD-L1. Additionally, *Haemophilus* was positively correlated with PD-L1, contrary to the negatively associated *Porphyromonas* and *Peptostreptococcus* [43].

5. Conclusions

This review provides an overview of the current knowledge about the airway bacterial microbiota of asthmatic children and preschool wheezers. As mentioned above, regardless of their location, the most dominant phyla of the airways are Proteobacteria, Firmicutes, Fusobacterium, Bacteroidetes and Actinobacteria. At the genus level, *Streptococcus*, *Moraxella* and *Haemophilus* were in most cases discriminant, indicating that their role is possibly far more important not only during various respiratory tract infections, but also as bacterial homeostatic and immunomodulatory factors. To assess whether asthma as a disease is characterized by higher bacterial diversity, further studies are needed because of the ambiguous outcomes of the collected data. For now, there is no particular known genus that highly discriminates asthmatics from healthy individuals; therefore, no biological interference in the microbiota of the airways is possible as a preventive intervention to reduce the disease prevalence. As seen in the prospective studies on infant wheezers, there are some visible dysbiosis clusters that may provoke the initiation of persistent wheezing/asthma in the future. Understanding the influence of microbiota on the integrity of the microbiome and immune responses in the airways is crucial for the future pharmacological/environmental implementation of certain strategies against the disease.

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8. DYSKUSJA

8.1. Praca oryginalna

Wyniki biostatystyczne i oznaczenia taksonomiczne naszej pracy wydają się zgodne z dotychczasowymi danymi. Dominującymi typami bakterii w wymazach z nosa okazywały się Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes i Fusobacteria [23]. Dzieci chorujące na astmę wykazywały mniejsze wartości alfa-różnorodności i różniły się względem różnorodności beta w wymazach z nosa, w stosunku do dzieci zdrowych [24]. W pracy Boutin i wsp. nie wykazano istotnej różnicy w wymazach z jamy ustno-gardłowej pomiędzy astmatykami a dziećmi zdrowymi [25], co również miało miejsce w przypadku naszych wyników. Podobne wyniki uzyskiwano również przy użyciu indukowanej plwociny jako materiału z dolnych dróg oddechowych u dorosłych. Do typów bakterii o największym udziale w próbkach należały Firmicutes, Proteobacteria, Actinobacteria [26], oraz dodatkowo Bacteroidetes [27]. W popłuczynach oskrzelowo-płucnych u astmatyków stwierdzano zwiększony udział rodzajów *Streptococcus* i *Prevotella*, członków Firmicutes i Bacteroidetes [28]. Stwierdzana w wieku 1 miesiąca życia w aspiracie z okolicy podgłośniowej zwiększona kolonizacja *Moraxella catarrhalis*, *Streptococcus pneumoniae* i *Haemophilus influenzae* zwiększała diagnozę astmy w wieku 5 lat [29]. W naszej pracy stwierdziliśmy zmniejszony udział Gammaproteobacteria i Bacilli, klas w których zawierają się wymienione patogenne bakterie, jednak ze względu na zbyt obszerną jednostkę taksonomiczną, nie możemy odnieść tych wyników do naszej publikacji.

Nie znaleźliśmy potwierdzenia w literaturze wpływy wziewnych alergii na bioróżnorodność bakteryjną dolnych dróg oddechowych. Wpływ wziewnych glikokortykosteroidów na mikrobiotę dróg oddechowych pozostaje niejasny. Wykazano zarówno brak wpływu na alfa-różnorodność mikrobioty bakteryjnej przy stosowaniu wziewnych GKS [30], jak i jej spadek u pacjentów stosujących dodatkowo doustne sterydy do terapii wziewnej, w porównaniu z pacjentami o astmie kontrolowanej samymi inhalacjami [31]. W pracy Zhang i wsp. wykazano zwiększony odsetek typu Firmictues i nieznaczny spadek Proteobacteria u pacjentów wymagających stosowania bardzo dużych dawek beklometazonu w stosunku do pacjentów z lepszą kontrolą choroby [32]. Dodatkowo wziewne glikokortykosteroidy okazały się być mniej skuteczne u pacjentów ze zwiększoną kolonizacją rodzaju Haemophilus związanego z astmą

[33]. W naszej pracy przyjmowanie leków przeciwastmatycznych, w tym wziewnych GKS w codziennym użyciu, było istotne statystycznie w odniesieniu do beta-różnorodności.

