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Charakterystyka składników lipidowych i analiza wrażliwości *Legionella gormanii* na apolipoforynę III *Galleria mellonella*

Characteristics of lipid components and analysis of Legionella gormanii sensitivity to Galleria mellonella apolipophorin III

Rozprawa doktorska

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a także Pani prof. dr hab. Teresie Urbanik-Sypniewskiej

za poświęcony czas, opiekę naukową, cenne uwagi merytoryczne,

okazaną mi pomoc oraz niezwykłą wyrozumiałość i życzliwość.

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Wyniki niniejszej rozprawy doktorskiej zostały opublikowane w trzech oryginalnych artykułach naukowych oraz pracy przeglądowej:

Publikacja 1

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Spis treści

2. Wykaz skrótów

AFM – (ang. *Atomic Force Microscope*) - mikroskop sił atomowych

apoLp-III – apolipoforyna III

BCYE - (ang. *Buffered Charcoal Yeast Extract*) - buforowany węglem drzewnym wyciąg drożdżowy

Cer - ceramidy

CL – kardiolipina

CMP – dwufosforan cytydyny

DG - dwuglicerydy

DMPE (dMePE) - dimetylo-N,N-fosfatydyloetanoloamina

Dot – (ang. *Defect for organelle trafficking*) - zaburzenia w kierowaniu organelli

GC/MS - (ang. *Gas Chromatography/Mass Spectrometry*) - chromatografia gazowa sprzężona ze spektrometrią mas

GlcNAc - N-acetyloglukozamina

Icm – (ang. *Intracellular multiplication*) - wewnątrzkomórkowe namnażanie

IL-6 - interleukina 6

IM – (ang. *Inner Membrane*) - wewnętrzna błona

Kdo - kwas 3-deoksy-D-*manno*-2-oktulozonowy

KEGG – (ang. *Kyoto Encyclopedia of Genes and Genomes*) – bazy danych Kyoto encyklopedia genów i genomów

lag-1 - gen kodujący O-acetylotransferazę

LPS – lipopolisacharyd

LpSpl - liaza 1-P-sfingozyny

MIC– (ang. *Minimum Inhibitory Concentration*) - minimalne stężenie hamujące

Man - mannoza

MMPE - monometylo-N-fosfatydyloetanoloamina

MSA – (ang. *Multiple Sequence Alignment*) – wielokrotne przyrównanie/dopasowanie sekwencji

OM – (ang. *Outer Membrane*) - zewnętrzna błona

OMV – (ang. *Outer Membrane Vesicles*) - pęcherzyki błony zewnętrznej

PAF – (ang. *Platelet-Activating Factor*) - czynnik aktywujący płytki krwi

PC – fosfatydylocholina

PCS – szlak syntazy fosfatydylocholiny

pcs – gen kodujący syntazę PC

PE – fosfatydyloetanoloamina

PG – fosfatydyloglicerol

pmtA – gen kodujący N-metylotransferazę

PMTA - szlak N-metylacji fosfatydyloetanoloaminy

PSI-BLAST – (ang. *Position-Specific Iterative Basic Local Alignment Search Tool*) – specyficzny dla pozycji, iterowany BLAST

QuiNAc - N-acetylochinowozamina

Rha – ramnoza

SAH - S-adenozylohomocysteina

SAM - S-adenozylometionina

SM – sfingomielinaza

SMase Cs - sfingomielinaza Cs

SMase Ds - sfingomielinaza Ds

SMPD1 - fosfodiesteraza-1 sfingomieliny

SPT - palmitoilotransferaza seryny

TLC – (ang. *Thin Layer-Chromatography*) - chromatografia cienkowarstwowa

TLR4 - (ang. *Toll-Like Receptor 4*) – receptor toll-podobny 4

TG – trójglicerydy

TNF-α – (ang. *Tumor Necrosis Factor α*) - czynnik martwicy nowotworu

UHPLC-MS/MS - (ang. *Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry*) - ultra wysokosprawna chromatografia cieczowa sprzężona ze spektrometrią mas

3. Streszczenie

Pałeczki *Legionella* są szeroko rozpowszechnione w naturalnych, słodkowodnych środowiskach oraz sztucznych systemach wodnych, zarówno jako wolnożyjące bakterie, wchodzące w skład złożonych struktur biofilmu, ale przede wszystkim jako wewnątrzkomórkowe patogeny organizmów eukariotycznych. Adaptacja do tak skrajnie odmiennych warunków, jakim jest środowisko wodne ubogie w składniki odżywcze i wnętrze komórki gospodarza, bogate w pokarm, wymaga uruchomienia przez te bakterie szeregu mechanizmów. W wyspecjalizowanych oddziaływaniach z komórkami gospodarza pałeczki *Legionella* wykorzystują lipidy. Lipidy *Legionella* pełnią zarówno ważne funkcje strukturalne, które pozwalają na stabilizację błon komórkowych oraz funkcjonują jako czynniki wirulencji, antygeny bądź wzorce molekularne rozpoznawane przez układ odpornościowy gospodarza. Podobnie jak w przypadku białek, bakterie *Legionella* mają zdolność dostosowywania składu lipidów błonowych w odpowiedzi na zmieniające się warunki środowiska. *L. gormanii* syntetyzuje glicerolipidy (trójglicerydy i dwuglicerydy), fosfolipidy (fosfatydyloetanoloamina, PE, fosfatydylocholina, PC, kardiolipina, CL, fosfatydyloglicerol, PG) oraz sfingolipidy (ceramidy i heksozylceramidy). Zastosowanie metod opartych na chemicznej analizie markerów strukturalnych osłon komórkowych pozwoliło na wykazanie, że w błonach *L. gormanii* występują związki, które charakteryzują różne gatunki *Legionella* takie jak PE15:0_15:0 oraz PC15:0_16:0. Natomiast PEcyklopropanowy17:0_16:0 oraz PCcyklopropanowy17:0_15:0 mogą stanowić chemiotaksonomiczne wyznaczniki gatunku *L. gormanii*. Zdolność do wykorzystywania zewnątrzkomórkowej choliny i syntezy PC w jednoetapowym szlaku katalizowanym przez syntazę fosfatydylocholiny potwierdzona dla *L. gormanii* wskazuje, że proces ten jest wspólny dla różnych gatunków *Legionella*. Syntaza PC może być obiecującym celem terapeutycznym osłabiającym zdolność do wewnątrzkomórkowego namnażania się tych patogenów. *L. gormanii* hodowana na podłożu z egzogenną choliną modyfikuje zawartość i rozmieszczenie lipidów w błonach, co wpływa na jej fizyko-chemiczne właściwości i determinuje wrażliwość tych bakterii na bójcze działanie apolipoforyny III wyizolowanej z hemolimfy gąsienic *Galleria mellonella*.

Słowa kluczowe: *Legionella gormanii*, choroba legionistów, lipidy, apolipoforyna III, *Galleria mellonella*

4. Summary

Legionella bacteria are widely distributed in natural freshwater environments and artificial water systems both as free-living bacteria that are part of complex biofilm structures and primarily as intracellular pathogens of eukaryotic organisms. Adaptation to such extremely different conditions as the aquatic environment, poor in nutrients, and the interior of the host cell, rich in food, requires the activation of several mechanisms by these bacteria. In specialized interactions with host cells, *Legionella* bacteria use lipids. *Legionella* lipids play essential structural functions that stabilize cell membranes and function as virulence factors, antigens, or molecular patterns recognized by the host's immune system. As in the case of proteins, *Legionella* bacteria can adapt the composition of membrane lipids in response to changing environmental conditions. *L. gormanii* synthesizes glycerolipids (triglycerides and diglycerides), phospholipids (phosphatidylethanolamine, PE, phosphatidylcholine, PC, cardiolipin, CL, phosphatidylglycerol, PG), and sphingolipids (ceramides and hexosylceramides). The use of methods based on the chemical analysis of structural markers of the cell envelope allowed demonstrating that the membranes of *L. gormanii* contain compounds that characterize various *Legionella* species, such as PE15:0_15:0 and PC15:0_16:0. On the other hand, PEcyclopropane17:0_16:0 and PCcyclopropane17:0_15:0 may be chemotaxonomic determinants of the *L. gormanii* species. The ability to use extracellular choline and synthesize PC in a one-step pathway catalyzed by phosphatidylcholine synthase confirmed for *L. gormanii* indicates that this process is common to different *Legionella* species. PC synthase may be a promising therapeutic target to impair the intracellular proliferation capacity of these pathogens. *L. gormanii* cultured on a medium with exogenous choline modifies the content and distribution of lipids in membranes, which affects its physicochemical properties and determines the sensitivity of these bacteria to the killing effect of apolipophorin III isolated from the hemolymph of *Galleria mellonella*.

Keywords: *Legionella gormanii*, Legionnaires' disease, lipids, apolipophorin III*, Galleria mellonella*

5. Wstęp

5.1. Charakterystyka bakterii z rodzaju *Legionella*

Gram-ujemne pałeczki z rodzaju *Legionella* należą do grupy wewnątrzkomórkowych bakteryjnych czynników zakaźnych, które powodują infekcje zarówno dróg oddechowych jak i infekcje pozapłucne, określane wspólną nazwą legionelozy. Bakterie występują w wodach naturalnych i sztucznych systemach dystrybucji wody. W środowisku naturalnym tzn. w wodach śródlądowych, morskich czy w glebie, bakterie ze względu na niską liczbę nie stanowią zagrożenia higienicznego. Duże niebezpieczeństwo dla zdrowia i życia ludzi stwarzają skolonizowane bakteriami instalacje wodne budynków użyteczności publicznej i obiektów szpitalnych. Na stopień zasiedlenia systemów wodnych wpływają zarówno czynniki abiotyczne, jak i biotyczne. Występowaniu pałeczek *Legionella* sprzyjają miejsca o niewielkiej recyrkulacji wody, bogate w składniki migrujące z elementów instalacji wodociągowej. Największym ryzykiem kolonizacji tymi bakteriami obarczone są instalacje wody ciepłej. Pałeczki z rodzaju *Legionella* bytują w systemach wodnych w zakresie temperatur od 25°C do 37°C. Drobnoustroje te są zdolne do przeżycia przez kilka godzin w temperaturze powyżej 50°C oraz namnażania się w temperaturze poniżej 20°C (Buse i in., 2012). Kluczowym czynnikiem wpływającym na występowanie tych bakterii w sztucznych systemach wodnych jest obecność pierwotniaków głównie z rodzajów *Vermamoeba*, *Acanthamoeba i Naegleria* (Atif Nisar i in., 2020). Bakterie *Legionella* nabyły rzadkiej wśród mikroorganizmów zdolności do aktywnego wnikania i namnażania się w komórkach pierwotniaków, które są nie tylko źródłem pokarmu dla tych bakterii ale chronią je przed działaniem niekorzystnych czynników środowiska, w tym środków dezynfekujących wodę (Atif Nisar i in., 2020). Wszystkie te czynniki stwarzają dogodne warunki do tworzenia biofilmów i rozwoju *Legionella*. Badania przeprowadzone w Polsce wykazały, że ponad 60% zbadanych prób wody przekraczało dopuszczalny poziom skażenia sieci wodnej bakteriami *Legionella*, co stwarza realne zagrożenie dla ludzi (Wojtyła-Buciora i in., 2013). Najczęstszym sposobem infekcji człowieka jest wdychanie skażonego bakteriami wodno-powietrznego aerozolu wytwarzanego przez klimatyzatory, fontanny, wanny z hydromasażem, urządzenia medyczne i tym podobne źródła (Van Heijnsbergen i in., 2015). Jednak około 60% zgłaszanych przypadków infekcji *Legionella* nie ma zidentyfikowanego źródła pochodzenia. Do zakażenia bakteriami *Legionella* dochodzi również przez aspirację tych

mikroorganizmów z błon śluzowych gardła oraz w rzadkich przypadkach w wyniku transmisji między ludźmi (Correia i in., 2016). Bakterie *Legionella* po przedostaniu się do organizmu człowieka wywołują szeroki zakres objawów chorobowych od grypo-podobnej infekcji zwanej gorączką Pontiak do ciężkiego zapalenia płuc (choroby legionistów), które należy do zapaleń atypowych, nie poddających się leczeniu antybiotykami z grupy penicylin oraz aminoglikozydów (Viasus i in., 2022). Bakterie *Legionella* mogą być również przyczyną pozapłucnych infekcji o bardzo ciężkim przebiegu ze strony układów: sercowo-naczyniowego (zapalenie mięśnia sercowego), nerwowego (ropień mózgu), pokarmowego (zapalenie trzustki), moczowego (ostra niewydolność nerek). Pałeczki *Legionella* są przyczyną zarówno sporadycznych przypadków, jak i ognisk epidemicznych zapalenia płuc nabytego w środowisku zamieszkania, pracy, rekreacji lub podczas pobytu w szpitalu. Ponad 70% przypadków zachorowań na chorobę legionistów jest sporadyczna i nabyta w środowisku pozaszpitalnym. Pozostałe infekcje związane są z podróżami (ponad 20%) lub z pobytem osób w placówkach opieki zdrowotnej (około 10%). Bakterie *Legionella* powodują od 2 do 15% wszystkich hospitalizowanych przypadków zapalenia płuc nabytego w środowisku zamieszkania i są drugą po *Streptococcus pneumoniae* najczęstszą przyczyną ciężkiego zapalenia płuc, które wymaga przyjęcia pacjenta na odział intensywnej terapii. Znane czynniki ryzyka rozwoju choroby legionistów można podzielić na czynniki związane z gospodarzem i środowiskowe. Czynniki związane z gospodarzem obejmują: starszy wiek (>50 lat), płeć męską, palenie tytoniu, przewlekłe choroby płuc, cukrzycę i wrodzony lub nabyty w wyniku przeszczepu i/lub chemioterapii niedobór odporności. U osób z obniżoną odpornością, biorców przeszczepów oraz dzieci poniżej pierwszego roku życia poziom śmiertelności jest wysoki i wynosi około 50%. Do środowiskowych czynników ryzyka związanych z epidemiami choroby legionistów zaliczamy podróże, pobyt w placówce służby zdrowia, bliskość wież chłodniczych, wanien z hydromasażem czy ozdobnych fontann (Alrahimi i in., 2022). Prawdopodobieństwo zakażenia zależy od dawki infekcyjnej i czasu ekspozycji na bakterie, skuteczności rozprzestrzeniania się drobnoustrojów, określonych predyspozycji gospodarza oraz gatunku, który spowodował infekcję. Do tej pory wyizolowano i opisano 69 gatunków i ponad 70 grup serologicznych, z których 28 pochodziło bezpośrednio od chorych (ECDC, 2022). Wśród szczepów gatunku *L. pneumophila*, które są przyczyną 80-90% rozpoznawanych przypadków legionelozy wyróżniono 16 grup serologicznych, z których serogrupa 1 jest najczęściej izolowana od chorych. Pozostałe serogrupy i gatunki są rzadziej izolowane od pacjentów

z wyjątkiem *L. longbeachae*, który odpowiada za 60% zakażeń występujących w Australii i Nowej Zelandii (Chambers i in., 2021).

Gatunek *L. gormanii* został po raz pierwszy wyizolowany od pacjentki z nietypowym zapaleniem płuc i współtowarzyszącymi chorobami (Griffith i in., 1988). Bakteria ta powoduje infekcje również u osób z prawidłowo funkcjonującym układem odpornościowym oraz u dzieci, chociaż *Legionella* rzadko powodują zapalenie płuc w tej grupie wiekowej pacjentów (Lei i in., 2022) (Greenberg i in., 2006). *L. gormanii* w odróżnieniu od innych gatunków rodzaju *Legionella* nie namnaża się w komórkach *A. castelanii* ale ma zdolność do adhezji, wnikania i replikacji w makrofagach linii THP-1. Analizy bioinformatyczne genomu *L. gormanii* wykazały obecność klastra genów kodujących białka IV systemu sekrecji Dot/Icm, który jest kluczowy w procesie tworzenia niszy umożliwiającej namnażanie się bakterii wewnątrz makrofagów człowieka (Peruski i in., 2017). Przełamanie mechanizmów bójczych i utworzenie stabilnej wakuoli replikacyjnej poprzez modyfikację środowiska gospodarza stanowi podstawę patomechanizmu *Legionella.* Skoordynowane w czasie i przestrzeni manipulowanie procesami komórkowymi gospodarza jest możliwe dzięki sprawnie funkcjonującej bakteryjnej ścianie komórkowej. *Legionella* nie wytwarzają otoczek oraz nie syntetyzują zewnątrzkomórkowych polisacharydów, dlatego makrocząsteczki ściany komórkowej (białka, lipidy, LPS) wchodzą w bezpośredni kontakt z komórką gospodarza i determinują przebieg różnych etapów cyklu życiowego tych drobnoustrojów. W cyklu życiowym *Legionella* występuje faza troficzna, która ma miejsce w wyspecjalizowanych wakuolach gospodarza i faza infekcyjna, w której bakterie wykazują dużą przeżywalność w warunkach abiotycznych. Przejście pałeczek *Legionella* ze środowiska wodnego, które nie stwarza warunków umożliwiających namnażanie się, do środowiska nieprzyjaznego, ale bogatego w składniki pokarmowe, jakim jest aktywnie metabolizująca komórka gospodarza wymaga modyfikacji struktury składników ściany komórkowej bakterii.

Budowa, struktura oraz znaczenie biologiczne lipidowych składników ściany komórkowej *Legionella* zostały przedstawione w pracy przeglądowej:

Kowalczyk, B., **Chmiel, E**., Palusińska-Szysz, M. (2021). The role of lipids in *Legionella*-host interaction. *Int. J. Mol. Sci.,* 2;22(3); 1487. doi: 10.3390/ijms22031487.

5.2. Lipidy ściany komórkowej *Legionella*

Osłona komórkowa stanowi złożoną, wielowarstwową, dynamiczną strukturę, która utrzymuje kształt i integralność komórki bakteryjnej. W ścianie komórkowej Gram-ujemnych bakterii występują dwie błony: wewnętrzna (Inner Membrane, IM) i zewnętrzna (Outer Membrane, OM). Błona wewnętrzna składa się z fosfolipidów, które u bakterii Gram-dodatnich i Gram-ujemnych są rozmieszczone dość równomiernie w obu monowarstwach tworząc symetryczną dwuwarstwę. W przeciwieństwie do niej OM ma układ asymetryczny: warstwa wewnętrzna składa się głównie z fosfolipidów a zewnętrzna z fosfolipidów, białek i glikolipidu-lipopolisacharydu (LPS). Białka transbłonowe OM mają w większości strukturę drugorzędową β-harmonijki, podczas gdy w błonie wewnętrznej w białkach transbłonowych dominują struktury α-helisy. Asymetryczna budowa i wyjątkowe właściwości LPS sprawiają, że OM funkcjonuje jako pierwsza linia obrony komórki bakteryjnej przed zewnętrznymi zagrożeniami biotycznymi i abiotycznymi, stanowi barierę przepuszczalności molekularnej, chroni przed fagocytozą i działaniem substancji przeciwdrobnoustrojowych (Silhavy i in., 2010).