Poród drogą cięcia cesarskiego jest uznanym czynnikiem zwiększającym ryzyko astmy [34,35]. W naszej pracy stwierdziliśmy u dzieci rodzonych siłami natury zwiększoną różnorodność bakteryjną dolnych dróg oddechowych, co może być czynnikiem ochronnym dla rozwoju choroby. Na uwagę zasługuje fakt, że w naszej grupie badanej zdecydowana większość dzieci z rozpoznaną astmą była urodzona siłami natury (n=13), jednak ze względu na stosunkowo małe grupy uczestników badania, nie można uznać tego jako zaprzeczenie powyższych tez.

Zamieszkiwanie na wsi ma udowodniony ochronny wpływ na ryzyko wystąpienia zarówno astmy [36,37], jak i innych chorób alergicznych [38]. W naszych materiale z kondensatów wyodrębniono dwie różne grupy w zakresie beta-różnorodności w zakresie miejsca zamieszkania uczestników.

Bierne palenie i narażenie na dym tytoniowy jest również czynnikiem mającym wpływ na mikrobiotę układu oddechowego. W badaniu z udziałem dzieci uczestnicy narażeni na bierne palenie byli statystycznie częściej skolonizowani potencjalnie patogennymi bakteriami, w wymazach z nosa i nosogardła [39]. U dzieci chorujących na alergiczny nieżyt nosa stwierdzano niższą alfa-różnorodność w przypadku takiego narażenia [40]. W naszym badaniu narażenie na bierne palenie było czynnikiem różnicującym w zakresie beta-różnorodności w materiale z dolnych dróg oddechowych u astmatyków.

Niewątpliwą zaletą niniejszego badania jest fakt, że według naszej wiedzy było to pierwsze na świecie badanie mikrobioty bakteryjnej dolnych dróg oddechowych w populacji pediatrycznej astmatyków, przy pomocy kondensatów powietrza wydychanego. Ze względu na niepowodzenie dotychczas opisywanej metodologii uzyskiwania i amplifikacji materiału genetycznego zastosowano pionierską metodę, której opis może pomóc w dalszym dążeniu do ustalenia jednoznacznych wytycznych. Udowodniliśmy, że pomimo trudnego materiału (w ponad 99,99% składającego się z wody) badania mikrobiologiczne są możliwe i w przyszłości może stanowić alternatywę dla badań inwazyjnych, czy indukowanej plwociny, której techniczne wykonanie sprawia trudność młodszym pacjentom.

8.2. Praca poglądowa

Obecna wiedza na temat mikrobiomu bakteryjnego w populacji dziecięcej astmatyków nie jest w stanie jednoznacznie odpowiedzieć na pytanie które rodzaje/gatunki bakterii mogłyby zostać użyte celem prewencji rozwoju astmy oskrzelowej lub zaostrzeń choroby. Wskazuje się na podobieństwo mikrobiomu osób chorych oraz nie-astmatyków względem udziału głównych typów bakterii w mikrobiomie dróg oddechowych, różnice są wykazywane w większości na poziomie ilościowym, a nie jakościowym.

9. WNIOSKI

1. Mikrobiota bakteryjna górnych dróg oddechowych u astmatyków w populacji dziecięcej nie różni się istotnie w stosunku do dzieci zdrowych

2. Mikrobiota bakteryjna w uzyskanych próbkach z dolnych i górnych dróg oddechowych różni się między sobą

3. Bakteryjna mikrobiota dolnych dróg oddechowych astmatyków w populacji dziecięcej charakteryzuje się większą alfa-różnorodnością oraz beta-różnorodnością względem dzieci zdrowych

4. Mikrobiota bakteryjna dolnych dróg oddechowych dzieci urodzonych siłami natury cechuje się większą alfa-różnorodnością od urodzonych drogą cięcia cesarskiego

5. Astmatyków z dodatnim wywiadem rodzinnym w kierunku chorób alergicznych cechuje większa alfa-różnorodność w obrębie kondensatów powietrza wydychanego w stosunku astmatyków bez takiego czynnika

6. Najliczniej prezentowanymi typami bakterii w materiale z górnych i dolnych dróg oddechowych były Proteobacteria, Firmicutes i Bacteroidetes

7. Liczebność klas Gammaproteobacteria i Bacilli była statystycznie niższa w kondensatach powietrza wydychanego u astmatyków w porównaniu do dzieci zdrowych

8. W obrębie kondensatów powietrza wydychanego astmatyków do czynników wpływających na wyodrębnienie różnić w zakresie beta-bioróżnorodności pomiędzy grupami należały ekspozycja na dym tytoniowy, obciążenie rodzinne chorobami alergicznymi, przyjmowanie leków przeciwastmatycznych oraz miejsce zamieszkania (miasto/wieś).

9. Kondensat powietrza wydychanego może stanowić alternatywę dla badań mikrobiomu dolnych dróg oddechowych u dzieci w stosunku do obecnie przyjętych metod inwazyjnych

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11. ZAŁĄCZNIKI

11.1 DOROBEK NAUKOWY DOKTORANTA

lek. Kamil Bar

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1 Publikacje w czasopiśmie z IF

Lp	Opis bibliograficzny	IF	Punkty
1	Pieniawska-Śmiech Karolina, **Bar Kamil**, Babicki Mateusz, Śmiech Karol, Lewandowicz-Uszyńska Aleksandra: Assessment of weight and height of patients with primary immunodeficiency disorders and group of children with recurrent respiratory tract infections, BMC Immunology, 2020, vol. 21, art.42 [10 s.], DOI:10.1186/s12865-020-00372-x	3,615	70
2	Połomska Joanna, **Bar Kamil**, Sozańska Barbara: Exhaled breath condensate - a non-Invasive approach for diagnostic methods in asthma, Journal of Clinical Medicine, 2021, vol. 10, nr 12, art.2697 [17 s.], DOI:10.3390/jcm10122697	4,964	140
3	**Bar Kamil**, Żebrowska Paulina, Łaczmański Łukasz, Sozańska Barbara: Airway bacterial biodiversity in exhaled breath condensates of asthmatic children - does it differ from the healthy ones?, Journal of Clinical Medicine, 2022, vol. 11, nr 22, art.6774 [18 s.], DOI:10.3390/jcm11226774	4,964*	140
4	**Bar Kamil**, Litera-Bar Maja, Sozańska Barbara: Bacterial microbiota of asthmatic children and preschool wheezers' airways - what do we know?, Microorganisms, 2023, vol. 11, nr 5, art.1154 [18 s.], DOI:10.3390/microorganisms11051154	4,926*	40
	Podsumowanie	18,469	390

** IF 2021*

1.2 Publikacje w czasopiśmie bez IF

Lp	Opis bibliograficzny	Punkty
1	Pieniawska Karolina, Śmiech Karol, **Bar Kamil**, Pawlas Krystyna: Zawód przed zawodem - czy wypalenie może objawiać się już na studiach? Badanie populacji polskich studentów medycyny, Medycyna Środowiskowa -Environmental Medicine, 2017, vol. 20, nr 2, s. 22-31, DOI:10.19243/2017203	8
2	Biela Mateusz, Chrostek Remigiusz, Pluta Agnieszka, Winiarski Jacek, Ostromęcka Urszula, **Bar Kamil**, Jaremków Aleksandra, Pawlas Krystyna: Środowisko górskie jako miejsce aktywnego wypoczynku i związane z tym niebezpieczeństwa z uwzględnieniem wypadków w Tatrach Polskich, Medycyna Środowiskowa -Environmental Medicine, 2018, vol. 21, nr 3, s. 43-49, DOI:10.19243/2018306	8
	Podsumowanie	16

2. Monografie naukowe

2.1 Książka autorska -
2.2 Książka redagowana -

2.3 Rozdziały

Lp	Opis bibliograficzny	Punkty
1	**Bar Kamil**: Konspekt zajęć [Badanie przedmiotowe szczegółowe - układ oddechowy], W: Nowe strategie w kształceniu studentów : dobre praktyki - rekomendacje, (red.) Żanetta Kaczmarek, Janusz Morbitzer, Wrocław 2018, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, s. 271-275, ISBN 978-83-7055-379-1	0
	Podsumowanie	0