5.2.1. Charakterystyka lipopolisacharydu *Legionella*

Lipopolisacharyd jest kluczowym składnikiem OM ściany komórkowej Gram-ujemnych bakterii. Bierze udział w stabilizacji jej struktury, a wprowadzony do organizmu wyższego staje się endotoksyną. LPS zbudowany jest z trzech kowalencyjnie połączonych strukturalnych domen: lipidu A, rdzenia oraz łańcucha cukrowego O-swoistego O-PS. Najlepiej poznanym LPS wśród gatunków *Legionella* jest LPS *L. pneumophila*. O-PS *L. pneumophila* szczep Philadelphia 1 jest silnie hydrofobowym homopolimerem składającym się z 10-75 podjednostek kwasu legionaminowego (5-acetamidino-7-acetamido-8-O-acetylo-3,5,7,9-tetradeoksy-L-glycero-D-galakto-non-2-ulozonowego) połączonego wiązaniami α (2-4). O-polimer jest przyłączony do rdzenia zewnętrznego zbudowanego z 7 reszt cukrowych: ramnozy (Rha), mannozy (Man), N-acetylochinowozaminy (QuiNAc) i N-acetyloglukozaminy (GlcNAc). Rdzeń zewnętrzny ma charakter hydrofobowy związany z obecnością grup metylowych 6-deoksycukrów oraz podstawników O-acetylowych. Rdzeń wewnętrzny jest natomiast hydrofilowy, składa się z 2 cząsteczek kwasu 3-deoksy-D-manno-2-oktulozonowego (Kdo) związanych ketozydowo (2-4) i cząsteczki D-mannozy przyłączonej do reszty Kdo w pozycji C8. Rdzeń wewnętrzny LPS *L. pneumophila* wyróżnia brak reszt heptozy oraz grup fosforanowych, które występują u wielu bakterii np. z rzędu *Enterobacterales*. Również

kilka innych niż *L. pneumophila* gatunków np.: *L. feeleii*, *L. jordanis*, *L. erythra*, *L. bozemanae*, *L. oakridensis* i *L. micdadei* posiada w LPS D- i L-glicero-D-mannoheptozę.

Wśród strukturalnie scharakteryzowanych lipopolisacharydów bakterii *Legionella*, tylko *L. pneumophila* syntetyzuje kwas legionaminowy. W części polisacharydowej LPS pozostałe *Legionella* zawierają znaczne ilości D-mannozy i D-glukozaminy oraz cukry specyficzne dla danego gatunku np. 3-amino-3,6-dideoksy-mannozę dla *L. israelensis*, kwas galakturonowy i galaktozaminę dla *L. hackeliae*, jersiniozę dla *L. micdadei* i *L. maceachernii*.

Z kwasem 3-deoksy-D-manno-oktulozonowym oligosacharydowego rdzenia za pomocą wiązania ketozydowego wiąże się lipid A, który osadzony jest w zewnętrznej warstwie błony zewnętrznej. Najbardziej konserwatywną i archetypową strukturą lipidu A jest dwucukrowy szkielet, który u *L. pneumophila* składa się z połączonych wiązaniem β glikozydowym (1→6) dwóch cząsteczek ufosforylowanej 2,3-diamino-2,3-dideoksy-D-glukozy (D-GlcpN3N). *L. israelensis* i *L. bozemanae* syntetyzują mieszany disacharyd zbudowany z GlcN3N i N-acetyloglukozaminy (GlcNAc). Grupy amidowe D-GlcpN3N lipidu A *L. pneumophila* są podstawione przez 3-hydroksy i 2,3-dihydroksy kwasy tłuszczowe. Z kolei grupy hydroksylowe tych kwasów są acylowane przez drugorzędowe kwasy tłuszczowe o różnej naturze, prostołańcuchowe, rozgałęzione (*izo* i *anteizo*) oraz długołańcuchowe. Do długołańcuchowych kwasów występujących w lipidzie A *L. pneumophila* należą: 28:0(27-OH), 28:0(27-okso), 30:0(29-okso) oraz (27-diowy i 29-diowy). Wyjątkową cechą lipidu A bakterii *Legionella* jest duża zawartość estrowo związanych kwasów tłuszczowych: hydroksy oraz długołańcuchowych (-ω)-okso, (-ω)-hydroksy i (-ω)-dikarboksylowych. W lipidzie A wszystkich gatunków występuje kwas 27-okso-oktakozanowy, który uważany jest za chemiotaksonomiczny marker tej grupy bakterii.

Lipid A zakotwicza cząsteczki LPS w błonie zewnętrznej poprzez oddziaływania hydrofobowe z łańcuchami acylowymi fosfolipidów, które budują wewnętrzną warstwę tej błony.

5.2.2. Budowa i właściwości fosfolipidów *Legionella*

Fosfolipidy stanowią główny składnik budulcowy lipidowych błon komórkowych. Składają się z rdzenia oraz przyłączonych do niego kwasów tłuszczowych, reszty kwasu

fosforowego oraz związanego z nią alkoholu: w przypadku gdy szkieletem jest glicerol, noszą nazwę glicerofosfolipidów, jeżeli szkielet jest nienasyconym aminoalkoholem, sfingozyną tworzy grupę sfingolipidów. Ceramidy należą do klasy sfingolipidów i są połączeniem długołańcuchowych kwasów tłuszczowych z grupami aminowymi szkieletu sfingozynowego.

W błonach *L. pneumophila*, *L. lytica*, *L. bozemanae, L. dumoffi*, *L. anisa*, *L. longbeacheae* zidentyfikowano następujące klasy fosfolipidów: fosfatydylocholinę (PC), fosfatydyloetanoloaminę (PE), kardiolipinę (CL) i fosfatydyloglicerol (PG). Głównymi fosfolipidami bakterii *Legionella* jest PE i PC. Fosfatydylocholina, która stanowi od 30% do 50% zawartości fosfolipidów jest syntetyzowana na drodze dwóch niezależnych szlaków: PMTA i PCS. W szlaku PMTA, występującym w komórkach eukariotycznych i prokariotycznych substratem jest PE, która jest trzykrotnie N-metylowana w wyniku czego powstaje PC, a produktami pośrednimi tych reakcji są monometylo-N-fosfatydyloetanoloamina (MMPE) i dimetylo-N,N-fosfatydyloetanoloamina (DMPE). Enzymem katalizującym reakcję metylacji jest kodowana przez gen *pmtA* N-metylotransferaza, która jako donor grupy metylowej wykorzystuje S-adenozylometioninę (SAM), ulegającą przekształceniu do S-adenozylohomocysteiny (SAH). Geny *pmtA Legionella* kodują małe cytozolowe białka o długości od 208 do 218 aminokwasów. Domena katalizująca tych białek zawiera charakterystyczny dla metylotransferaz motyw o długości 9 aminokwasów: V/ILE/DXGXGXG, który wiąże S-adenozylometioninę (SAM).

Drugi szlak PCS jest jednoetapową reakcją polegającą na bezpośredniej kondensacji choliny z CDP-diacyloglicerolem z utworzeniem PC i dwufosforanu cytydyny (CMP). Proces ten jest katalizowany przez występującą wyłącznie w bakteriach syntazę fosfatydylocholiny, kodowaną przez gen *pcsA*. Analiza porównawcza sekwencji nukleotydowych genów *pcsA* wykazała wysoką identyczność sekwencji tych genów wśród *Legionella* (od 64% do 98%).

Syntazy fosfatydylocholiny różnych gatunków *Legionella* mają zbliżoną długość wynoszącą 254 aminokwasy i zawierają wysoce konserwatywny region: DGX2ARX8PX3GX3DX3D. Białka Pcs wykazują charakter hydrofobowy i posiadają do ośmiu transbłonowych helis z N- i C-końcami zlokalizowanymi w cytoplazmie. Region N-końcowy białka zawiera domenę odpowiedzialną za aktywność enzymatyczną.

Obecność tego enzymu w komórkach *Legionella* umożliwia bakteriom syntezę PC z choliny pobieranej z komórki gospodarza.

5.2.3. Znaczenie składników lipidowych *Legionella* w oddziaływaniu z komórkami gospodarza

Modulacja struktury składników lipidowych (fosfolipidów, LPS) umożliwiła pałeczkom *Legionella* skuteczną ochronę przed działaniem komórek i czynników układu immunologicznego. LPS jest głównym antygenem rozpoznawanym przez komórki zakażonego organizmu. Antygen jest wydalany do moczu już od pierwszego dnia wystąpienia objawów klinicznych, co stanowi podstawę testu diagnostycznego, który jest najczęściej stosowany do wykrywania zakażeń spowodowanych przez *L. pneumophila* sg 1. Przeciwciała anty-LPS *L. pneumophila* są wykrywane w surowicy krwi pacjentów. Dzięki opracowaniu panelu diagnostycznego z przeciwciałami monoklonalnymi rozpoznającymi epitopy w O-PS (panel Drezdeński) istnieje możliwość rozróżnienia 15 grup i 9 podgrup serologicznych w obrębie serogrupy 1 *L. pneumophila*. Badania prowadzone w Europie i Azji wykazały, że szczepy *L. pneumophila* sg 1 będące nosicielami genu *lag-1* kodującego O-acetylotransferazę, która odpowiada za przenoszenie grup O-acetylowych na kwas legionaminowy, były częściej izolowane ze szczepów klinicznych niż środowiskowych. Jest to związane z wysokim stopniem O-acetylacji LPS, a co za tym idzie wysoką hydrofobowością powierzchni *Legionella*, która wspomaga mechanizm koncentracji bakterii w aerozolu, dzięki któremu patogen może rozprzestrzeniać się. Rola LPS w infekcji gospodarzy jest szczególnie ważna w początkowych etapach zakażania jako jeden z głównych czynników warunkujących adhezję do błony komórkowej makrofagów i ameb. Po sfagocytowaniu przez makrofagi *Legionella* uwalniają LPS, który znacząco opóźnia dojrzewanie wypełnionych bakteriami fagolizosomów. Ponadto bakterie modyfikują skład glikokoniugantów zawartych w pęcherzykach membrany zewnętrznej (OMV) uwalnianych z powierzchni bakterii. W fazie transmisyjnej bakterii cząsteczki LPS ulegają deacetylacji i „wydłużaniu" do form wysokocząsteczkowych, co niezależnie od białek efektorowych IV systemu sekrecji hamuje dojrzewanie makrofagów. W fazie replikacyjnej stopień acetylacji O-PS wzrasta, co wiąże się ze zwiększoną tolerancją na warunki środowiska wewnątrz makrofagów.

Podobnie jak u większości wewnątrzkomórkowych patogenów, LPS *L. pneumophila* jest mniej toksyczny i znacznie słabiej indukuje cytokiny prozapalne w porównaniu z pirogennie działającym LPS przedstawicieli *Enterobacteriaceae*. Aktywność toksyczną

LPS determinuje głównie ilość i długość reszt kwasów tłuszczowych acylujących szkielet cukrowy lipidu A. W przypadku *L. pneumophila* długość kwasów przekracza dwukrotnie długość kwasów występujących w toksycznych LPS: C12, C12OH, C14 i C14OH, co jest sferyczną przeszkodą w efektywnym wiązaniu LPS do głównego receptora LPS CD14 (błonowego i rozpuszczalnego). Receptor CD14 pośredniczy w rozpoznaniu lipidu A *L. pneumophila* przez receptor TLR4 zakotwiczony w błonie komórkowej makrofagów. Natomiast białko adaptorowe TRAM zaangażowane jest w rozpoznanie lipidu A przez receptor TLR4 zlokalizowany w membranach endosomów makrofagów. W wyniku pobudzenia tych receptorów przez LPS *L. pneumophila* dochodzi do aktywacji wewnątrzkomórkowej kaskady sygnalizacyjnej, czego następstwem jest indukcja prozapalnych cytokin (np.: IL-6 i TNF-α). Poziom wydzielanych przez makrofagi cytokin pod wpływem LPS *L. pneumophila* jest ponad tysiąc razy niższy w porównaniu do poziomu indukowanego przez *Salmonella enterica* serovar Minnesota, co jest jednym z mechanizmów ucieczki *Legionella* spod kontroli układu immunologicznego gospodarza. W modulacji odpowiedzi immunologicznej na zakażenie, *L. pneumophila* wykorzystuje podobieństwo strukturalne kwasu legionaminowego do kwasu neuraminowego, który występując na powierzchni komórek ssaczych uczestniczy w oddziaływaniach między komórkami.

Z jednej strony unikalna struktura LPS *L. pneumophila* zapewnia bakteriom skuteczną ochronę przed działaniem czynników układu immunologicznego, z drugiej zaś LPS jest rozpoznawany przez naturalnie występujące w organizmie człowieka przeciwdrobnoustrojowe białka i peptydy. Zaangażowane w nieswoistą odpowiedź odpornościową, występujące w płucach kolektyny (hydrofilowe białka A i D) wiążą LPS *L. pneumophila* promując lokalizację bakterii w kwaśnym środowisku lizosomów, w ten sposób osłabiając ich zdolność do wewnątrzkomórkowego namnażania się. Zewnątrzkomórkowe pęcherzyki (OMV) zawierające LPS w wyniku endocytozy przedostają się do środowiska wewnątrzkomórkowego mysich makrofagów, gdzie dochodzi do uwolnienia LPS *L. pneumophila* z endosomów do cytozolu przez białka GBP i aktywacji pyroptozy zależnej od kaspazy 11. Ludzka apolipoproteina E wiąże LPS *L. pneumophila* powodując zmianę struktury powierzchni bakterii co może osłabiać zdolność wnikania pałeczek do makrofagów. Micella utworzona przez 12-29 cząsteczek LPS *L. dumoffii* wiąże dwie cząsteczki wyizolowanej z hemolimfy *Galleria mellonella* apolipoforyny III (apoLp-III), homologa ludzkiej apolipoproteiny E. ApoLp-III wykazuje silniejsze działanie bakteriobójcze w stosunku do bakterii *L. dumoffii* wyrosłych na

podłożu z egzogenną choliną, co wskazuje, że poza LPS również PC zaangażowana jest w interakcję z białkiem. *L. dumoffii* hodowana na podłożu z dodatkiem egzogennej choliny syntetyzuje o 12% więcej PC a mniej PE w stosunku do bakterii hodowanych na podłożu bez dodatku choliny co ułatwia bakteriom oddziaływanie z makrofagami. Komórki *L. dumoffii* o zwiększonej zawartości PC znacznie łatwiej są internalizowane przez makrofagi linii THP-1 (komórki ludzkiej białaczki monocytarnej). PC *L. pneumophila* umożliwia bakteriom przyłączenie się do makrofagów za pośrednictwem receptora czynnika aktywującego płytki krwi (PAF), a skuteczność procesu jest uzależniona od zawartości tego fosfolipidu. Adhezja *L. pneumophila* do makrofagów jest blokowana przez antagonistę receptora PAF. Mutanty *L. pneumophila* defektywne w syntezie PC wykazują słabą zdolność przyłączania się do makrofagów i funkcjonowania IV systemu sekrecji. Ze względu na chemiczne podobieństwo PC i PAF, fosfatydylocholina może naśladować PAF i wiążąc się z jego receptorem zwiększać szansę na wychwyt bakterii przez makrofagi.

Zawartość i struktura fosfatydylocholin syntetyzowanych w szlaku PCS wpływa na poziom indukcji prozapalnych cytokin. Bakterie *L. anisa, L. longbeachae, L. gormanii* i *L. pneumophila* hodowane na pożywce z egzogenną choliną indukowały w makrofagach linii THP-1 znacznie niższą produkcję TNF-α w porównaniu z bakteriami hodowanymi bez dodatku choliny. Dodatek choliny do podłoża wzrostowego *L. pneumophila, L. longbeachae* i *L. anisa* wpłynął na zmniejszenie produkcji IL-6 przez makrofagi stymulowane tymi bakteriami. Pałeczki *Legionella* w warunkach dostępności choliny słabiej pobudzają syntezę prozapalnych cytokin, zatem są mniej rozpoznawalne dla układu immunologicznego.

5.2.4. Analiza lipidomowa w chemiotaksonomii i identyfikacji *Legionella*

Szczególną cechą błon *Legionella* jest wysoka zawartość rozgałęzionych (*i*, *izo* i *a*, *anteizo*) kwasów tłuszczowych. Grupa metylowa występująca w rozgałęzionych kwasach zakłóca upakowanie łańcucha acylowego wpływając na płynność błony. Bakterie, które syntetyzują rozgałęzione kwasy tłuszczowe modyfikują stosunek kwasów *izo* i *anteizo* rozgałęzionych w zależności od temperatury i czynników środowiska w celu utrzymania płynności błony. Pałeczki *Legionella* syntetyzują od 39% dla *L. oakridgensis* do 91% dla *L. jordanis* rozgałęzionych kwasów tłuszczowych. Do dominujących kwasów należą 14-metylopentadekanowy (*i*16:0), 12-metyloteradekanowy (*a*15:0) oraz 14-metyloheksadekanowy (*a*17:0). Na podstawie różnic w zawartości komórkowych kwasów

tłuszczowych *i*16:0, 16:0 oraz *a*15:0 *Legionella* zostały podzielone na 3 grupy: 16C, A15 i A15/16C. Bakterie należące do grupy 16C charakteryzują się wysoką zawartością kwasów 16:0 lub 16:1 albo obu kwasów. Grupa A15 obejmuje gatunki o wysokiej zawartości kwasu *a*15:0 w ilości około dwukrotnie większej niż kwasu *i*16:0. Do grupy A15/16C zaliczono bakterie u których dominowały kwasy *a*15:0 i *i*16:0. Analiza struktury fosfolipidów *Legionella* wykazała, że np.: PC16:0_15:0 i PE15:0_15:0 są wspólne dla *L. bozemanae*, *L. lytica* i *L. dumoffii*. W klasie fosfatydylocholin PC17:0_15:0 jest charakterystyczna dla *L. bozemanae*, PC16:0_14:0 i PC18:0_16:1 dla *L. lytica* i PC16:0_17:1 (lub cyklopropanowy 17:0) dla *L. dumoffii*. W klasie fosfatydyloetanoloamin PE16:1_15:0 dominuje u *L. bozemanae*, PE14:0_14:0 i PE15:0_14:0 u *L. lytica* i PE15:0_15:1 u *L. dumoffii*.

Różnice w zawartości komórkowych kwasów tłuszczowych oraz kwasów zawartych w poszczególnych fosfolipidach mogą być pomocne w różnicowaniu gatunków *Legionella* między sobą, a także w odróżnianiu od innych bakterii.

6. Hipoteza i cel pracy

Hipoteza badawcza zakłada, że skład i struktura lipidów błonowych wpływa na fizyko-chemiczne właściwości *L. gormanii* i determinuje oddziaływanie z apolipoforyną III.

Dlatego celem pracy było:

1. szczegółowa analiza składu i struktury lipidów wyizolowanych z zewnętrznej (OM) i wewnętrznej (IM) membrany *L. gormanii* hodowanych na podłożu z dodatkiem i bez dodatku egzogennej choliny,
2. określenie fizyko-chemicznych właściwości modelowych błon utworzonych z fosfolipidów wyizolowanych z bakterii hodowanych na podłożu z dodatkiem i bez dodatku egzogennej choliny,
3. badanie oddziaływania apolipoforyny III wyizolowanej z hemolimfy *Galleria mellonella* na bakterie *L. gormanii* hodowane na podłożu z dodatkiem i bez dodatku egzogennej choliny.

W szerszym znaczeniu badania miały na celu poszerzenie stanu wiedzy o lipidomie *L. gormanii* i wyznaczeniu chemiotaksonomicznych markerów identyfikacji tego gatunku oraz potencjalnych celów terapeutycznych w zakażeniach wywoływanych przez *Legionella*.