3. Abstrakty

Lp	Opis bibliograficzny
1	**Bar Kamil**: Anty-koncepcje, czyli niefortunne próby przeciwstawienia się rozrodczości, W: Konferencja "Interdyscyplinarność przyszłością nauki". Zieleniec, 13-15 kwietnia 2018. Księga abstraktów 2018, s. 13
2	**Bar Kamil**: You know nothing, doctor Snow - ostre stany pediatryczne w Centrum Symulacji Medycznych, W: Interdyscyplinarność przyszłością nauki. Zieleniec, 12-14 kwietnia 2019. Księga abstraktów 2019, s. 68, [[Dostęp 13.09.2019]. Dostępny w: http://www.doktoranci.umed.wroc.pl/wp-content/uploads/2019/09/Wiosna-2019-Biomed-1.pdf]

Impact factor: **18,469**

Punktacja ministerialna: **406,0**

28.04.2023 Piotr Owczarek

11.2 OŚWIADCZENIA O WSPÓŁAUTORSTWIE

mgr Paulina Żebrowska-Różańska

Laboratorium Genomiki i Bioinformatyki

Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda

Polskiej Akademii Nauk we Wrocławiu

Wrocław, 17.04.2023

OŚWIADCZENIE

Oświadczam, że w pracy :

Bar, K.; Żebrowska, P.; Łaczmański, Ł.; Sozańska, B. Airway Bacterial Biodiversity in Exhaled Breath Condensates of Asthmatic Children—Does It Differ from the Healthy Ones? *J. Clin. Med.* **2022**, *11*, 6774. https://doi.org/10.3390/jcm11226774

mój udział polegał na doborze metodologii badawczej, przygotowaniu materiału biologicznego, tj. ekstrakcji DNA oraz przygotowaniu bibliotek DNA do sekwencjonowania, wykonaniu sekwencjonowania DNA, analizie bioinformatycznej i statystycznej otrzymanych wyników, tworzeniu manuskryptu oraz akceptacji jego ostatecznej wersji.

P. Żebrowska-Różańska

.....

(podpis współautora)

Dr hab. Łukasz Łaczmański, prof. IITD PAN

Laboratorium Genomiki i Bioinformatyki

Instytut Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk we Wrocławiu

Wrocław, 17.04.2023

OŚWIADCZENIE

Oświadczam, że w pracy :

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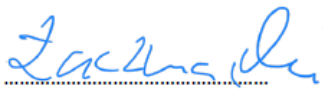
mój udział polegał na tworzeniu metodologii pracy oraz akceptacji ostatecznej wersji manuskryptu.



(podpis współautora)

Wrocław, 30.03.2023

Dr hab. n. med. Barbara Sozańska, prof. UM

I Katedra i Klinika Pediatrii, Alergologii i Kardiologii

Uniwersytet Medyczny we Wrocławiu

Oświadczenie

Oświadczam, że w pracy :

Bar K, Żebrowska P, Łaczmański Ł, Sozańska B. Airway Bacterial Biodiversity in Exhaled Breath Condensates of Asthmatic Children-Does It Differ from the Healthy Ones? J Clin Med. 2022 Nov 16;11(22):6774. doi: 10.3390/jcm11226774. PMID: 36431251; PMCID: PMC9698396.

Mój udział polegał na kierowaniu projektem naukowym STM.A220.20.057., obejmującym prowadzenie badań, które są opisane w tej pracy, w szczególności na udziale w tworzeniu koncepcji projektu naukowego, metodologii pracy, nadzorze nad realizacją pracy i opracowywaniu wyników a także tworzeniu oraz weryfikacji ostatecznej wersji manuskryptu.