7. Omówienie wyników z uwzględnieniem użytych metod badawczych

7.1. Publikacja 3

Chmiel, E., Galuska, C.E., Koper, P., Kowalczyk, B., Urbanik-Sypniewska, T., Palusińska-Szysz, M., Fuchs, B. (2022). Unusual lipid components of *Legionella gormanii* membranes. *Metabolites,* 6;12(5); 418. doi: 10.3390/metabo12050418.

7.1.1. Metody

Bakterie *L. gormanii* zostały namnożone na podłożu BCYE bez dodatku oraz z dodatkiem 100 µg/ml chlorku choliny. Po degradacji enzymatycznej komórek oraz dezintegracji w prasie Frencha zawiesina bakteryjna została naniesiona na siedmiostopniowy (70%, 64%, 58%, 52%, 48%, 42%, 36%) gradient sacharozy, a następnie ultrawirowana przez 20 godzin przy 114 000 x g, w temperaturze 4°C. Zebrano 1 ml frakcje z gradientu, w których oznaczono zawartość białka, aktywność NADH oksydazy oraz esterazy. Połączono frakcje o najwyższej aktywności NADH oksydazy, która jest markerem błony wewnętrznej oraz aktywności esterazy, która jest markerem błony zewnętrznej. Błony OM i IM osadzono przez ultrawirowanie (100 000 x g), a następnie dwukrotnie przepłukano w buforze HEPES o pH 7,4. Z uzyskanych błon bakteryjnych wyekstrahowano lipidy metodą Bligh i Dyer (1959). Szczegółową analizę lipidów *L. gormanii* wykonano za pomocą wysokosprawnej chromatografii cieczowej sprzężonej ze spektrometrią mas. Fosfolipidy zostały rozdzielone na poszczególne klasy w jednokierunkowym układzie rozpuszczalników chloroform:metanol:kwas octowy (13:5:2, v/v/v) na płytkach TLC (Silica gel 60). Do elucji fosfolipidów z płytek TLC zastosowano chloroform:metanol (1:1, v/v). Kwasy tłuszczowe zostały uwolnione z fosfolipidów za pomocą saponifikacji (0.8 M NaOH w 50% metanolu, 1,5 h, 80°C), wyekstrahowane mieszaniną chloroformu z wodą (1:2, v/v), a następnie przekształcone w estry metylowe (1M HCl/met, 1h, 85°C). Profil kwasów tłuszczowych w poszczególnych fosfolipidach został oznaczony za pomocą chromatografii gazowo-cieczowej sprzężonej ze spektrometrią mas (GC/MS). Lipidy analizowano za pomocą ultra wysokosprawnej chromatografii cieczowej sprzężonej ze spektrometrią mas (UHPLC-MS/MS)

7.1.2. Wyniki

Lipidy *L. gormanii*, które stanowią około 4% suchej masy bakterii obejmują zarówno glicerolipidy (trójglicerydy, TG i dwuglicerydy, DG), fosfolipidy (PE, PC, CL, PG) oraz sfingolipidy (ceramidy, Cer i heksozylceramidy). Fosfolipidy były dominującą frakcją lipidów, a wśród nich PE i PC występowały w największej ilości zarówno w OM i IM. Każda klasa fosfolipidów miała charakterystyczny dla niej wzór kwasów tłuszczowych. W klasie fosfatydyloetanoloamin prawie połowę kwasów stanowiły kwasy o rozgałęzionym łańcuchu: *i*16:0, *a*15:0 i *a*17:0. Frakcja ta charakteryzowała się wysoką zawartością kwasu cyklopropanowego 17:0 (cis-9,10-metylenoheksadekanowego) (18%) oraz heksadekanowego (17%). W dMePE ponad 65% kwasów stanowiły heksadekanowy i oktadekanowy. We frakcji tej występował również nienasycony kwas 18:1Δ^9 oraz długołańcuchowe 22:0 i 24:0 kwasy tłuszczowe. Dominującym kwasem frakcji fosfatydylocholin był kwas cyklopropanowy 17:0 (22%). Frakcja ta zawierała zarówno kwasy rozgałęzione (*i*16:0, *a*15:0), jak i prostołańcuchowe (16:0, 18:0). Klasa fosfatydylogliceroli charakteryzowała się duża ilością rozgałęzionych kwasów tłuszczowych: *a*15:0, (17%); *i*16:0, (19%); *a*17:0, (9%). W porównaniu z innymi fosfolipidami kardiolipiny zawierały największą ilość nienasyconych kwasów 16:1Δ^9, 18:1Δ^9 oraz długołańcuchowych kwasów tłuszczowych (od 20 do 24 atomów węgla).

Analiza profilu molekularnego fosfolipidów wykazała występowanie charakterystycznych dla poszczególnych klas związków. W klasie PE dominowały fosfatydyloetanoloaminy o strukturze: PE15:0_15:0 (16% w OM i 18% w IM), PE15:0_16:0 (13% w OM i 23% w IM) oraz PEcyklopropanowy17:0_16:0 (9% w OM i 11% w IM). Fosfatydylocholina charakteryzowała się występowaniem w dużej ilości PC15:0_15:0 (22% w OM i 20% w IM), PC15:0_16:0 (19% w OM, 22% w IM), PCcyklopropanowy17:0_16:0 (15% w OM, 20% w IM) i PCcyklopropanowy17:0/15:0 (10% w OM i 9% w IM). Występowanie tych lipidów było podobne w obu błonach. Charakterystycznym związkiem w klasie PG był PG16:1_16:1. Zewnętrzna membrana zawierała więcej PG15:0_15:0, PG16:1_14:0 i PG15:0_14:0 w porównaniu do wewnętrznej membrany, natomiast wewnętrzna membrana miała większą zawartość PG15:0_16:0, PG15:0_17:0 i PG16:1_17:0. Analiza profilu kardiolipin wykazała, że dominowały związki: CL66:1 i CL64:2. Kardiolipina 66:1 występowała w obu błonach a w IM stanowiła około 87% wszystkich kardiolipin.

Badania lipidomiczne wykazały, że *L. gormanii* syntetyzuje PC w dwóch niezależnych szlakach: PMTA i PCS. Obecność metylowanych pochodnych PE w błonach

L. gormanii wskazuje na występowanie szlaku PMTA syntezy PC. W membranie zewnętrznej dominującymi dimetylopochodnymi PE były: dMePE cyklopropanowy17:0_17:0 (77%), dMePE16:0/16:1 (11%). W błonie wewnętrznej w największej ilości występowały dMePE16:0_16:1 (44%), dMePE cyklopropanowy17:0_16:1 (39%). Dimetylopochodne PE występowały również w lipidach wyizolowanych z IM i OM bakterii hodowanych na podłożu z dodatkiem choliny co wskazuje, że u *L. gormanii* mogą jednocześnie funkcjonować dwa niezależne szlaki syntezy PC.

Bakterie *L. gormanii* hodowane na podłożu z egzogenną choliną syntetyzowały o 25% więcej PC w OM i 28% w IM w porównaniu do bakterii hodowanych na podłożu bez dodatku egzogennej choliny. Zawartość PE w OM była o 18% a w IM o 32% mniejsza niż w lipidach błon bakterii, których podłoża hodowlanego nie suplementowano choliną. Analiza porównawcza profilu fosfatydylocholin syntetyzowanych w dwóch szlakach syntezy PC wykazała różnice ilościowe. Zawartość PCcyklopropanowy17:0_15:0 w lipidach IM była dwukrotnie wyższa u bakterii hodowanych z dodatkiem egzogennej choliny. PCcyklopropanowy17:0_16:0 występowała w większej ilości w lipidach IM wyizolowanej z bakterii hodowanych na podłożu z dodatkiem choliny. Lipidy zewnętrznej membrany bakterii hodowanych z egzogenną choliną charakteryzowały się większą zawartością PC15:0_15:0 w porównaniu do lipidów uzyskanych z OM bakterii wyrosłych na podłożu bez dodatku choliny. Ponadto zawartość PE15:0_15:0, PE15:0_16:0, PE16:0_16:1 była wyższa w lipidach zawartych w IM wyizolowanej z bakterii hodowanych z dodatkiem choliny. Nie występowały różnice w zawartości fosfatydyloetanoloamin występujących w lipidach OM bakterii hodowanych z dodatkiem i bez dodatku egzogennej choliny.

Drugą pod względem zawartości frakcją lipidów występujących w komórkach *L. gormanii* były glicerolipidy (TG i DG), które charakteryzowały się dużą różnorodnością strukturalną. Lipid TG18:0_16:0_16:0 zlokalizowany głównie w OM stanowił ponad 50% wszystkich zidentyfikowanych trójglicerydów. Nie występowały wyraźne różnice w zawartości trójglicerydów w błonach izolowanych z bakterii pochodzących z różnych warunków hodowli z wyjątkiem 3-krotnie większej zawartości TG18:0_16:0_16:0 w lipidach IM wyizolowanej z bakterii hodowanych bez dodatku choliny.

Wśród dwuglicerydów w IM dominowały DG18:0_16:0 (20%) i DG18:0_18:0 (14%) natomiast w OM w największej ilości występowały DG15:0_16:0 (15%) i DG16:0_16:0 (15%).

L. gormanii syntetyzuje również ceramidy podstawione nasyconymi i jednonienasyconymi kwasami o długości od 12 do 24 atomów węgla w cząsteczce oraz utlenowane ceramidy i bardziej złożone heksozyloceramidy. Wszystkie rodzaje ceramidów występowały w OM i IM przy czym wystąpiły różnice ilościowe w ich rozmieszczeniu w błonach. Cer(t16:1_12:0) stanowił 66% ceramidów w OM i 44% w IM bakterii hodowlanych bez suplementacji choliną. Bakterie hodowane na podłożu z egzogenną choliną zawierały więcej cer(t16:1_12:0) w OM, natomiast zawartość tego lipidu w IM była podobna, niezależnie od warunków hodowli. Drugim pod względem ilości ceramidem był cer(t18:1_12:0) zlokalizowany głównie w IM bakterii hodowanych na podłożu z choliną i bez dodatku choliny. Zawartość tego lipidu w OM była o połowę niższa w porównaniu do zawartości w IM niezależnie od warunków hodowli bakterii.

W lipidach *L. gormanii* zidentyfikowano trzy główne heksozyloceramidy. Dominujący Hex1Cer22:1/18:1 stanowił 76% wszystkich heksozyloceramidów w IM bakterii hodowanych w obecności choliny. W OM jego zawartość była niższa i wynosiła 54%. Suplementacja choliną podłoża wzrostowego *L. gormanii* powodowała spadek ilości tego lipidu w obu błonach.

W celu znalezienia genów kodujących funkcjonalne enzymy zaangażowane w szlaki syntezy ceramidów w genomie *L. gormanii* ATCC 33279 wykorzystano oprogramowanie bioinformatyczne KofamKOALA, będące częścią pakietu KEGG (Kyoto Encyclopedia of Genes and Genomes). Punktem odniesienia była mapa metabolizmu sfingolipidów obejmująca szlak syntezy ceramidów (map00600). W tym szlaku zmapowano enzymy kodowane w genomie *L. gormanii* ATCC 33279, którym przypisano identyfikatory KO (KEGG Orthology). Pierwszym etapem biosyntezy bakteryjnych sfingolipidów jest kondensacja L-seryny i palmitoilo-CoA, w wyniku której powstaje 3-ketodihydrosfingozyna. Reakcję katalizuje zależna od fosforanu pirydoksalu palmitoilotransferaza seryny (SPT). W oparciu o standardowe kryteria wyszukiwana homologii, stosowane w narzędziach KEGG, sekwencje kodujące SPT nie zostały odnalezione ale dodatkowe przeszukiwania z zastosowaniem programu PSI-BLAST i sekwencji referencyjnej SPT pochodzącej z genomu *Sphingomonas paucimobilis* pozwoliły na wykrycie dwóch CDS-ów, kodujących hipotetyczne białka w genomie *L. gormanii* ATCC 33297, identyczne odpowiednio w 31% i 25%. Zweryfikowano automatyczną adnotację obydwu sekwencji, uzyskaną z użyciem narzędzi NCBI (PGAP) i PATRIC (RASTtk). Sugerowała ona odpowiednio enzymy należące do kategorii ligaz i syntaz, z tej samej rodziny białek co SPT. Analiza porównawcza MSA (Multiple

Sequence Alignment) razem z sekwencją SPT, wykazała obecność konserwatywnych motywów w analizowanych białkach.

Ceramidy mogą być syntetyzowane *de novo* z seryny i palmitynianu bądź powstawać wskutek rozpadu sfingomielin z udziałem sfingomielinaz: SMase Cs, (SMPD1) i SMase Ds. Sfingomielinaza SMPD1 hydrolizuje wiązanie estrowe między ceramidem a fosforylocholiną. Sfingomielinaza SMase Ds. hydrolizuje wiązanie fosfodiestrowe między cer-1-fosforanem i choliną. W wyniku przeszukiwania genomu *L. gormanii* ATCC 33297 z zastosowaniem standardowych progów i ustawień narzędzia KofamKOALA otrzymano wynik negatywny dla sekwencji kodującej SMPD1. Jednak, w przeciwieństwie do palmitoilotransferazy serynowej, geny kodujące sfingomielinazy wykrywano w innych gatunkach *Legionella*: *L. pneumophila* szczep Paris i *L. longbeachae* szczep NSW150. Przeszukując sekwencje kodujące, odnalezione w genomie *L. gormanii*, z użyciem narzędzia blastp i stosując, jako zapytanie sekwencję białkową SMPD1 pochodzącą z genomu *L. pneumophila* szczep Paris odnaleziono homolog SMPD1, charakteryzujący się 61% identyczności ze sfingomielinazą *L. pneumophila*. Hipoteza została potwierdzona analizą syntenii w obrębie 4 genów kodujących białko partycyjne ParB, karboksypeptydazę D-ala-D-ala, sfingomielinazę i rodzinę hydrolaz SGNH/GDSL. Wskazuje to na wspólne ewolucyjne pochodzenie tego bloku genów.

Lipidy błon *L. gormanii* charakteryzuje obecność fosfatydylocholiny i ceramidów, co pozwoliło zakwalifikować te bakterie do wąskiej grupy mikroorganizmów syntetyzujących lipidy typowe dla komórek eukariotycznych. Na podstawie przeglądu danych literaturowych otrzymane wyniki stanowią pierwsze doniesienie o występowaniu ceramidów w komórkach *Legionella*. Szczegółowa analiza lipidów wykazała dużą różnorodność syntetyzowanych związków, których występowanie w błonach *L. gormanii* może zostać wykorzystane w chemiotaksonomii tej grupy bakterii. *L. gormanii* syntetyzuje PC w dwóch niezależnych szlakach: PCS i PMTA. Bakterie hodowane na podłożu z choliną charakteryzują się różnym wzorem lipidów budujących OM i IM w porównaniu do bakterii hodowanych na podłożu bez dodatku egzogennej choliny. Struktura cząsteczek lipidów ma bezpośredni wpływ na parametry fizyczne błony biologicznej takie jak: gęstość upakowania, grubość, ściśliwość i właściwości fazowe membrany.

7.2. Publikacja 4

Pastuszak, K., **Chmiel, E.**, Kowalczyk, B., Tarasiuk, J., Jurak, M., Palusińska-Szysz, M. (2023). Physicochemical characteristics of model membranes composed of *Legionella gormanii* lipids. *Membranes*, 13(3); 356. doi. 10.3390/membranes13030356.

W pracy tej analizowano fizyko-chemiczne właściwości monowarstw utworzonych z fosfolipidów wyekstrahowanych z bakterii *L. gormanii* hodowanych z dodatkiem oraz bez dodatku egzogennej choliny.

7.2.1. Metody

Lipidy *L. gormanii* zostały wyizolowane z bakterii hodowanych na podłożu z dodatkiem chlorku choliny oraz bez dodatku tego związku metodą Bligh i Dyer, a następnie rozdzielone na poszczególne klasy za pomocą chromatografii cienkowarstwowej. Kwasy tłuszczowe zostały uwolnione z fosfolipidów za pomocą saponifikacji (0.8 M NaOH w 50% metanolu, 1,5 h, 80°C), wyekstrahowane mieszaniną chloroformu z wodą (1:2, v/v), a następnie przekształcone w estry metylowe za pomocą 0.02 M trimetylsilyl diazometanu. Skład kwasów tłuszczowych w poszczególnych klasach fosfolipidów został oznaczony za pomocą chromatografii gazowo-cieczowej sprzężonej ze spektrometrią mas (GC/MS).

Fosfolipidy uzyskane z bakterii hodowanych na podłożu z i bez dodatku egzogennej choliny zostały poddane badaniom przy użyciu techniki monowarstw Langmuira sprzężonej z modułem do pomiaru potencjału powierzchniowego oraz mikroskopią kąta Brewstera. Z chloroformowo-metanolowych roztworów fosfolipidów o stężeniu 1mg/ml utworzone zostały monomolekularne modelowe błony bakteryjne na granicy faz ciecz/powietrze w temperaturze 20°C i 37°C. W ramach pomiarów zostały wyznaczone izotermy ciśnienia (π) i zmian potencjału powierzchniowego (ΔV) w funkcji powierzchni (A) przypadającej na cząsteczkę fosfolipidu oraz morfologia błon.

7.2.2. Wyniki

W pierwszym etapie badań określono skład kwasów tłuszczowych zawartych w poszczególnych klasach fosfolipidów wyizolowanych z bakterii z różnych warunków hodowli. PC wyizolowana z bakterii hodowanych na podłożu z choliną zawierała 2,4 razy więcej długołańcuchowych (od C19 do C21) kwasów tłuszczowych, zaś mniej nasyconych

kwasów oraz kwasów o długości łańcucha acylowego od C14 do C18 w porównaniu do bakterii hodowanych bez dodatku choliny. *L. gormanii* niezależnie od warunków hodowli miały podobny wzór kwasów tłuszczowych w klasie PE. Dodatek choliny do podłoża wzrostowego spowodował, że bakterie syntetyzowały około 3% mniej długołańcuchowych kwasów tłuszczowych i 3% więcej kwasów tłuszczowych o długości łańcucha acylowego od C14 do C18 w klasie CL. Natomiast w klasie PG bakterie hodowane z dodatkiem choliny zawierały mniej długołańcuchowych kwasów, a więcej nienasyconych oraz kwasów o długości łańcucha od C14 do C18 w porównaniu do bakterii, które nie były suplementowane choliną.

Monowarstwy utworzone z lipidów wyizolowanych z *L. gormanii* hodowanych z dodatkiem egzogennej choliny były bardziej uporządkowane i gęściej upakowane w temperaturze 20°C i 37°C w porównaniu do monowarstw utworzonych z bakterii hodowanych bez dodatku choliny na co wskazują większe wartości modułu ściśliwości tych monowarstw. Ściślejsze upakowanie cząsteczek przyczyniło się do zwiększonego uporządkowania łańcuchów acylowych. Wyższy stopień kondensacji cząsteczek w monowarstwach utworzonych z lipidów *L. gormanii* hodowanych na podłożu z dodatkiem choliny wynikał z korzystnych interakcji za pośrednictwem wiązań wodorowych i sił Lifshitza-van der Waalsa odpowiednio między grupami polarnymi fosfolipidów i długimi łańcuchami kwasów tłuszczowych C19-C21. Wyższy stopień upakowania tych monowarstw może wynikać z wyższej zawartości PC, która ma cylindryczny kształt.

Obrazy morfologii otrzymane przy pomocy mikroskopu kąta Brewstera wykazały, że monowarstwy utworzone z lipidów wyizolowanych z *L. gormanii* hodowanej na podłożu z egzogenną choliną były bardziej jednorodne niż monowarstwy utworzone z lipidów bakterii hodowanych bez dodatku egzogennej choliny. Monowarstwy lipidów wyekstrahowanych z bakterii hodowanych na podłożu bez dodatku choliny wykazywały zwiększoną elastyczność wynikającą z obecności krótszych łańcuchów (C14–C18) nienasyconych kwasów tłuszczowych, a także wyższej zawartości kardiolipiny (21%), prowadząc do tworzenia niejednorodności (domen).

Plejotropowy charakter wpływu lipidów na biologię błony jest definiowany przez modulację domen lipidowych, a także bezpośrednią interakcję lipidów z białkami oraz związkami o charakterze antybiotycznym.

7.3. Publikacja 1

Chmiel, E., Palusińska-Szysz, M., Zdybicka-Barabas, A., Cytryńska, M., Mak, P. (2014).The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. *Acta Biochim. Pol.*, 61(1); 123-127.

W pracy tej koncentrowano się na określeniu działania ekstraktu z hemolimfy gąsienic *Galleria mellonella* oraz wyizolowanego z niej białka apolipoforyny III (apoLp-III) na bakterie *Legionella gormanii*.

7.3.1. Metody

Preparaty immunizowanej hemolimfy otrzymano przez iniekcje larw zawiesiną żywych Gram-ujemnych (*Escherichia coli* D31) i Gram-dodatnich (*Micrococcus luteus*) bakterii. Izolację apoLp-III z hemolimfy przeprowadzono według procedury opisanej przez Cytryńska i in., 2007 (Cytryńska i in., 2007). Białko apoLp-III oczyszczano metodą HPLC, a stopień czystości i homogenności analizowano za pomocą elektroforezy oraz sekwencjonowania w automatycznym sekwenatorze aminokwasów.

Badania aktywności przeciwbakteryjnej immunizowanej hemolimfy i apoLp-III wykonywano po inkubacji hodowli *L. gormanii* (OD=0.1) z ekstraktem hemolimfy (końcowe stężenie białka 0,025–0,8 mg/ml) lub preparatu apoLp-III (końcowe stężenie białka 0,025–0,2 mg/ml) w temperaturze 37°C przez 1 h. Po tym czasie bakterie z próby kontrolnej oraz potraktowanej ekstraktem hemolimfy lub apoLp-III wysiano na podłoże BCYE. Po czterech dniach inkubacji w 37°C obliczono liczbę wyrosłych kolonii bakterii. Powierzchnię bakterii potraktowanych apoLp-III analizowano w mikroskopie sił atomowych (AFM). W tym celu zawiesinę *L. gormanii* (OD=0.2) kontrolną i inkubowaną z apoLp-III (końcowe stężenie 0,2 mg/ml) w 37°C przez 1 h odwirowano a bakteryjny osad zawieszano w wodzie, a następnie naniesiono na krążki z miki. Obraz powierzchni *L. gormanii* analizowano w mikroskopie NanoScope V AFM (Veeco, USA) w trybie "PeakForce QNM".

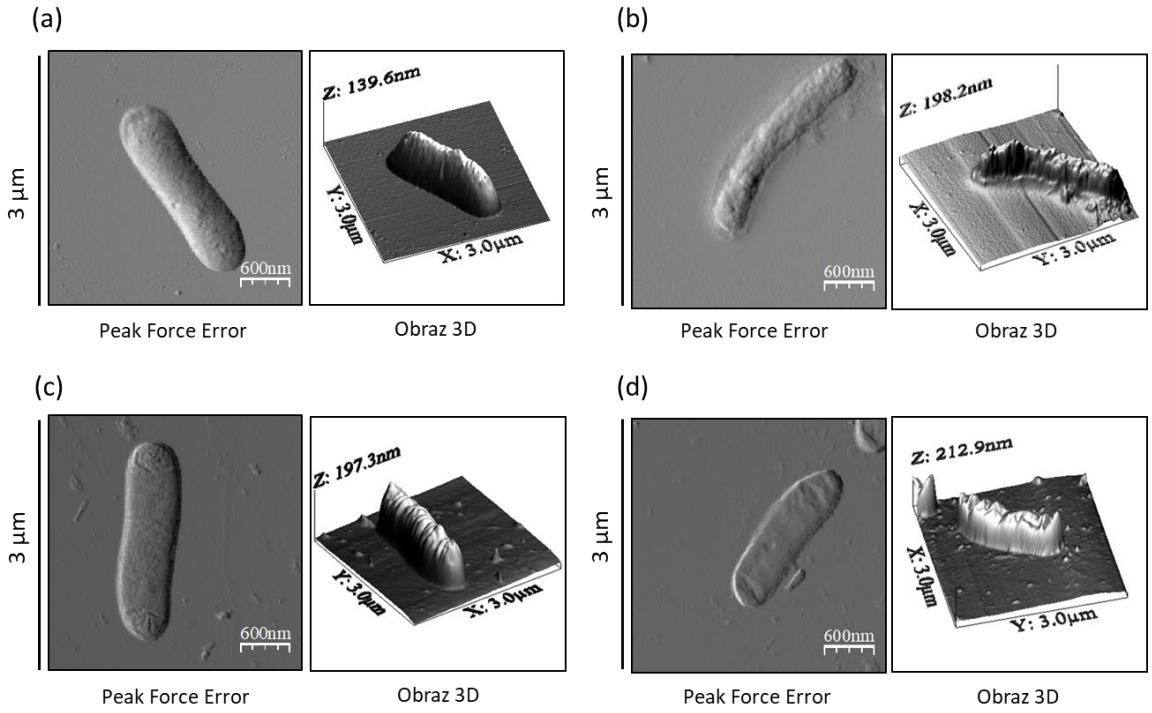
Statystyczną analizę wyników opracowano przy użyciu testu Wilcoxsona dla par obserwacji. Dane przedstawiano jako ± odchylenia standardowego (SD) z trzech niezależnych doświadczeń.

7.3.2. Wyniki

Ekstrakt hemolimfy *G. mellonella* immunizowany Gram-ujemnymi (*E. coli* D31) i Gram-dodatnimi (*M. luteus*) bakteriami wykazywał zależne od stężenia bakteriobójcze działanie w zakresie 0,025–0,8 mg/ml. Po 1 godzinie inkubacji efekt bakteriobójczy ekstraktu hemolimfy w stężeniu 0,025 mg/ml wynosił 75% w stosunku do kontroli nietraktowanej ekstraktem. Bakteriobójcze, zależne od stężenia działanie na *L. gormanii* wykazywała również apoLp-III – wielofunkcyjne białko, które uczestniczy w transporcie lipidów, rozpoznawaniu determinant molekularnych drobnoustrojów i aktywacji układu immunologicznego. Preparat apoLp-III w stężeniu 0,2 mg/ml powodował 50% spadek przeżywalności *L. gormanii*. Analiza w mikroskopie sił atomowych wykazała zmiany w topografii oraz właściwościach mechanicznych powierzchni komórki *L. gormanii* po zadziałaniu preparatu apoLp-III. Pod wpływem tego białka na powierzchni bakterii pojawiły się liczne, o różnej wielkości kuliste struktury, a tzw. szorstkość, parametr będący miarą heterogenności powierzchni, była 1,44 razy większa w porównaniu do bakterii nietraktowanych apoLp-III.

Pałeczki *L. gormanii* hodowane na podłożu z choliną wykazywały większą wrażliwość na apoLp-III w porównaniu do bakterii hodowanych bez dodatku choliny. Stężenie apoLp-III 0,05 mg/ml powodowało 60% śmiertelność bakterii. Szorstkość bakterii hodowanych z dodatkiem choliny po zadziałaniu apoLp-III w stężeniu 0,1 mg/ml była 1,22 razy wyższa w porównaniu do bakterii hodowanych bez dodatku choliny traktowanych białkiem (dane niepublikowane) [Ryc. 1].

Większa wrażliwość na apoLp-III bakterii hodowanych z dodatkiem egzogennej choliny może wskazywać, że zmiana zawartości fosfolipidów, która wpływa na zmianę fizyko-chemicznych właściwości komórek *L. gormanii*, decyduje o silniejszym oddziaływaniu tego białka z powierzchnią bakterii.



Ryc. 1. Zdjęcia z mikroskopu AFM bakterii *L. gormanii*: a/ kontrola, - cholina b/ - cholina po działaniu apoLp-III, c/ kontrola, + cholina, d/ + cholina po działaniu apoLp-III [dane niepublikowane].

8. Dyskusja

L. gormanii jest jednym z 28 gatunków bakterii *Legionella* wywołujących nietypowe zapalenie płuc u ludzi w tym relatywnie często u dzieci (Greenberg i in., 2006). Chorobotwórczość tej bakterii jest determinowana m.in. wysoką wydajnością degradacji surfaktantu płucnego porównywalną do aktywności *L. pneumophila*. Bakterie *L. gormanii* produkują fosfolipazy A (PlaB) i lizofosfolipazy, których substratem jest PC stanowiąca 80% zawartości surfaktantu płuc (Bender i in., 2009). Zdolność wykorzystywania lipidów eukariotycznych jest specyficzną i oryginalną strategią opracowaną przez bakterie *Legionella* do adaptacji do środowiska gospodarza. Przebadane do tej pory gatunki *Legionella* mają zdolność do wykorzystywania egzogennej choliny do syntezy PC. Analiza lipidomiczna *L. gormanii* wykazała, że spośród czterech eksperymentalnie potwierdzonych u bakterii szlaków syntezy PC dwa szlaki: PCS i PMTA funkcjonowały jednocześnie. *L gormanii* hodowana na podłożu z egzogenną choliną syntetyzowała o 21% więcej PC a 12% mniej PE i 9% mniej CL w stosunku do bakterii hodowanych na podłożu bez dodatku egzogennej choliny (Palusińska-Szysz i in., 2019). *L. pneumophila* hodowana na podłożu z choliną wytwarzała o 6% więcej PC i 3% więcej PE natomiast 9% mniej CL. W przypadku innych gatunków *L. anisa*, *L. longbeachae*, *L. micdadei* bakterie hodowane na podłożu z dodatkiem i bez dodatku egzogennej choliny miały taką samą zawartość PC (Palusińska-Szysz i in., 2019). Dominującym szlakiem syntezy PC u *L. micdadei* był szlak PCS, a fosfolipid ten był wbudowywany głównie do membrany zewnętrznej. Dodatek choliny do podłoża spowodował zwiększenie zawartości PC w obu błonach *L. gormanii*, przy czym większy wzrost wystąpił w błonie wewnętrznej. Analiza porównawcza profilu fosfatydylocholin *L. gormanii* oraz *L. micdadei* wykazała, że jest on charakterystyczny zarówno dla błony zewnętrznej jak i błony wewnętrznej, a dodatek choliny nie wpływał na zmianę długości i stopnia nienasycenia kwasów tłuszczowych budujących PC ale decydował o różnicach ilościowych (Palusińska-Szysz i in., 2022). *L. gormanii* hodowana na podłożu z choliną w IM zawierała dwa razy więcej PC cyklopropanowy17:0/15:0 niż w IM izolowanej z bakterii hodowanych bez dodatku choliny. Lipidy OM izolowane z bakterii wyrosłych na podłożu z choliną miały więcej PC15:0_15:0 niż lipidy OM bakterii hodowanych bez dodatku choliny. Każda klasa fosfolipidów *L. gormanii* miała charakterystyczny skład kwasów tłuszczowych z wysoką zawartością kwasów rozgałęzionych (*a*15:0, *i*16:0). W klasie PE rozgałęzione kwasy stanowiły około 49% a w klasie PC 31% wszystkich kwasów. Występowanie kwasów o rozgałęzionych

łańcuchach acylowych jest jedną z cech, która odróżnia membrany *Legionella* od innych bakterii. Kwasy te występują głównie u bakterii Gram-dodatnich oraz nielicznych Gram-ujemnych z rodzajów *Flavobacterium*, *Bacteroides* i *Desulfovibrio* (Kaneda, 1991). Charakterystyczna dla wszystkich klas fosfolipidów *L. gormanii* była wysoka zawartość kwasu cyklopropanowego 17:0. Obecność pierścienia cyklopropanowego sprawia, że kwas ten jest bardziej trwały w porównaniu z kwasami nienasyconymi, co zwiększa stabilność błon *L. gormanii*. Zawartość i struktura lipidów budujących błony komórkowe wpływa na ich fizyko-chemiczne właściwości. Monowarstwy utworzone z lipidów bakterii hodowanych na podłożu z choliną charakteryzowały się większym uporządkowaniem, były bardziej upakowane i homogenne w porównaniu do monowarstw utworzonych z lipidów bakterii, których podłoże wzrostowe nie było suplementowane choliną.

Wrażliwość bakterii *Legionella* na białka i peptydy przeciwdrobnoustrojowe może być związana ze składem kwasów tłuszczowych oraz ich rozmieszczeniem (pozycja *sn-1*/*sn-2*) w strukturze fosfolipidów błonowych. Bogatym źródłem białek i peptydów odpornościowych jest hemolimfa gąsienic *Galleria mellonella*. *L. gormanii* była wrażliwa zarówno na ekstrakt hemolimfy oraz wyizolowane z hemolimfy białko apoLp-III. Testy ilościowe wykazały, że apoLp-III w stężeniu 0,025 mg/ml powodowała około 20% spadek żywotności bakterii a użyty w tym samym stężeniu pełny (niefrakcjonowany) ekstrakt hemolimfy był bardziej efektywny w zabijaniu *L. gormanii* powodując około 75% śmiertelność komórek. Świadczy to o synergistycznym działaniu innych komponentów immunizowanej hemolimfy takich, jak białka i peptydy obronne oraz związki niebiałkowe. Bakteriobójcza dawka apoLp-III była 8-krotnie niższa dla *L. gormanii* w porównaniu do *L. dumoffii* a gatunek *L. micdadei* nie był wrażliwy na działanie apoLp-III (Palusińska-Szysz i in., 2012; 2022). Wyższa wrażliwość *L. gormanii* na apoLp-III w porównaniu z innymi gatunkami *Legionella* wskazuje, na zróżnicowaną podatność tych bakterii na naturalne, przeciwdrobnoustrojowe białka, wynikająca z różnic w strukturze i fizyko-chemicznych właściwościach składników powierzchniowych komórek *Legionella*. Komórki *L. gormanii* po zadziałaniu apoLp-III wykazywały wzrost chropowatości powierzchni, której przyczyną mogło być pęknięcie komórki. Zaburzenia w regularnej strukturze powierzchni bakterii prawdopodobnie wynikały z wiązania się apoLp-III z fosfolipidami i/lub LPS *L. gormanii*. Tworzenie takich kompleksów zostało wykazane dla *L. dumoffii* (Palusińska-Szysz i in., 2020)

Unikalną cechą błon *L. gormanii* jest obecność ceramidów. Sfingolipidy (SphL) występują głównie w warstwie zewnętrznej błon komórek eukariotycznych i odgrywają

kluczową rolę w sygnalizowaniu i tworzeniu tratw lipidowych (Nelson i Cox, 2017). W komórkach bakteryjnych sfingolipidy są bardzo rzadko spotykane. Występujące w błonach bakteryjnych odpowiadają za interakcję z eukariotycznymi systemami sygnalizacyjnymi (*Sphingomonas*, *Bacteroides*), mają znaczenie dla przetrwania drobnoustrojów w stacjonarnej fazie wzrostu (*Bacteroides*, *Porphyromonas*), zapewniają odporność na podwyższoną temperaturę (*Acetomonas*, *Bacteroides*), kwaśne środowisko (*Acetomonas*) i stres oksydacyjny (*Bacteroides*, *Porphyromonas*) (Geiger, 2019). U Gram-ujemnych bakterii pozbawionych LPS takich jak *Sphingomonas* (Kawasaki i in., 1994) lub *Sorangium* (Keck i in., 2011) sfingolipidy funkcjonalnie zastępują ten składnik błony zewnętrznej. Bakterie posiadające zarówno LPS, jak i sfingolipidy w odpowiedzi na stres abiotyczny (kwasowość lub podwyższoną temperaturę) syntetyzują więcej sfingolipidów lub ceramidów np. w komórkach *Acetobacter malorum* (Ogawa, 2010). W badanych do tej pory gatunkach bakterii sfingolipidy są zlokalizowane w warstwie zewnętrznej OM. Natomiast u *L. gormanii* obecność ceramidów stwierdzono w obu błonach, przy czym cer(16:1_12:0) dominował w OM. Kwasy tłuszczowe ceramidów *L. gormanii* były parzystowęglowe, nasycone lub jednonienasycone o długości od 12 do 24C i strukturalnie były podobne do ceramidów występujących w komórkach eukariotycznych. Przeszukiwania za pomocą narzędzi bioinformatycznych genów kodujących białka szlaku syntezy sfingolipidów wykazały, że w genomie *L. gormanii* występują białka o identyczności 31% i 25% z palmitoilotransferazą seryny (SPT) *Sphingomonas paucimobilis*. Alternatywnym szlakiem wytwarzania ceramidów jest rozkład sfingomieliny (SM) przez sfingomielinazy. *L. gormanii* koduje hipotetyczną sfingomielinazę o poziomie podobieństwa wynoszącym 61% z sfingomielinazą *L. pneumophila*. *L. pneumophila* wytwarza również liazę 1-P-sfingozyny (LpSpl), której aktywność hamuje wzrost stężenia sfingozyny w makrofagach zakażonych bakteriami, prowadząc do zahamowania procesu autofagii (Rolando i in., 2016). Degradacja sfingolipidów komórek gospodarza może przeciwdziałać odpowiedzi obronnej i umożliwiać wewnątrzkomórkową replikację. Ponieważ sfingolipidy i enzymy metabolizujące sfingolipidy są ważnymi mediatorami patogeniczności drobnoustrojów mogą stanowić potencjalne cele dla opracowywania nowych strategii terapeutycznych. Jednak biologiczne znaczenie ceramidów w komórkach *L. gormanii* pozostaje do wyjaśnienia.

9. Podsumowanie i wnioski

Bakterie *Legionella* są klinicznie ważnymi drobnoustrojami i cennym modelem badania mechanizmów interakcji między patogenem a gospodarzem oraz wrodzonej odpowiedzi immunologicznej na zakażenie. Unikalne składniki ściany komórkowej bakterii *Legionella* odpowiadają za odziaływanie z komórkami fagocytującymi na każdym etapie cyklu rozwojowego tych bakterii. W błonach *L. gormanii* są obecne składniki typowe dla błon komórek eukariotycznych takie jak fosfatydylocholina i ceramidy. Duża różnorodność strukturalna w obrębie lipidów pozwoliła na wyodrębnienie markerów zarówno wspólnych dla bakterii *Legionella* jak i charakterystycznych dla *L. gormanii*. Do adaptacji do środowiska gospodarza bakterie *Legionella* wykształciły oryginalną strategię opartą na zdolności do wykorzystywania lipidów gospodarza. *L. gormanii* syntetyzuje PC z zewnątrzkomórkowej choliny oraz CDP-diacyloglicerolu w jednoetapowym szlaku katalizowanym przez występującą wyłącznie u bakterii syntazę fosfatydylocholiny. Bakterie syntetyzują równocześnie PC w szlaku trzykrotnej metylacji PE. Porównanie profilu lipidów syntetyzowanych w tych dwóch niezależnych szlakach wskazuje na różnice w ilości i rozmieszczeniu lipidów w błonie OM i IM. Modyfikacje w strukturze lipidów wpływają na fizyko-chemiczne właściwości błon bakterii i mogą decydować o mechanizmie oddziaływania z przeciwbakteryjnymi białkami na co wskazuje większa wrażliwość na działanie apolipoforyny III *L. gormanii* hodowanej na podłożu z egzogenną choliną.