Barbara Sozańska

Podpis współautora

Wrocław, 28.04.2023

Dr hab. n. med. Barbara Sozańska, prof. UM

I Katedra i Klinika Pediatrii, Alergologii i Kardiologii

Uniwersytet Medyczny we Wrocławiu

Oświadczenie

Oświadczam, że w pracy :

Bar, K.; Litera-Bar, M.; Sozańska, B. Bacterial Microbiota of Asthmatic Children and Preschool Wheezers' Airways—What Do We Know? *Microorganisms* 2023, *11*, 1154. https://doi.org/10.3390/microorganisms11051154

mój udział polegał na przygotowaniu tekstu, korekcie manuksryptu, jego ostatecznej akceptacji.

Uniwersytet Medyczny we Wrocławiu
I KATEDRA I KLINIKA PEDIATRII, ALERGOLOGII
I KARDIOLOGII
Kierownik
dr hab. n. med. Barbara Sozańska, profesor uczelni

Podpis współautora

Wrocław, 28.04.2023

lek. Maja Litera-Bar

Uniwersytecki Szpital Kliniczny we Wrocławiu

Oświadczenie

Oświadczam, że w pracy :

Bar, K.; Litera-Bar, M.; Sozańska, B. Bacterial Microbiota of Asthmatic Children and Preschool Wheezers' Airways—What Do We Know? *Microorganisms* **2023**, *11*, 1154. https://doi.org/10.3390/microorganisms11051154

mój udział polegał na przygotowaniu tekstu oraz zaakceptowaniu ostatecznej wersji manuskryptu.

Maja Litera-Bar

Podpis współautora

11.3 ZGODA KOMISJI BIOETYCZNEJ

KOMISJA BIOETYCZNA
przy
Uniwersytecie Medycznym
we Wrocławiu
ul. Pasteura 1; 50-367 WROCŁAW

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 175/2019

Komisja Bioetyczna przy Uniwersytecie Medycznym we Wrocławiu, powołana zarządzeniem Rektora Uniwersytetu Medycznego we Wrocławiu nr 133/XV R/2017 z dnia 21 grudnia 2017 r. oraz działająca w trybie przewidzianym rozporządzeniem Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. (Dz.U. nr 47, poz. 480) na podstawie ustawy o zawodzie lekarza z dnia 5 grudnia 1996 r. (Dz.U. nr 28 z 1997 r. poz. 152 z późniejszymi zmianami) w składzie:

dr hab. Jacek Daroszewski, prof. nadzw. (endokrynologia, diabetologia)
prof. dr hab. Krzysztof Grabowski (chirurgia)
dr Henryk Kaczkowski (chirurgia szczękowa, chirurgia stomatologiczna)
mgr Irena Knabel-Krzyszowska (farmacja)
prof. dr hab. Jerzy Liebhart (choroby wewnętrzne, alergologia)
ks. dr hab. Piotr Mrzygłód, prof. nadzw. (duchowny)
mgr Luiza Müller (prawo)
dr hab. Sławomir Sidorowicz (psychiatria)
dr hab. Leszek Szenborn, prof. nadzw (pediatria, choroby zakaźne)
Danuta Tarkowska (pielęgniarstwo)
prof. dr hab. Anna Wiela-Hojeńska (farmakologia kliniczna)
dr hab. Andrzej Wojnar, prof. nadzw. (histopatologia, dermatologia) przedstawiciel Dolnośląskiej Izby Lekarskiej)
dr hab. Jacek Zieliński (filozofia)

pod przewodnictwem
prof. dr hab. Jana Kornafela (ginekologia i położnictwo, onkologia)

Przestrzegając w działalności zasad Good Clinical Practice oraz zasad Deklaracji Helsińskiej, po zapoznaniu się z projektem badawczym pt.:

„Odrębności bakteryjnej mikrobioty dróg oddechowych w wymazie z jamy ustno-gardłowej oraz kondensatach powietrza wydychanego w przebiegu astmy oskrzelowej w populacji dziecięcej"

zgłoszonym przez **lek. Kamila Bara** uczestnika studiów doktoranckich w I Katedrze i Klinice Pediatrii, Alergologii i Kardiologii Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła **wyrazić zgodę** na przeprowadzenie badania w Klinice Pediatrii, Alergologii i Kardiologii USK; w NZOZ „Twój Lekarz" w Kobierzycach oraz Instytucie Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk we Wrocławiu pod nadzorem dr hab. Barbary Sozanskiej **pod warunkiem zachowania anonimowości uzyskanych danych**.