Wnioski

1. Lipidy *L. gormanii* obejmują glicerolipidy (TG i DG), fosfolipidy (PE, PC, CL, PG) oraz sfingolipidy (ceramidy i heksozylceramidy), które są charakterystyczne zarówno dla błony zewnętrznej (OM) jak i wewnętrznej (IM).
2. *L. gormanii* syntetyzuje PC w dwóch niezależnych szlakach: PCS i PMTA.
3. Porównanie profilu lipidów syntetyzowanych w dwóch szlakach wskazuje na różnice w ilości i rozmieszczeniu lipidów w błonie OM i IM.
4. Monowarstwy utworzone z lipidów bakterii hodowanych na podłożu z choliną były bardziej uporządkowane, gęściej upakowane i tworzyły mniej domen w porównaniu do monowarstw lipidów bakterii hodowanych na podłożu bez dodatku choliny.

5. *L. gormanii* wykazywała zależną od dawki wrażliwość na ekstrakt hemolimfy gąsienic *Galleria mellonella* oraz jej głównego składnika apolipoforynę III.
6. Bakterie hodowane na podłożu z egzogenną choliną były bardziej wrażliwe na apolipoforynę III w porównaniu do bakterii hodowanych bez dodatku egzogennej choliny.

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The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*

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The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*

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Among *Legionella* species, which are recognized to be pathogenic for humans, *L. gormanii* is the second prevalent causative agent of community-acquired pneumonia after *L. pneumophila*. Anti-*L. gormanii* activity of *Galleria mellonella* hemolymph extract and apolipophorin III (apoLp-III) was examined. The extract and apoLp-III at the concentration 0.025 mg/ml caused 75% and 10% decrease of the bacteria survival rate, respectively. The apoLp-III-induced changes of the bacteria cell surface were analyzed for the first time by atomic force microscopy. Our studies demonstrated the powerful anti-*Legionella* effects of the insect defence polypeptides, which could be exploited in drugs design against these pathogens.

Key words: *Legionella gormanii*, *Galleria mellonella*, apolipophorin III, Atomic Force Microscopy

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INTRODUCTION

Currently, members of the family *Legionellaceae* comprise 58 described species that are highly successful in colonizing natural aquatic environments (Euzeby, 2013). A particular hallmark of these bacteria is their dual host system allowing intracellular growth in protozoa (from the genera *Acanthamoeba*, *Naegleria*, *Hartmanella*) and in human alveolar macrophages during infection (Fields *et al.*, 2002). The ability to survive and replicate inside unicellular organisms contributed to the acquisition of virulence factors that enable *Legionella* to overcome the antimicrobial activities of human macrophages. The clinical manifestations of *Legionella* infections are primarily related to the respiratory tract. The most common presentation is acute pneumonia, which varies in severity from mild illness to fatal multilobar pneumonia. *Legionella* is widely recognised as an important source of community- and hospital-acquired pneumonia. The second form of a respiratory illness is called Pontiac fever, which is a flu-like infection (Palusinska-Szysz & Cendrowska-Pinkosz, 2009). The first described *Legionella* species, *L. pneumophila*, is unceasingly the dominating species among clinical isolates, whereas *L. gormanii* is the second prevalent causative agent of community-acquired pneumonia (Lode, 1987). *Legionella* infections are difficult to treat because of their intracellular localization in phagocytic cells. Moreover, bacteria released from *A. castellanii* are more resistant to chemical disinfectants and antibiotics used to treat pneumonia in comparison with those residing outside amoebae in the environment or laboratory cultured on *Legionella* artificial medium. A combination of fluoroquinolones with macrolides is an effective method of Legionnaires' disease treatment. However, the mortality rate among patients with hospital-acquired pneumonia is 14%, and with community-acquired pneumonia ranges from 5 to 10% (Benin *et al.*, 2002; Palusinska-Szysz, 2011). Therefore, the development of new antibacterial agents for *Legionella* infections is urgently needed.

A rich source of natural defence peptides (antimicrobial peptides; AMPs) with different biochemical properties and antimicrobial activity is insect's hemolymph (Bulet *et al.*, 2004; Bulet & Stöcklin, 2005). Due to amino acid composition, amphipathicity, cationic charge, and molecular size, AMPs can interact with microbial cell membranes forming toroidal or barrel-stave pores, or micelles composed of peptides and the membrane phospholipid molecules. Disturbing a proper structure of a cell membrane leads to depolarization, increased permeabilization, and even membrane fragmentation, which results in death of the invading microbes (Bulet *et al.*, 2004). These properties and selective toxicity towards some pathogens make them the patterns for designing drugs alternative to antibiotics.

A great arsenal of defence peptides of different biochemical and antimicrobial properties has been described in *Galleria mellonella* immune hemolymph. In addition, lysozyme and apolipophorin III (apoLp-III) play important role in the insect immune response against bacteria and fungi (Hultmark, 1996; Weers & Ryan, 2006; Cytryńska *et al.*, 2007; Brown *et al.*, 2008; 2009; Zdybicka-Barabas *et al.*, 2013). ApoLp-III, an abundant hemolymph protein, is involved in lipid transport and immune reactions. Because of lipopolysaccharide (LPS), lipoteichoic acid (LTA), and β-1,3-glucan binding ability, the protein is considered as an important pattern recognition receptor (Halwani *et al.*, 2000; Pratt & Weers, 2004; Whitten *et al.*, 2004; Zdybicka-Barabas & Cytryńska, 2013). The defence proteins and peptides isolated in our laboratory from immune hemolymph of *G. mellonella* larvae exhibit antimicrobial activity against different fungi, Gram-positive and Gram-negative bacteria

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Abbreviations: ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; AFM, atomic force microscopy; AMP, antimicrobial peptide; apoLp-III, apolipophorin III; BCYE, buffered charcoal yeast extract; LPS, lipopolysaccharide; LTA, lipoteichoic acid; RMS roughness, root-mean-square roughness; TFA, trifluoric acid.

(Cytryńska *et al.* 2007; Zdybicka-Barabas & Cytryńska, 2011; Zdybicka-Barabas *et al.*, 2012a; 2012b; 2013). Recently, the greater wax moth *G. mellonella* has been successfully used to study pathogenesis and infection by different human pathogenic bacteria and fungi, including *L. pneumophila* (Harding *et al.*, 2012; 2013a; 2013b). In our previous study, anti-*L. dumoffii* activity of *G. mellonella* defensin and apoLp-III has been documented indicating promising potential of the insect defence factors in fighting *Legionella* spp. (Palusinska-Szysz *et al.*, 2012).

In order to test antimicrobial activity of *G. mellonella* defence compounds against other species of *Legionella*, the effects of hemolymph methanolic extracts and the purified apoLp-III on *L. gormanii* cells were investigated. In addition, the apoLp-III-induced changes of the bacteria cell surface topography and properties were imaged and analyzed by atomic force microscopy (AFM). Our study is an attempt at assessment of the potential of the new agents in elimination of *L. gormanii*.

MATERIALS AND METHODS

Microorganisms and growth conditions. *L. gormanii* strain NCTC 11401 (Health Protection Agency Culture Collections, Salisbury, UK) was cultured on buffered charcoal-*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) yeast extract agar plates (BCYE; pH 6.9) (Oxoid, Basingstoke, UK) for 72 h at 37°C in a humidified incubator (5% CO_2) (Feeley *et al.*, 1979). *Micrococcus luteus* ATCC 10240 and *Escherichia coli* D31 (Boman *et al.*, 1974) were grown in 2.5% Luria-Bertani (LB) medium at 28°C and 37°C, respectively.

Insects immunization and preparation of hemolymph methanolic extracts. The larvae of *G. mellonella* (Lepidoptera: Pyralidae) were reared on honeybee nest debris (a natural diet) at 30°C in the dark. The immune challenge was performed by puncturing of the last instar larvae with a needle dipped into a pellet containing live *E. coli* D31 and *M. luteus* cells. The immune hemolymph was collected 24 h after the challenge. The methanolic extracts containing antimicrobial peptides and proteins below 30 kDa were prepared from the hemocyte-free hemolymph and deprived of lipids as described earlier (Cytryńska *et al.*, 2007). The protein concentration was determined by a Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Purification of apolipophorin III. *G. mellonella* apoLp-III was purified from the immune hemolymph extract as described in our previous study (Zdybicka-Barabas *et al.*, 2013). Briefly, the freeze-dried hemolymph extract dissolved in 0.1% trifluoroacetic acid (TFA) was subjected to the HPLC chromatography using a Discovery Bio Wide Pore C18 4.6 mm × 250 mm column (Sigma-Aldrich, USA) and two buffer sets, A: 0.1% TFA (v/v), B: 0.07% TFA, 80% acetonitrile (v/v). A linear gradient from 30 to 70% of buffer B over 35 min and 1 ml/min flow rate was applied. The homogenous fraction containing apoLp-III was freeze-dried, subjected to weighting, redissolved in sterile deionized water, and stored at –80°C until use. The homogeneity and identity of apoLp-III was confirmed by SDS-PAGE electrophoresis (Schägger & von Jagow, 1987) and by N-terminal sequencing on an automatic protein sequencer (Procise 491, Applied Biosystems).

Antimicrobial assays. The activity of immune hemolymph extract and apoLp-III against *L. gormanii* was carried out using a colony counting assay as described previously (Palusinska-Szysz *et al.*, 2012). Briefly, 10 µl of a bacterial suspension (obtained by 2×10^{-4} dilution of a suspension with OD_{620} = 0.1) was incubated without (control) or with the extract (final protein concentrations 0.025–0.8 mg/ml) or apoLp-III (final protein concentrations 0.025–0.2 mg/ml) at 37°C for 1 h. Then, the incubation mixtures were spread onto BCYE medium plates, incubated for 4 days at 37°C, and the number of the colony-forming units (CFU) was determined. The minimal inhibitory concentration (MIC) was defined at the concentration which yielded 95% inhibition of bacterial growth. The data were calculated from three independent experiments, each performed in triplicate.

Atomic force microscopy imaging of *L. gormanii*. Forty µl of a water suspension (OD_{620}=0.2) containing the *L. gormanii* cells grown on BCYE medium were incubated without (control) and in the presence of purified *G. mellonella* apoLp-III (final concentration 0.2 mg/ml) at 37°C for 1 h. After centrifugation (8000 × *g*, 10 min., 4°C) the bacteria were suspended in 5 µl of pyrogen-free water, applied on the surface of mica discs and allowed to dry overnight at room temperature.

L. gormanii cell surface imaging was carried out using NanoScope V AFM (Veeco, USA) in "PeakForce QNM" operation mode with a NSG 30 silicon tip (spring constant of 20N/m; NT-MDT, Russia) (Analytical Laboratory, Faculty of Chemistry, UMCS, Lublin, Poland). The data were analyzed with Nanoscope Analysis ver. 1.40 software (Veeco, USA). Two fields on each mica disc were imaged. The roughness values were measured over the entire bacterial cell surface on 3 µm×3 µm areas. The average surface root-mean-square (RMS) roughness was calculated from forty fields (265 nm×265 nm).

Statistics. Statistical analysis was performed using the Wilcoxon's paired test. The data were presented as ± standard deviation (SD) from three independent experiments.

RESULTS AND DISCUSSION

The antibacterial activity of *G. mellonella* immune hemolymph extract against *L. gormanii* was evaluated. The results showed dose-dependent killing of *L. gormanii* cells by the extract (Table 1). The incubation of *L. gormanii* in the presence of the extract at the concentration 0.025 mg/ml for 1 h caused more than 75% decrease of the bacteria survival rate compared to the control,

Table 1. The growth inhibition of *L. gormanii* by *G. mellonella* immune hemolymph extract.

Concentration of hemolymph extract (mg/ml)	Survival rate (%)
0	100 (%)
0.025	23.8 (±1.5) (%)
0.05	15 (±0.6) (%)
0.1	11 (±0.6) (%)
0.2	7 (±0.6) (%)
0.4	6 (±0.6) (%)
0.8	5 (±0.6) (%)

L. gormanii was incubated with the *G. mellonella* immune hemolymph extract at the concentrations of 0–0.8 mg/ml at 37°C for 1 h. The bacteria were then seeded on BCYE agar plates and the colonies were counted after four days incubation at 37°C in a 5% CO_2 atmosphere. Data were expressed as means ±S.D. of three independent experiments.

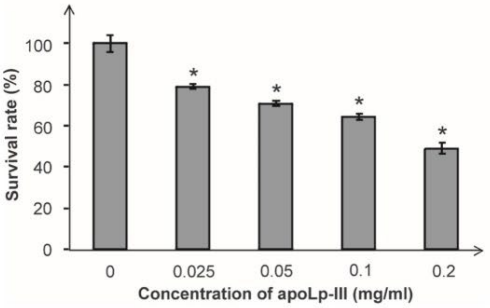

Figure 1. The effect of *G. mellonella* apoLp-III on *L. gormanii* survival rate.
The bacteria were incubated with the *G. mellonella* apoLp-III at the concentrations of 0–0.2 mg/ml at 37°C for 1 h. Cells were then plated on BCYE agar and the number of colonies was counted after four days incubation at 37°C in a 5% CO_2 atmosphere. Experimental results were mean ±S.D. of three independent experiments. The Wilcoxon's paired test was used for comparisons within groups. *p* values *$p \leq 0.001$ were considered.

i.e. non-treated bacteria. The MIC value defined as the concentration yielding at least 95% inhibition of bacterial growth for the extract was determined to be 0.8 mg/ml. However, the extract used at the concentration 0.4 mg/ml reduced the bacteria survival rate to almost the same extent, i.e. by 94%. Recently, we have reported that antimicrobial proteins and peptides of the *G. mellonella* immune hemolymph extract inhibited *L. dumoffii* growth. When the extract was used at the concentration 0.4 mg/ml, ca. 50% decrease of *L. dumoffii* survival rate was observed (Palusinska-Szysz *et al.*, 2012).

A main protein component of *G. mellonella* hemolymph extract is apoLp-III (Cytryńska *et al.*, 2007). Hence, the effect of apoLp-III on *L. gormanii* survival rate was studied. A colony counting assay using apoLp-III at the concentrations 0.025–0.2 mg/ml revealed that *L. gormanii* cells were sensitive to apoLp-III. The protein at the concentration 0.2 mg/ml caused ca. 50% decrease of the bacteria survival rate (Fig. 1). Interestingly, when apoLp-III was used at the concentration 0.025 mg/ml, the survival rate of *L. gormanii* decreased only by 10%, whereas the hemolymph extract at the same concentration caused 75% reduction of bacterial survivability (Fig. 1). The results indicated that although *L. gormanii* was susceptible to apoLp-III action, other proteinaceous (e.g. defence proteins and peptides) and non-proteinaceous compounds present in *G. mellonella* hemolymph extract were probably also involved in killing of the bacteria. Increasing of the hemolymph extract and apoLp-III concentrations above 0.8 mg/ml and 0.2 mg/ml, respectively, did not reduce further *L. gormanii* survival rate (data not shown). However, the explanation of this fact needs further investigations.

In our previous papers, usefulness of an atomic force microscopy for analysis of an influence of *G. mellonella* defence factors on bacterial and fungal cell surface has been demonstrated (Zdybicka-Barabas *et al.*, 2011; 2012a; 2012b; 2013). In this study, AFM was used for examining of the *G. mellonella* apoLp-III effect on *L. gormanii* cell surface. AFM imaging revealed that the surface of *L. gormanii* control cells was covered with small uniform granules (Figs. 2, 3). On the surface of some control cells few shallow depressions were also visible.

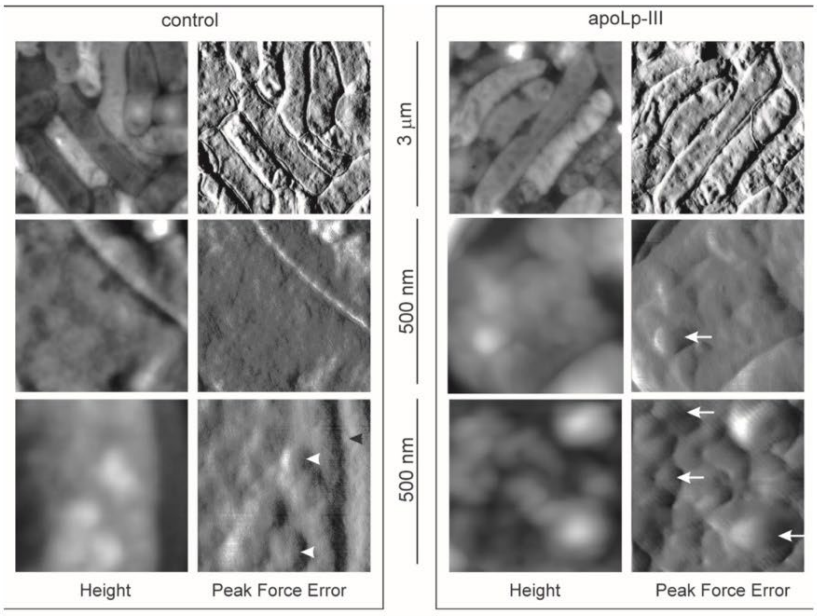

Figure 2. The effect of *G. mellonella* apoLp-III on *L. gormanii* cell surface topography.
The cells were incubated without (control) or in the presence of apoLp-III (0.2 mg/ml) at 37°C for 1 h and then imaged by AFM. The height and "peak force error" images are presented. The white and black arrowheads indicate granules and furrows, respectively, on the control cells surface. The white arrows mark bubble-like features appearing on apoLp-III-treated cells.

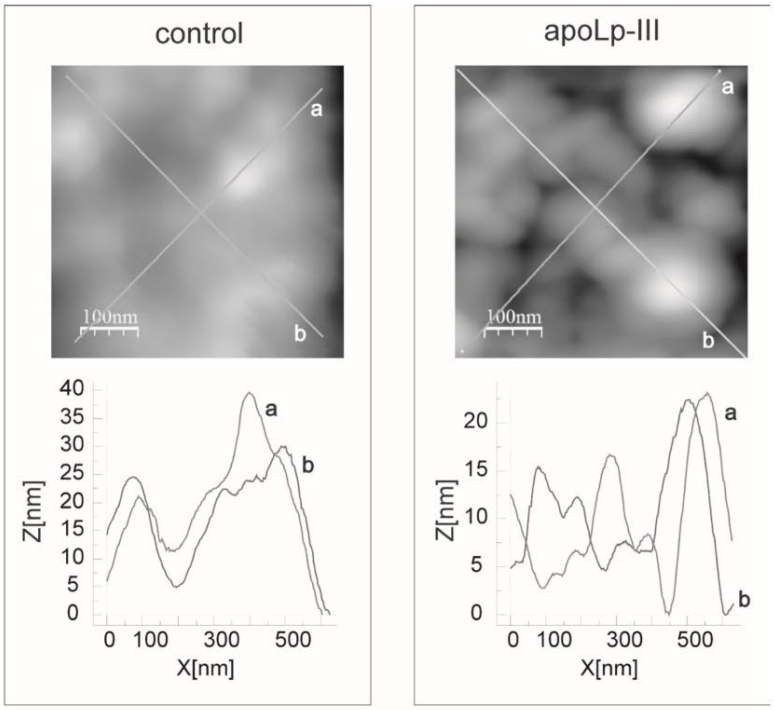

Figure 3. Profile section analysis of *L. gormanii* cell surface.
The cells were incubated without (control) or in the presence of apoLp-III (0.2 mg/ml) at 37°C for 1 h and then imaged by AFM. The upper panels present the height images of the cell surface. The bottom panels demonstrate the section profiles corresponding to the lines (**a**, **b**) shown in the height images.