Uwaga: Badanie to zostało objęte ubezpieczeniem odpowiedzialności cywilnej Uniwersytetu Medycznego we Wrocławiu z tytułu prowadzonej działalności.

Pouczenie: W ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej UM we Wrocławiu.

Opinia powyższa dotyczy projektu badawczego będącego podstawą rozprawy doktorskiej.

Wrocław, dnia 8 stycznia 2019 r.

Uniwersytet Medyczny w [illegible]awiu
KOMISJA BIOETYCZNA
przewodniczący
prof. dr hab. Jan Kornafel

11.4 ANKIETA DLA RODZICÓW W RAMACH PRACY ORYGINALNEJ

*Zwracam się do Pani/Pana z uprzejmą prośbą o wzięcie udziału w poniższej ankiecie. Udział w badaniu jest dobrowolny, a ankieta jest w pełni **anonimowa**. Zebrane informacje zostaną wykorzystane **wyłącznie w celach naukowych.***

Z góry bardzo dziękuję!

1. Inicjały pacjenta :

2. Płeć : K ☐ M ☐

3. Wiek :

4. Wzrost :

5. Masa ciała :

6. Miejsce zamieszkania : wieś/miasto do 50 tys. mieszkańców/ miasto od 50 do 100 tys. mieszkańców/ miasto powyżej 100 tys. mieszkańców

7. Czy w miejscu stałego zamieszkania (dom, mieszkanie) dziecko ma kontakt z :

- wilgocią ☐nie ☐tak
- pleśnią ☐nie ☐tak
- zwierzętami ☐nie ☐tak

(jeśli tak, z jakimi - .....)

8. Czy wśród osób mieszkających na stałe z dzieckiem są osoby palące?

☐nie ☐tak (Jeśli tak, kto .....)

7. Urodzony/a : o czasie/ przedwcześnie/ po terminie (o czasie - pomiędzy ukończonym 37 tygodniem życia do końca 41 tygodnia)

8. Poród drogą : naturalną / cięcie cesarskie

9. Liczba pkt w skali Apgar w 1 minucie życia (0-10) :

10. Czy ma rozpoznaną astmę oskrzelową? ☐nie ☐tak

11. Czy ktoś w rodzinie (rodzice, dziadkowie, rodzeństwo) choruje na :

- astmę oskrzelową ☐nie ☐tak
- atopowe zapalenie skóry ☐nie ☐tak
- alergiczne zapalenie spojówek ☐nie ☐tak
- alergiczny nieżyt nosa ☐nie ☐tak

12. Czy zdiagnozowano u dziecka alergię? ☐nie ☐tak

(Jeśli tak - jaką .....)

13. Czy choruje na coś przewlekle (poza astmą oskrzelową)?

☐nie ☐tak

(jeśli tak - na co .....)

14. Czy przyjmuje na stałe leki? ☐nie ☐tak

(jeśli tak - jakie?).....

15. Czy w przeciągu ostatnich 4 tygodni stosowano u dziecka antybiotykoterapię? ☐nie ☐tak

16. (Jeśli w 15 zaznaczono "tak) Proszę wskazać grupę antybiotyków, które przyjmował w tym czasie syn/córka:

☐ peniciliny (Ospen, Amoksiklav, Augmentin)

☐ cefalosporyny (Zinnat, Biofuroksym)

☐ makrolidy (Sumamed, Klacid, Klabax)

☐ Biseptol

Inne (wpisać jakie) .....

17. Czy w przeciągu ostatniego miesiąca wystąpiło u dziecka :

- zapalenie oskrzeli ☐nie ☐tak
- zapalenie zatok ☐nie ☐tak
- nieżyt górnych dróg oddechowych ("przeziębienie") ☐nie ☐tak
- grypa ☐nie ☐tak
- zapalenie płuc ☐nie ☐tak
- angina paciorkowcowa ☐nie ☐tak
- zapalenie ucha środkowego ☐nie ☐tak
- inne zakażenie .....

18. Czy dziecko ma zdiagnozowany niedobór odporności?

☐nie ☐tak

19. Czy dziecko jest szczepione zgodnie z kalendarzem szczepień ?

☐nie ☐tak