In addition, the cells with uneven surface covered with furrows, some of them 10–20 nm deep and even 200 nm wide, were detected in the control samples. In contrast, the most of the apoLp-III-exposed cells were decorated with numerous rounded bubble-like features of different size, 15–20 nm in height and 100–200 nm in diameter (Figs. 2, 3). The alterations of the *L. gormanii* cell surface caused by apoLp-III were also reflected by 1.44-fold increase in cell surface roughness, the parameter used to describe the structural heterogeneity of the bacterial cell surface. The RMS roughness values for the control and apoLp-III-treated cells were calculated as 2.935 nm (±1.016) and 4.2305 nm (±1.003; p=0.00103), respectively. One of the reasons of the increase of the surface roughness could be rupturing of bacterial cell following the apoLp-III binding to LPS and phospholipids, which was reported by Pratt & Weers (2004).

Although both *L. dumoffii* and *L. gormanii* were sensitive to *G. mellonella* apoLp-III, the 8-fold lower concentration of apoLp-III was sufficient to exert the same bactericidal effect on *L. gormanii* in comparison to *L. dumoffii* (Palusinska-Szysz *et al.*, 2012). The high sensitivity of *L. gormanii* to apoLp-III in comparison with *L. dumoffii* indicates that different *Legionella* species could exhibit diverse susceptibility to the insect-derived antimicrobial factors, possibly reflecting differences in the cell surface properties.

Searching for antimicrobials effective against *L. gormanii* is especially important since this species was isolated from peadiatric cases. Moreover, antimicrobial therapy commonly used in empirical pneumonia treatment in young patients is not effective against *Legionella* spp. (Ephros *et al.*, 1989; Greenberg *et al.*, 2006).

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Publikacja 2 – praca przeglądowa

Kowalczyk Bożena, **Chmiel Elżbieta**, Palusińska-Szysz Marta

The role of lipids in *Legionella*-host interaction

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Review

The Role of Lipids in *Legionella*-Host Interaction

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Abstract: *Legionella* are Gram-stain-negative rods associated with water environments: either natural or man-made systems. The inhalation of aerosols containing *Legionella* bacteria leads to the development of a severe pneumonia termed Legionnaires' disease. To establish an infection, these bacteria adapt to growth in the hostile environment of the host through the unusual structures of macromolecules that build the cell surface. The outer membrane of the cell envelope is a lipid bilayer with an asymmetric composition mostly of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. The major membrane-forming phospholipid of *Legionella* spp. is phosphatidylcholine (PC)—a typical eukaryotic glycerophospholipid. PC synthesis in *Legionella* cells occurs via two independent pathways: the *N*-methylation (Pmt) pathway and the Pcs pathway. The utilisation of exogenous choline by *Legionella* spp. leads to changes in the composition of lipids and proteins, which influences the physicochemical properties of the cell surface. This phenotypic plasticity of the *Legionella* cell envelope determines the mode of interaction with the macrophages, which results in a decrease in the production of proinflammatory cytokines and modulates the interaction with antimicrobial peptides and proteins. The surface-exposed O-chain of *Legionella pneumophila* sg1 LPS consisting of a homopolymer of 5-acetamidino-7-acetamido-8-O-acetyl-3,5,7,9-tetradeoxy-L-glycero-D-galacto-non-2-ulosonic acid is probably the first component in contact with the host cell that anchors the bacteria in the host membrane. Unusual in terms of the structure and function of individual LPS regions, it makes an important contribution to the antigenicity and pathogenicity of *Legionella* bacteria.

Keywords: *Legionella*; phosphatidylcholine; lipopolysaccharide

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1. Introduction

Bacteria from the family *Legionellaceae* are Gram-negative bacilli, which are part of the natural microflora of aquatic and soil environments, as well as anthropogenic ecosystems. From the microbiological point of view, the ecology of *Legionella* bacilli is highly complex. *Legionella* bacteria survive in a temperature range between 5 °C and 65 °C and at pH 7–9; however, due to their specific nutritional requirements and a narrow range of environmental conditions necessary for their growth, they are unable to compete with other bacteria [1]. The bacteria require specific physicochemical conditions for development, which can only be fulfilled inside the eukaryotic host. Protozoa, commonly found in natural ecosystems, are an important link in the food chain and exert a significant impact on bacterial populations. However, not all bacteria are ingested, killed, and digested by protozoa. The outcome of the *Legionella*–protozoa interaction is associated with the host species. In some cases, *Legionella* resists digestion and kills the host, and in others, the protist digests the bacterium [2]. A total of 61 different *Legionella* species and more than 70 serogroups have been identified [3]. An in-silico analysis of the *Legionella pneumophila* genome identified several genomic islands indispensable for bacterial growth within protozoa, which differ among the various amoeba species [4]. Free-living amoebae from the genus *Acanthamoeba*, *Naegleria*, and *Vermamoeba* (*Hartmanella*) and ciliates from the genus *Tetrahymena* provide

an intracellular niche in which *Legionella* can proliferate [5,6]. The intracellular growth of the bacteria has been associated with the enhanced environmental survival, virulence, and antibiotic resistance of the bacteria [7,8]. *L. pneumophila*, i.e., the most pathogenic species of this family, use many molecular and cellular aspects of intracellular proliferation inside protozoa in patho-adaptation to human cells [9]. *Legionella* enter the human organism from the natural environment via contaminated aerosols generated by cooling towers, large air-conditioning systems, fountains, showers, groundwater used for sprinkler irrigation, and similar sources [10,11]. This mode of bacterial spread is regarded as the major infection route, although bacterial transmission from person to person has been reported as well [12]. In the human organism, the bacteria cause respiratory infections with varying severity: from a flu-like infection called Pontiac fever, which does not require specialised treatment, to an acute, multi-lobar pneumonia called Legionnaires' disease, which may result in death [13]. The bacteria proliferate robustly in lung alveolar macrophages, leading to tissue damage and the subsequent development of the disease. The macrophage resistance of *Legionella* spp. is a prerequisite for their virulence. Bacterial survival in contact with eukaryotic cells—in particular, those predisposed for killing bacteria—depends on many determinants of adaptation to an environment that is extremely hostile but rich in nutrients. The bacteria overcome the killing mechanisms of phagocytes by means of specialised protein translocation systems: type II Lsp and type IVB Dot/Icm. The type II system controls the secretion of enzymes (lipases, protease, and RNase), thus promoting growth, intracellular replication, and virulence [14]. The Dot/Icm transporter delivers substrates that modulate multiple host cell processes, resulting in the biogenesis of the *L. pneumophila*-containing vacuole (LCV) permissive for intracellular bacterial replication. *L. pneumophila* achieve host infection by exporting approximately 300 substrates of the Dot/Icm system across one or two cell membranes to the site of action [15]. The precise delivery of the effectors to the host cell in a timely manner and space is possible thanks to an efficiently functioning bacterial cell envelope. The cell envelope of *L. pneumophila* is typical for Gram-negative bacteria and consists of two distinct membranes, the inner (IM) and outer membrane (OM), separated by the periplasm. The periplasm contains a relatively thin layer of strongly crosslinked peptidoglycan and various proteins. Peptidoglycan is composed of muramic acid, glucosamine, glutamic acid, alanine, and meso-diaminopimelic acid in a molar ratio of 0.8:0.8:1.1:1.7:1 [16]. The OM is asymmetric, with an inner leaflet mostly composed of phospholipids and an outer leaflet mostly comprising lipopolysaccharides (LPS). This asymmetry is critical for maintaining the OM permeability barrier. LPS consists of three regions: O-antigen, core, and lipid A. The lipid A region anchors LPS molecules to the outer membrane through hydrophobic interactions with the acyl chains of the phospholipids (PLs) constituting the inner layer of this membrane [17]. In addition to proteins, which have a fundamental importance for various aspects of cell physiology, including the infection of a host organism, lipids are involved in the highly specific interactions with the host cell. Three types of lipid-containing molecules are present in the cell envelope of *L. pneumophila*: phospholipids (PL), lipopolysaccharide (LPS), and lipoproteins (Figure 1).

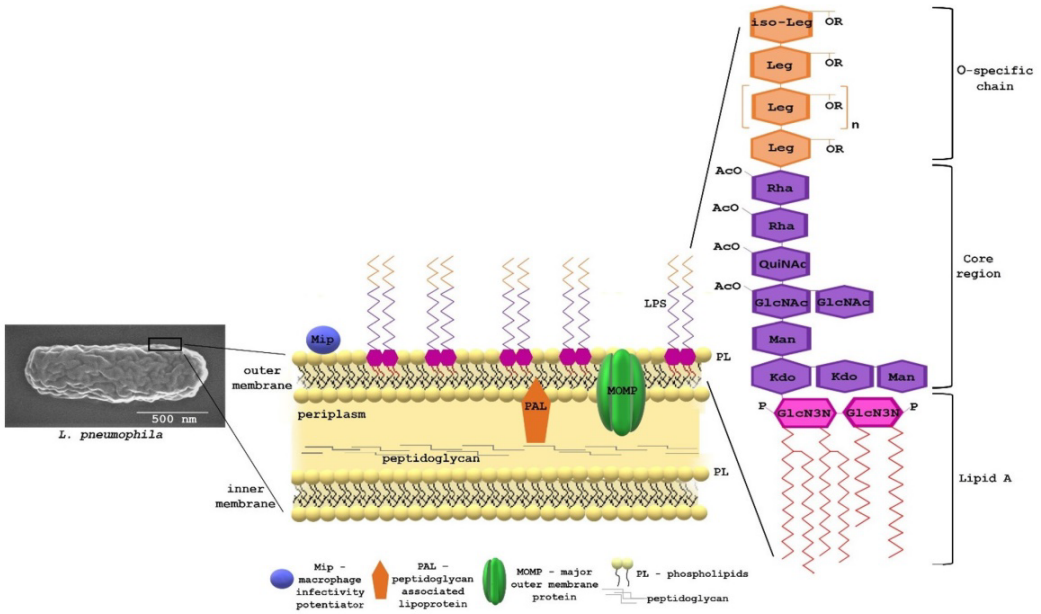

Figure 1. Scanning electron microscopy image of *Legionella pneumophila*, and model of the *L. pneumophila* cell envelope. Structure of lipopolysaccharide (LPS) (modified from [16]). Leg., legionaminic acid; Rha, rhamnose; OAc, O-acetyl; QuiNAc, acetylquinovosamine; GlcNAc, acetylglucosamine; Man, mannose; Kdo, 3-deoxy-d-manno-oct-2-ulosonic acid; P, phosphate; and GlcN3N, 2,3 diamino-2,3-dideoxy-D-glucose.

2. Characteristics of *Legionella* Phospholipids

Most bacterial membranes are formed by amphiphilic lipids, mainly glycerophospholipids. Phospholipids are composed of a variable hydrophilic head group, two fatty acids esterified in the *sn-1* and *sn-2* positions of the glycerol moiety, which form a hydrophobic tail, and a phosphate group in the *sn-3* position. Phospholipids fulfil a passive role as building blocks of the lipid bilayer and stimulate cell functions, e.g., targeted protein transport, DNA replication, or signal transduction. The composition and distribution of phospholipids in the membrane, their ability to undergo hydrolysis, and the modifications of the phospholipid "head-groups" constitute a specific "molecular language" in the interactions with other cell molecules and the environment [18].

2.1. Phospholipids of Legionella spp.

The most typical bacterial phospholipids include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Zwitterionic PE is found in many groups of Gram-negative bacteria (e.g., *Enterobacteriaceae*) and in several Gram-positive bacteria, while anionic PG and CL are common in Gram-positive bacteria such as enterococci, streptococci, and staphylococci [19,20].

The following phospholipid classes have been identified in the membranes of *L. pneumophila*, *L. lytica*, *L. bozemanae*, *L. dumoffii*, *L. anisa*, *L. gormanii*, and *L. longbeachae*: phosphatidylcholine (PC), PE, CL, and PG [21–25] (Figure 2A). PC and PE are the major *Legionella* phospholipids. The relative amount of PC in the different species ranges from 30% to 50% of all PLs. PE dominates in the lipids of *L. anisa*, *L. gormanii*, and *L. longbeachae* [25]. The distribution of phospholipids in the particular layers of the *L. pneumophila* bacterial envelope is as follows: the outer layer of the cytoplasmic membrane (CM-1) contains ca.

43% PC, 45% PE, and 12% CL, while the inner layer (CM-2) has 35% PC, 42% PE, 14% CL, and 8% PG. PE dominates in the outer (OM-1) and inner layers (OM-2) (ca. 50% in each layer), whereas PC constitutes 27% and 33%, respectively, CL represents 10% and 6%, respectively, and PG accounts for ca. 13% [26].

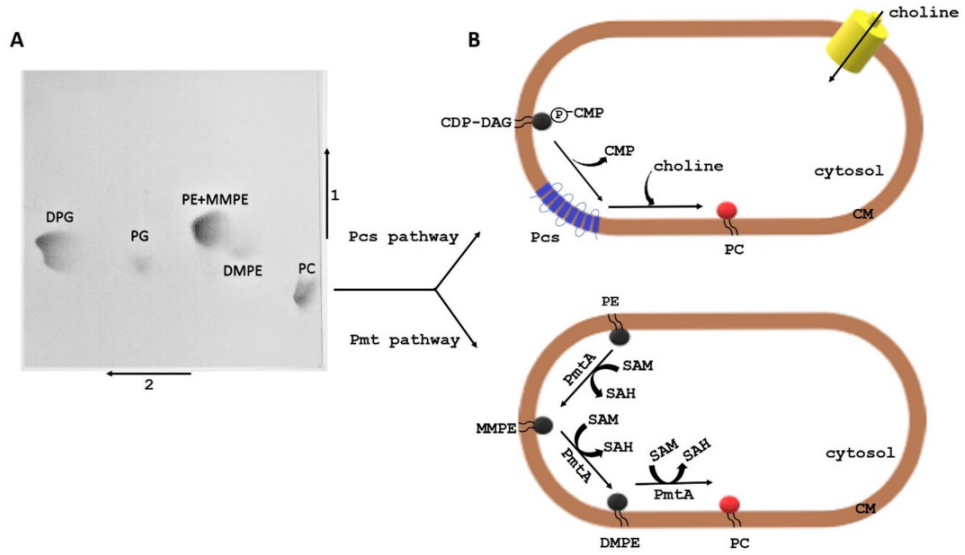

Figure 2. (**A**) Two-dimensional thin-layer chromatogram of phospholipids from *Legionella gormanii*. PC—phosphatidylcholine, DMPE—phosphatidyl-*N*,*N*-dimethylethanolamine, PE—phosphatidylethanolamine, MMPE—phosphatidyl-*N*-monomethylethanolamine, PG—phosphatidylglycerol, and DPG—diphosphatidylglycerol (cardiolipin). Solvent system: (1) first dimension—chloroform/methanol/water (14:6:1, *v*/*v*/*v*) and (2) second dimension—chloroform/methanol/glacial acetic acid (13:5:2, *v*/*v*/*v*). (**B**) PC biosynthesis pathways in *L. pneumophila*. CDP, diacylglycerol; Pcs, phosphatidylcholine synthase; PmtA, phospholipid *N*-methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; and CM, cytoplasmic membrane.

2.2. Fatty Acid Composition of Legionella spp.

The major fatty acyl residues in *Escherichia coli* phospholipids are palmitic, stearic, *cis*-vaccenic, and cyclopropane-containing lactobacillic acids [19]. An analysis of the fatty acid (FA) composition of *Legionella* spp. showed the presence of cyclopropyl heptadecanoic acid and a high content of branched (*iso* and *anteiso*) FAs (40–90%). The cellular FAs are dominated by 14-methylpentadecanoic (i16:0), 12-methyltetradecanoic (a15:0), and 14-methylhexadecanoic (a17:0) acids. On the basis of the differences in the relative amounts of 14-methylpentadecanoic (il6:0), hexadecenoic (16:0), and 12-methyltetradecanoic (a15:0) acids, Lambert and Moss divided the investigated *Legionella* species into three major groups (16C, A15, and A15/16C). The 16C group includes species with a high content of i16:0 or 16:1 (with one unsaturated bond) or both fatty acids. The A15 group includes species with a high content of a15:0 at concentrations approximately twice that of i16:0. The third group comprises species with a15:0 and i16:0 as major fatty acids in approximately equal amounts [27]. An analysis of the fatty acid contents in the individual *L. dumoffii* lipid classes showed that PC and PE contained high amounts of a15:0 and cyclopropyl 17:0 fatty acids. The PC fraction was also characterised by the presence of unbranched hexadecenoic acid, while PE had a17:0 fatty acid. Similar to PC and PE, CL contained significant amounts of

a15:0 and branched i16:0. Saturated and unbranched hexadecenoic and octadecanoic fatty acids dominated in the PG fraction [28].

Detailed information on the structure of phospholipids, i.e., the distribution of fatty acids in the *sn-1* and *sn-2* positions of the glycerol backbone, was provided by liquid chromatography (LC) coupled with mass spectrometry (MS). Besides components that were common to *L. bozemanae*, *L. lytica*, and *L. dumoffii*, e.g., PC16:0_15:0 and PE15:0_15:0, the species differed in the composition of fatty acids bound with different classes of phospholipids. In the phosphatidylcholine class, PC17:0_15:0 was characteristic for *L. bozemanae*, PC16:0_14:0 and PC18:0_16:1 for *L. lytica*, and PC16:0_17:1 (or cyclopropyl 17:0) for *L. dumoffii*. PE16:1_15:0 dominated in *L. bozemanae*, PE14:0_14:0 and PE15:0_14:0 in *L. lytica*, and PE15:0_15:1 in *L. dumoffii*.

The differences in the composition of fatty acids contained in the phospholipids are an important chemotaxonomic feature and may have practical relevance in the diagnostics of this bacterial group at the species level. Moreover, the aforementioned *Legionella* species synthesised straight-chain acids besides the branched ones, in contrast to *L. pneumophila*, whose phospholipid molecules contained only branched fatty acids [21–25]. The LC-MS/MS method based on the structural analysis of PLs can be applied in *Legionella* diagnostics, but a greater number of species should be examined to create PL databases allowing the identification of *Legionella* species.

L. pneumophila change their fatty acid compositions, depending on the growth phase. In the stationary growth phase, the proportion of branched-chain fatty acids rises to over 60%, and the average length of fatty acids in phospholipid molecules decreases, compared to the exponential growth. Since acyl chains attached to phospholipids determine various membrane properties, e.g., fluidity and sensitivity to antibiotics, Verdon et al. showed that changes in the pattern of *L. pneumophila* fatty acids between the growth phases led to an increased tolerance to the antimicrobial peptide warnericin RK [29].

PC is the main phospholipid of *Legionella* bacteria. This zwitterionic phospholipid, characteristic of eukaryotic cells and a narrow group of bacteria, is responsible for many biological functions, including interactions with the host cell.

2.3. PC Synthesis Pathways

Among the four experimentally confirmed PC synthesis pathways in bacterial cells, *Legionella* spp. use the phosphatidylethanolamine (PE) methylation (Pmt) pathway and the PC synthase (Pcs) pathway [30] (Figure 2B). In the *N*-methylation pathway, PE is *N*-methylated three times, which leads to the formation of PC. Monomethyl-*N*-phosphatidylethanolamine (MMPE) and dimethyl-*N*,*N*-phosphatidylethanolamine (DMPE) are intermediate compounds in this process. The pathway is catalysed by phospholipid *N*-methyltransferases (PmtA). The enzyme uses *S*-adenosylmethionine (SAM) as the methyl donor, converting it to *S*-adenosylhomocysteine (SAH) [31]. The Pcs pathway is a single-step reaction between choline and CDP-diacylglycerol to form PC [32]. The reaction is catalysed by the phosphatidylcholine synthase (Pcs) present exclusively in bacteria [33].

2.4. Genetic Diversity of Legionella pcs and pmtA Genes

As demonstrated by genetic analyses, *Legionella* species contain genes (*pcs* and *pmtA*) encoding enzymes involved in the synthesis of PC via two independent pathways. A comparative analysis of the nucleotide sequences of the *pcs* gene encoding Pcs has shown the high sequence identity of these genes among members of the *Legionellaceae* family (in a range from 64% to 98%). *L. bozemanae*, *L. anisa*, *L. gormanii*, *L. parisiensis*, and *L. tusconensis* were found to form one clade on the phylogenetic tree, which indicates that they are closely related. The three strains of *L. pneumophila*, *L. fallonii*, and *L. micdadei* exhibit a considerable phylogenetic distance from all non-*L. pneumophila* species [25]. The *pcs* genes in all analysed *Legionella* spp. exhibit a high conservation degree, although the genome of these bacteria is highly heterogeneous, with over 60% species-specific genes [34]. *Legionella* Pcs proteins encoded by *pcs* genes have a similar length of 254 amino acids the exceptions

are *L. pneumophila* (255 aa) and *L. longbeachae* (253 aa). These proteins contain a highly conserved 27-aa-long motif $DGX_2ARX_8PX_3GX_3DX_3D$, and the amino acid motif is shared with the CDP-alcohol phosphotransferase superfamily [35]. The Pcs proteins are highly hydrophobic. They contain up to eight transmembrane helices with *N*- and *C*-termini in the cytoplasm. Their *N*-terminal region has a domain responsible for enzymatic activity.

In comparison with the *pcs* genes, *Legionella pmtA* genes encoding PmtA enzymes exhibit higher sequence diversity and lower sequence identity to each other. As revealed by comparative sequence studies of the *pmtA* genes, *L. longbeachae* is closely related to *L. sainthelensi* (92% sequence identity), *L. cincinnatiens* (89%), *L. santicrucis* (91%), and *L. gratiana* (87%). However, no *pmtA* sequences for *L. drancourtii*, *L. gormanii*, *L. dumoffii*, and *L. micdadei* have been identified with the use of degenerative primer pairs homologous to *Legionella*, *Rhodobacter*, and *Rhizobium* bacteria. PmtA sequences have been found to diverge substantially among *Legionella* spp. at the amino acid level. The PmtA protein of *L. pneumophila* exhibited only 65–70% aa sequence identity to the proteins from other *Legionella*. The *pmtA* genes encode small 208–218-aa-long cytosolic proteins. The catalytic domain of these proteins includes a 9-aa-long motif: V/ILE/DXGXGXG. It is predicted to bind the methyl donor S-adenosylmethionine (SAM) and is characteristic for methyltransferases [36]. At least two PmtA families have been described in bacterial cells. One family is similar to the PmtA of *Rhodobacter sphaeroides* (Rs-PmtA), whereas the other type is similar to the PmtA of *Ensifer meliloti* (Sm-PmtA) [37]. The *Legionella* PmtA enzymes exhibit homology to the *Rhodobacter* Pmt-type enzyme, which, in turn, is homologous to UbiE (ubiquinone/menaquinone biosynthesis methyltransferase). The single PmtA enzyme in *Legionella* spp. catalyses successive PE *N*-methylation via the MMPE and DMPE intermediates to form PC. Interestingly, as shown by an analysis of the composition of *L. dumoffii* phospholipids, the bacterium produces methylated derivatives of PE, which provides evidence for the presence of the Pmt pathway in PC synthesis. However, the absence of a *pmtA* homologue in the *L. dumoffii* genome may suggest the presence of a new type of enzyme with PmtA activity, different from Sm-PmtA or Rs-PmtA.

A method for identification and discrimination between *Legionella* species in the set of duplex-PCR was specified based on the *16S rRNA* gene and the *pcs* and *pmtA* genes encoding PC synthase and PmtA, respectively [38].

2.5. Utilisation of Exogenous Choline for PC Synthesis by Legionella spp.

The use of exogenous choline by *Legionella* spp. in the Pcs pathway leads to a change in the contents of their individual classes of phospholipids. ^{31}P nuclear magnetic resonance (NMR) spectroscopy showed that *L. gormanii* cultured on a choline-supplemented medium synthesised by 21% and 12% higher PC and PE contents, respectively, and by 9% lower CL levels in comparison with bacteria grown on the medium without choline [25]. A comparative analysis of the contents of each PL class revealed that *L. dumoffii* grown on choline synthesised by 12% higher PC levels and by 12% lower PE amounts than bacteria cultured without the addition of choline [28]. *L. pneumophila* grown on the medium with choline produced by 6% and 3% higher quantities of PC and PE, respectively, and by 9% lower amounts of CL compared with bacteria grown on the medium without choline. However, the contents of individual classes of PLs in extracts from *L. anisa* and *L. longbeachae* cells were unchanged, irrespective of the culture conditions [25]. *L. bozemanae* and *L. dumoffii* grown on BCYE medium supplemented with *N*-nonadeuterotrimethyl-choline chloride synthesised labelled PC species. The occurrence of labelled PC such as d_9-PC16:0_15:0, d_9-PC15:0/15:0, d_9-PC17:0_15:0, d_9-PC16:1_15:0, d_9-PC17:0_17:0 cyclopropyl, and d_9-PC15:1_15:0 in *L. bozemanae* phospholipids, as well as d_9-PC14:0_16:0, d_9-PC16:0_17:1 (or 17:0 cyclopropyl), and d_9-PC15:0_15:0 in *L. dumoffii* phospholipids, indicated the utilisation of exogenous choline by these bacteria. An analysis of the distribution of labelled PC in the outer (OM) and inner (IM) membranes in *L. dumoffii* revealed that phosphatidylcholines derived from the Pcs pathway are located in both membranes. The selective MRM (multiple reaction monitoring) method showed greater amounts of PC in the inner membrane. A comparison

of ion intensities in the mass spectra (Matrix-Assisted Laser Desorption/Ionization/Time-of-Flight, MALDI-TOF) of PC (derived via the Pcs pathway) with unlabelled PC (derived via the Pmt pathway) indicated that both PC synthesis pathways operate simultaneously in *L. bozemanae* and *L. dumoffii* cells. The Pcs pathway was found to be a dominant, and probably more energy-efficient pathway [23,24,28]. The *L. pneumophila pmtA* mutant, in which the Pcs pathway depends on extracellular choline, synthesised almost all the PC, while the *pcs* mutant with the functioning Pmt pathway produced only 6% of PC [39].

The utilisation of extracellular choline was found to influence the composition of *L. gormanii* fatty acids. The bacterium cultured on choline nonsupplemented medium synthesised more a15:0 fatty acid and larger amounts of n16:0, 16:1, and cyclopropyl 17:0 fatty acids when grown on the medium with choline [25]. However, the addition of choline to the growth medium did not induce qualitative and quantitative changes in the fatty acid profiles in *L. pneumophila*, *L. anisa*, and *L. longbeachae* cells [25].

Exogenous choline induced changes not only in lipids and the fatty acid composition but, also, in proteins, as shown by FTIR (Fourier-Transform Infrared with Attenuated Total Reflection) spectroscopy. After choline supplementation, higher concentrations of proteins represented by the amide I and amide II bands in *L. anisa*, *L. gormanii*, and *L. longbeachae* were detected [25].

2.6. Biological Importance of Legionella PC

Legionella rods belong to the narrow group of bacteria synthesizing PC, which, in addition to membrane formation, plays important roles in bacterial–host interactions ranging from symbiosis to pathogenesis [40]. The presence of PC in cell membranes is specific to pneumonia-causing bacteria. The lung surfactant covering the small airways, bronchioles, and alveolar surface is composed of approximately 10% of protein and 90% of lipids, with approximately 80% of the lung surfactant lipids representing PC. *P. aeruginosa* utilises lung surfactant PC as a primary carbon and energy source, which facilitates high-cell density replication during lung infection [41]. In cystic fibrosis patients, in whom *P. aeruginosa* is a common cause of pulmonary infections, choline, i.e., a soluble product of PC and sphingomyelin hydrolysis, is a putative osmoprotectant in the high osmolarity environment of the lung [42]. The PC synthesis pathway (Pcs) can play the role of an environmental sensor, since the biosynthetic precursors present in lung tissue may exert an effect on *L. pneumophila* virulence via PC incorporation into the bacterial cell envelope [39]. Rapid PC synthesis might be required in *Legionella* spp. to quickly adjust their membrane physiology to new environmental conditions [43]. A PC-deficient *L. pneumophila* strain showed attenuated binding to macrophages and a defect in the functioning of the IV secretion system, which is essential for effector transport and formation of the intracellular replication niche in alveolar macrophages. The lack of PC in *L. pneumophila* membranes resulted in reduced cytotoxicity and impaired capability of the intracellular proliferation of these bacteria, which indicates an important function of this phospholipid in the interaction with the host cell [39]. The utilisation of extracellular choline and incorporation of PC into *Legionella* membranes lead to changes in the compositions (lipids and proteins) of cell membranes, which influences their physicochemical properties and interactions with external factors (other cells and antimicrobial peptides). Atomic force microscopy measurements revealed changes in the nanomechanical properties of the cell surface of *L. dumoffii* grown with extracellular choline, i.e., a two-fold increase in the Derjaguin–Muller–Toporov (DMT) modulus reflecting elasticity. A considerably more effective binding of apolipophorin III (apoLp-III, an insect homologue of human apolipoprotein E) to bilayers composed of PLs extracted from choline-grown *L. dumoffii*, thus enriched with PC, was observed [28]. This is in line with the study conducted by Zhang, which demonstrated a stronger binding of apoLp-III to PC compared to PE, indicating an important role of cell membrane PC in the interaction with apoLp-III [44]. Interestingly, the elasticity of the cell surface of choline-cultured *L. dumoffii* treated with apoLp-III did not change in contrast to the six-fold increase in the cell envelope elasticity of these bacteria grown without choline after incubation

with apoLp-III [45]. Another possible explanation for the stronger binding of apoLp-III to lipids isolated from *L. dumoffii* grown on choline is the change in the ratio between the PC and PE contents in these bacteria and the resulting alterations in the membrane architecture. PC formed a bilayer structure, whereas PE made the membrane more rigid. The 12% reduction in the PE content in membranes of choline-cultured *L. dumoffii* may be the cause of the less-tight membrane packing and easier apoLp-III binding. Similar to apoLp-III, defensin isolated from *Galleria mellonella* haemolymph was over three times more active against *L. dumoffii* when the bacterial cells were cultured in the presence of exogenous choline [46]. However, the differences in the morphology of the cells visualised with the use of the electron microscope, resulting from the action of apoLp-III and defensin, indicate a different mechanism of their interaction with *L. dumoffii*.

PC may serve as a specific recognition molecule. *L. pneumophila* PC is required for the binding of these bacteria to macrophages via the platelet-activating factor receptor (PAF receptor), and the efficiency of this process depends on the content of PC. The adhesion of *L. pneumophila* to macrophages was successfully blocked by a PAF receptor antagonist [39]. Chemically, PAF is 1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine, and, due to this structural similarity, *L. pneumophila* PC can mimic PAF and, by binding to its receptor, increase the chance of bacterial uptake by macrophages. *L. dumoffii* cells with increased PC contents underwent internalisation by macrophages of the THP-1 (the human monocytic leukemia cells) line more readily than bacteria with a reduced content of this phospholipid [24]. *Legionella* PC may influence the modulation of the inflammatory functions of macrophages measured by the level of proinflammatory cytokines (tumour necrosis factor, TNF-α and interleukin-6, IL-6). These cytokines are involved in the protective immune response controlling the early stages of *L. pneumophila* infection. The treatment of animals with either TNF-α or IL-6 resulted in a significant reduction in mortality caused by *L. pneumophila* infection.

Human phagocytic THP-1 cells, which differentiate into macrophages after treatment with phorbol 12-myristate 13 acetate (PMA), have been investigated. PMA-triggered THP-1 differentiation causes a rearrangement of the macrophage-specific kinome towards a more proinflammatory phenotype [47]. *L. dumoffii* grown on a choline-supplemented medium at a dose of 10 and 100 MOI (multiplication of infection) induced over a three-fold lower level of TNF-α in THP-1 macrophages than bacteria cultured without the addition of choline. Similarly, the inner membrane of *L. dumoffii* isolated from bacteria grown on the choline-supplemented medium was a weaker TNF-α inducer than the membrane of bacteria grown without choline. Statistically significant results were obtained in the presence of 100 ng/mL of the inner membrane. In turn, the same dose of the outer membrane of bacteria cultured with choline was a better cytokine inducer than the outer membrane of bacteria cultured without choline supplementation [24]. *L. anisa*, *L. longbeachae*, *L. gormanii*, and *L. pneumophila* grown on a medium with choline caused much lower TNF-α production, with the highest decrease in the TNF-α level for *L. longbeachae*, compared to those bacteria grown without the choline addition [25]. The supplementation of growth medium with choline was also the cause of a decrease in IL-6 production after the incubation of macrophages with *L. pneumophila*, *L. longbeachae*, and *L. anisa*, with the highest decrease by 52% in the level of IL-6 for *L. anisa* [25]. The exact mechanism of the weaker induction of proinflammatory cytokines by *Legionella* spp. grown on the choline-supplemented medium compared to those cultured without exogenous choline requires elucidation. A still open question is whether there are different mechanisms responsible for the attenuated production of proinflammatory cytokines, since there are differences among the various *Legionella* spp. in the PC structure and content, depending on culture conditions.

Changes in the contents and structures of membrane PCs may affect the contents and activity of other cell components (e.g., other classes of phospholipids and proteins), and the observed decrease in the proinflammatory cytokine production may result from the effect of various factors on the macrophages. PC synthesis in the Pcs pathway can lead to a change in the proportion between zwitterionic (PC and, PE) and anionic (CL and PG) lipids, resulting in an imbalance in the electrostatic charge of the cell membrane. Since such

a balance is required for many integral membrane proteins to adopt the correct topology in the cell membrane, changes in mutual relations between PLs can lead to modifications in the structure of the transmembrane α-helices of membrane proteins, altering the packing of these helices [48,49]. All of this induces surface changes in bacterial ligands and may lead to altered interactions with macrophages.

The less potent proinflammatory cytokine induction under the influence of bacteria grown on a choline-supplemented medium, in comparison with that induced by bacteria grown without a choline addition, may be one of the ways for bacterial evasion of the host immune system. To survive and multiply in host cells, *Legionella* rods have developed sophisticated adaptation mechanisms such as the ability to utilise extracellular choline sources. Selective blocking of phosphatidylcholine synthase can become a target in the specific therapeutic effect supporting the treatment of Legionnaires' disease. *Legionella* spp. are able to synthesise PC via two independent pathways, and potential drugs that block the activity of phosphatidylcholine synthase may contribute to the attenuation of the virulence trait.

3. Structure and Significance of *Legionella* Lipopolysaccharide

3.1. Chemical Structure of Legionella LPS

Lipopolysaccharide is a glycolipid composed of three covalently linked regions: lipid A, a core oligosaccharide featuring an outer and inner region, and an O-specific chain. Although LPS isolated from various *Legionella* species share common features in their basic architecture, the LPS of *L. pneumophila* differs significantly in the chemical structure and biological significance of the individual parts. The O-specific chain of the *L. pneumophila* Philadelphia strain consists of a homopolymer of α-(2-4)-linked 5-acetamidino-7-acetamido-8-O-acetyl-3,5,7,9-tetradeoxy-L-glycero-D-galacto-non-2-ulosonic acid [50]. This unusual sugar, termed legionaminic acid, is highly hydrophobic due to the presence of acetyl and acetimidoyl groups. The O-specific chain is bound to a seven-sugar outer core composed of rhamnose (Rha), mannose (Man), acetylquinovosamine (QuiNAc), and acetylglucosamine (GlcNAc) in the molar ratio of 2.1:1.1:1:1.4 [51]. The presence of *N*-acetyl groups (QuiNAc and GlcNAc); methyl groups of 6-deoxy sugars (Rha and QuiNAc); and *O*-acetyl groups at C2 of Rha, C4 of QuiNAc, and C3 of GlcNAc makes the outer core highly hydrophobic. The hydrophilic inner core consists of two molecules of 3-deoxy-D-manno-2-octulosonic acid bound by a 2→4 ketosidic linkage and one molecule of D-mannose connected to the C8 of Kdo present in the inner core [52]. A characteristic feature of the inner core of LPS from *L. pneumophila* is the absence of heptoses and phosphate residues. The carbohydrate backbone of *L. pneumophila* lipid A consists of a bisphosphorylated disaccharide of the 2,3 diamino-2,3-dideoxy-D-glucose (GlcN3N) amide linked with 3-hydroxy and 2,3-dihydroxy fatty acids [53,54]. The hydroxyl groups of the fatty acids linked directly to the sugar backbone are acylated by straight, branched (*iso* and *anteiso*), and long-chain fatty acids. Lipid A of *L. pneumophila* contains eight nonhydroxylated acids from 14 to 20 carbon atoms and five long-chain fatty acids (28:0(27-OH), 28:0(27-oxo), 30:0(29-oxo), 27-dioic, and 29-dioic) [55].

The high diversity among *Legionella* spp. is also expressed in the structures of the lipopolysaccharides, including the conserved region of lipid A. The lipid A moiety of LPS from most *Legionella* spp. comprises disaccharide GlcN3N. However, a mixed disaccharide backbone containing GlcN3N and glucosamine was found in *L. israelensis* and *L. bozemanae* [56,57]. An unusual feature of lipid A from *Legionella* spp. is the high content of non-hydroxy fatty acids (ester-linked) and long-chain (-ω)-oxo, (-ω)-hydroxy, and (ω)-dioic fatty acids (ester linked) [58]. 27-oxo-octacosanoic acid is generally present in all *Legionella* species studied; therefore, it serves as a useful marker of this bacterial group. An analysis of the sugar components of LPS from different *Legionella* species showed that all structurally characterised lipopolysaccharides displayed high contents of D-mannose and D-glucosamine. In addition, *Legionella* LPS contain species-specific sugars (3 amino-3, 6-dideoxy-mannose in *L. israelensis*, galacturonic acid and galactosamine in *L. hackeliae*,

quinovose in *L. feeleii*, and a rare sugar yersinose in *L. micdadei* and *L. maceachernii*) [59,60]. Unlike *L. pneumophila*, LPS isolated from several other species, e.g., *L. feeleii*, *L. jordanis*, *L. erythra*, *L. bozemanae*, *L. oakridensis*, and *L. micdadei* contain D- and L-glycero-D-manno-heptose [56,60,61].

The chemical composition and structure of *Legionella* LPS, especially the presence of various decorative substituents, exerts a strong influence on its biological activity in all stages of host cell infection.

3.2. Biological Significance of Legionella LPS

L. pneumophila LPS is the main antigen recognised by antibodies contained in the serum of patients and the thermostable antigen excreted in urine [62]. The urinary antigen test is the most commonly used method for the diagnosis of Legionnaires' disease [63]. The use of monoclonal antibodies (mAb) of the Dresden panel, recognising epitopes located in the highly heterogeneous O-specific chain of *L. pneumophila* LPS, facilitated distinguishing 15 serogroups and nine subgroups within serogroup 1 [64]. The two antibodies (mAb3/1 and mAb8/5) that recognise the virulent strains responsible for the majority of laboratory-confirmed cases of Legionnaires' disease turned out to be valuable in epidemiological studies and for clinical purposes. The characterisation of the LPS biosynthesis loci of *L. pneumophila* serogroup 1 strains revealed two major regions: a specific 18-kb region and a conserved 15-kb region containing genes found in serogroup 1 and non-serogroup 1 strains. The most variable region is involved in O-antigen modification [65]. Antibody mAb3/1 recognises an epitope associated with the 8-O-acetyl group in legionaminic acid. The O-acetyl-transferase enzyme encoded by the *lag-1* gene is responsible for the transfer of the O-acetyl group to legionaminic acid [66]. Studies carried out in Europe and Asia have shown that the *lag-1* gene was harboured by a significantly higher number of clinical isolates of *L. pneumophila* sg1 compared with environmental isolates [67,68].

The LPS of *L. pneumophila* is highly hydrophobic due to the presence of deoxy groups and *N*- and *O*-acyl substituents in legionaminic acid, but the highest degree of hydrophobicity is exhibited by *lag-1* strains producing 8-*O*-acetyl groups, which most likely contributes to the transmissibility of these bacteria in aerosols and adhesion to host cells. A TF3/1 mutant in the *lag-1* gene defective in the synthesis of 8-*O*-acetyl substituents, not recognised by the mAb3/1 antibody, adhered less strongly to the macrophages of the THP-1 line and to *A. castellanii* cells, compared to the wild-type strain [69]. This mutant also failed to produce high-molecular-weight long-chain O-polysaccharide [70]. Comparative studies of the kinetics of interactions between the host cell and the *L. pneumophila* wild-type strain or the mutant showed a higher efficiency of binding to the amoeba surface in bacteria with the full-length O-chain and 8-O-acetyl groups. However, both strains multiplied inside the host cells successfully, irrespective of the differences in the length and structure of the polysaccharide part of LPS. Previous studies also showed that *L. pneumophila* strains lacking 8-*O*-acetyl substituents were as effective in infecting amoebae and macrophages as strains that expressed this LPS motif [70]. Thus, the LPS of *L. pneumophila* plays a critical role in the early stages of infection, anchoring the bacteria to the host cell's membrane. Disturbances in the synthesis of the polysaccharide region of *L. pneumophila* LPS exert an effect on the composition and structure of phospholipids and proteins. The TF3/1 mutant showed differences in the structures of the PC and PG species, compared to the wild-type strain. Moreover, the mutant strain was synthesised by 11 mol% lower amounts of branched fatty acids and approximately two-fold higher amounts of long-chain fatty acid (20:0) than the wild-type strain. The changes in the surface components determined the cell surface topography of these bacteria and their nanomechanical properties. The TF3/1 mutant had grooves on the cell surface and did not produce as many outer membrane vesicles (OMV) as the wild-type strain [69]. Spherical bilayers consisting of LPS, phospholipids, outer membrane proteins, and periplasmic components are naturally secreted from the cell envelope of *L. pneumophila*. OMVs play a significant role in the pathogenesis of these bacteria, simultaneously delivering multiple virulence factors to host cells and

tissues [71]. The release of the vesicles from *L. pneumophila* is developmentally regulated, i.e., the vesicles are connected to the cell wall but shed LPS in the replicative phase and are profusely released in the transmissive phase [72]. One of the functions of *L. pneumophila* OMVs is the inhibition of phagosome–lysosome fusion during the intracellular infection of macrophages. This capability was correlated with growth phase-dependent modifications of the composition of glycoconjugates contained in the vesicles. The LPS of the bacteria in the transmissive phase of growth is deacetylated and elongated to a form that effectively blocks the fusion between phagosomes and lysosomes and, thus, independently of the effectors of the IV secretion system, inhibits the maturation of macrophages [73]. In turn, during the replicative phase, the degree of acetylation of the LPS O-chain increases, which probably contributes to the increased tolerance to the hostile conditions of the intracellular environment of macrophages [73]. Seeger et al. showed that LPS fractions below 300 kDa, not associated with OMV, significantly delayed phagolysosomal maturation one hour after phagocytosis, regardless of the bacterial growth phase [74].

LPS expressed by *L. pneumophila* in the transmissive phase binds to a sialic acid-specific lectin more strongly than LPS from bacteria in the replicative phase of growth. The ability of the bacteria to bind to this receptor correlates with the effectiveness of macrophage infection [73]. Legionaminic acid of *L. pneumophila* sg1 shares the same D-glycero-D-galacto absolute configuration as 5-acetamido neuraminic acid (Neu5Ac, sialic acid). Neu5Ac, located on the surface of mammalian cells, is involved in cell–cell interactions and the immune response [75]. The molecular mimicry of eukaryotic cell macromolecules is one of the strategies used by *Legionella* bacteria to colonise host cells. The structural similarity of legionaminic and neuraminic acid may be an example of mimicry to the host cell used not only in the adhesion process but, also, in modulation of the immune response to infection. The LPS of *L. pneumophila* is less toxic and less potent in its ability to induce proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) from Mono Mac 6 cells, compared to the highly pyrogenic LPS from *Enterobacteriaceae* members [76]. The bioactive centre of LPS is lipid A, whose toxicity is directly influenced by the length and number of groups of the fatty acids attached to its glycosidic backbone. The presence of fatty acid chains twice the length of the corresponding chains found in the majority of toxic lipids A containing C12, C12OH, C14, and C14OH account for the low endotoxic activity of *L. pneumophila* LPS due to a failure to interact with receptor CD14 (a glycosylphosphatidylinositol-anchored protein) and with its soluble form [76]. Host pattern recognition receptors such as Toll-like receptors (TLRs) are involved in the process of recognition of LPS. TLR4 functions as a sensor of LPS on the OM in Gram-negative bacteria, promptly inducing the production of antibacterial cytokines. The lipid A region of *L. pneumophila* LPS was a weak TLR4 agonist [77]. Additionally, macrophages of TLR4-deficient mice infected by *L. pneumophila* were not defective in the production of cytokines, and the rate of bacterial clearance from the lungs of these mice was similar to that in wild-type mice [78,79]. However, the TLR2-deficient mice were more sensitive to *L. pneumophila* than TLR2-sufficient mice [79,80]. These results indicated that LPS from *L. pneumophila* was recognised by TLR2, which is a typical receptor for peptidoglycan [77]. This unusual detection pattern of switching TLR4 to TLR2 is related to the presence of substituent (a ketone group at C27) or branch on the penultimate carbon of different fatty acids of *L. pneumophila* lipid A [77]. Upon recognition by TLR2, LPS triggers signalling pathways controlled by the MyD88 adaptor protein, which results in the production of inflammatory cytokines and subsequent clearance of *L. pneumophila* from the lungs [81]. The level of inflammatory cytokine production was reduced in TLR2 and MyD88 gene knockdown macrophages of the U937 cell line infected by *L. pneumophila* [82]. Additionally, human macrophages induced by *L. pneumophila* OMVs synthesised IL-8 relying on TLR2-dependent signalling pathways [71].

The structure of lipid A composed of GlcN3N and long-chain fatty acids also provides a protective mechanism against the degradation of lipid A/LPS by amidases and/or esterases of amoebae during the intracellular growth of *L. pneumophila* [54].

L. pneumophila LPS is subject to phase variations correlated with the attenuation of virulence traits such as the ability to multiply within macrophage-like HL60 cells or *A. castellanii* [83]. The molecular mechanism responsible for this variability consists in the chromosomal insertion and excision of an unstable 30-kb genetic element, which does not harbour genes related to LPS biosynthesis [84]. Phase variation in the RC1 strain of the *L. pneumophila* sg 1 subgroup OLDA influences the O-specific chain structure and the fatty acid profile of lipid A. The phase-variant strain 811 with the 29-kb element excised from the chromosome is devoid of *N*-methylation in legionaminic acid and contains shorter 3-hydroxy (16:0 and 18:0) fatty acids in lipid A [85,86].

L. pneumophila strain PtVFX/2014, associated with the first evidence of person-to-person transmission, i.e., a strain that was able to overcome the transmission barrier of human innate immunity, carried eight horizontally transferred regions encompassing genes involved in, e.g., LPS biosynthesis [87]. Epidemiological studies showed that the genes of the LPS cluster determining sg 1 of *L. pneumophila* were present in highly diverse genomic backbones of the strains responsible for the largest outbreaks of Legionnaires' disease described so far and that it probably constitutes a major determinant of human disease itself [87].

On the one hand, the LPS composed of three distinct regions in terms of structure and biological properties is an important factor in the virulence of *Legionella* bacteria, participating in complex mechanisms of the induction of lesions. On the other hand, LPS is a ligand recognised by proteins involved in the response to infection. During the infection of macrophages with *L. pneumophila*, host guanylate binding proteins (GBPs), triggered by cytoplasmic LPS derived from the bacteria, induce caspase-11-dependent pyroptosis [88,89]. The LPS of *L. pneumophila* is a ligand for collectins, which play an important role in the innate immunity of the lung. After the direct binding of LPS, hydrophilic proteins A and D promote the localisation of *L. pneumophila* in the acidic environment of the lysosomes, thus attenuating the intracellular multiplication of the phagocytosed bacteria [90]. Additionally, human apolipoprotein E binds to *L. pneumophila* LPS, which results in disturbances in the normal structure of the bacterial surface, and these changes may lead to impaired penetration of the host cells [91]. Two molecules of apoLp-III bind to a single micelle of *L. dumoffii* LPS formed from 12 to 29 monomeric LPS molecules, pointing to new strategies for anti-*Legionella* therapies [45].

Legionella spp. are important aetiological agents of pneumonia. They are responsible for 2–8% of community-acquired pneumonia cases [92]. A review of 46 CAP (community acquired pneumonia) studies from European countries has indicated that *Legionella* spp. are particularly frequent among patients who require admission to an intensive care unit (ICU) [93]. In the USA, the reported cases of Legionnaires' disease increased from 2.301 in 2005 to 7.104 in 2018. In Europe, the number of known cases of Legionnaires' disease has almost doubled (from 1.3/100,000 people in 2014 to 2.3/100,000 people in 2018) [94]. The increase in cases may be related to the changing environmental conditions, which favour the growth of *Legionella* bacteria, as well as the increasing number of people susceptible to infection, such as the elderly and immunocompromised subjects. The closure of public buildings related to the SARS-CoV-2 pandemic has led to the long-term stagnation of water in installations and created optimal conditions for intensive multiplication of the bacteria, which substantially increased the risk of *Legionella* infections [95]. *L. pneumophila* produces a variety of cytosolic and cell envelope-associated lipids with a unique structure that plays an important role in its physiology and promotes bacterial adhesion and adaptation to the host's intracellular environment.

Although more than 60 different *Legionella* species are known, with approximately half of the number of species isolated from clinical specimens, the complete structure of LPS is only known in *L. pneumophila*. The analysis of the genomes of various *Legionella* species has shown that these bacteria contain genes whose products are involved in the synthesis of lipids characteristic for eukaryotic cells, such as PC, or, e.g., hopanoids in *L. falonii* [96]. Lipid components, especially the unique ones, can serve as important biomarkers used in

diagnostics and in taxonomic studies of this bacterial group. Bacterial lipid membranes are targets for the development of new antimicrobial drugs and a promising object of research in the fight against resistant bacteria [97]. Therefore, the elucidation of the complex lipid structure of the cell envelope of various *Legionella* spp. using advanced lipidomic technologies may be important for the development of new strategies for the prevention and treatment of *Legionella* infections.

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Publikacja 3

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Unusual lipid components of *Legionella gormanii* membranes

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Unusual Lipid Components of *Legionella gormanii* Membranes

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Abstract: *Legionella* spp. cause Legionnaires' disease with pneumonia as the predominant clinical symptom. *L. gormanii* is the second most prevalent causative agent of community-acquired pneumonia after *L. pneumophila*. The study aimed to characterize the lipidome of *L. gormanii* membranes and the importance of these analyses in bacterial chemotaxonomy. Lipidomic analyses based on ultra-high performance liquid chromatography-mass spectrometry allowed the detection of individual molecular species of a wide range of *L. gormanii* membrane lipids contained in the outer (OM) and inner membranes (IM). The lipid profile comprised glycerolipids (triglycerides, diglycerides), phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, cardiolipin), and sphingolipids (ceramides, hexosylceramides). The most abundant lipid fraction in the IM and OM were phospholipids. The lipidomic analysis showed that two independent phosphatidylcholine (PC) synthesis pathways operating in *L. gormanii*: the PE-methylation (PmtA) pathway and the PC synthase (Pcs) pathway. Comparison of the molecular profile of PC species contained in the lipids of *L. gormanii* membranes cultured on the medium, with and without exogenous choline, showed quantitative differences in the PC pool. An unusual feature of the *L. gormanii* lipids was the presence of ceramides and hexosylceramides, which are typical components of eukaryotic cells and a very small group of bacteria. To the best of our knowledge, this is the first report of the occurrence of ceramides in *Legionella* bacteria.

Keywords: *Legionella gormanii*; phosphatidylcholine; ceramides; lipidomic analysis

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1. Introduction

Legionellosis is the common name for disease syndromes caused by exposure to *Legionella* bacteria. The disease varies in severity from a mild febrile illness (Pontiac fever) to a serious and fatal form of pneumonia (Legionnaires' disease, LD). *Legionella* species are environmental Gram-negative bacteria with the ability to survive and replicate within protozoan hosts. This adaptation of the bacteria to the intracellular niche in nature leads to invasion and growth within human alveolar macrophages [1]. Among the 69 *Legionella* spp. described, *L. pneumophila* is the leading cause of LD, and *L. pneumophila* serogroup 1 is associated with almost 85–90% of the cases worldwide [2]. However, non-pneumophila species of *Legionella* are also important human pathogens that pose a particular risk to immunocompromised and splenectomized individuals [3].

L. gormanii is one of the 28 *Legionella* species that have been associated with human disease. It was first isolated clinically from a 64-year-old pneumonia-affected woman with systematic lupus erythematosus and adenocarcinoma [4]. Although *Legionella* species are rarely isolated from children, *L. gormanii* has been found in this patient group [5,6]. In the process of lung tissue invasion and colonization, lipases and phospholipases are important

virulence factors of *Legionella* spp., as they degrade the surfactant covering small airways, bronchioles, and the alveolar surface. The lung surfactant is composed of approximately 10% of protein and 90% of lipids. Approximately 80% of these lipids are accounted by phosphatidylcholine (PC), which represents a good target for bacterial phospholipase A (PlaB) in the destruction of this important layer, thus contributing to impairment of lung function. *L. gormanii* showed cell-associated phospholipase A and lysophospholipase A activities in quantities comparable to *L. pneumophila* [7].

The cell envelope of Gram-negative bacteria consists of two distinct layers, the outer (OM) and the inner (IM) membranes, separated by the periplasm with a thin layer of peptidoglycan. The inner membrane contains of phospholipids covering the inner and the outer leaflets. The OM is a highly asymmetric lipid bilayer, comprising an inner leaflet enriched in phospholipids and an outer leaflet containing lipopolysaccharide (LPS). This strict OM asymmetry is important for the proper functioning of the bacterial cell, as it constitutes a permeability barrier, protects against the host's immune system and prevents the penetration of toxic compounds into the cell. Additionally, the *Legionella* OM mediates direct contact with other organisms, determining highly specific interactions with the host cell.

Unlike in the Gram-negative model bacterium *Escherichia coli* and the Gram-positive *Bacillus subtilis*, but similar to eukaryotic cells, *Legionella* membranes are rich in PC and this phospholipid is necessary for the full virulence of these pathogens. *L. pneumophila* mutant defective in PC synthesis showed a poorly functioning type IV secretion system (Dot/Icm), which delivers effectors required for intracellular multiplication in the cytosol of infected host cells [8]. Synthesis of PC in *Legionella* cells occurs via two independent biosynthesis pathways, i.e., the phosphatidylethanolamine (PE) methylation pathway and the PC synthase (Pcs) pathway. In the methylation pathway, PE is methylated three times to form PC via phospholipid *N*-methyltransferase (PMT). This enzyme is a small cytosolic protein encoded by the *pmtA* gene. In the Pcs pathway, choline is condensed directly with CDP-diacylglyceride to produce PC in a reaction catalyzed by the phosphatidylcholine synthase (Pcs), which occurs exclusively in bacteria [9]. The synthase is a highly hydrophobic protein containing up to eight transmembrane helices with *N*- and *C*-termini located inside the bacterial cell. A comparative analysis of the nucleotide sequences of *pcs* showed that these genes share a high sequence identity among *Legionella* spp.; therefore, they can be used to identify this bacterial group [10]. Lipids of *L. gormanii* were estimated to account for 4% of dry weight, with PE (50%) and PC (26%) representing the major glycerophospholipid classes. Other phospholipids that build the membranes of the bacterium were cardiolipin (CL, 21%) and phosphatidylglycerol (PG, 9%). Similar to another *Legionella* spp., *L. gormanii.* utilizes exogenous choline in the Pcs pathway to synthesize PC. These bacteria cultured on the medium with choline synthesized by 21% more PC and 12% PE and by 9% lower CL levels, compared with the bacteria grown on a medium without the addition of choline [10]. The supplementation of the growth medium with choline resulted in a higher concentration of proteins in *L. gormanii*, which indicated that the phospholipid environment affected the assembly of membrane proteins [10]. The synthesis of PC from an exogenous precursor influences the phospholipid and protein *Legionella* components and the interactions between these components lead to changes in sensitivity to antimicrobial protein and peptides. Our previous study showed that *L. dumoffii* cultured in the presence of choline was more sensitive to the antimicrobial peptide defensin and apolipophorin III (apoLp-III) isolated from *Galleria mellonella* hemolymph [11]. ApoLp-III, i.e., an insect homolog of human apolipoprotein E exerted a bactericidal effect on *L. gormanii* cells at an 8-fold lower concentration, compared to *L. dumoffii*, possibly reflecting differences in the cell surface properties [12].

In this study, the remarkable lipid components of *L. gormanii* membranes are reported and this strain is, therefore, a novel representative of a narrow group of bacteria containing phosphatidylcholine and ceramides.

2. Results

2.1. Composition of Fatty Acids of Individual Classes of Phospholipids

The fatty acid compositions of phosphatidylethanolamine (PE), phosphatidylcholine (PC), dimethylphosphatidylethanolamine (dMePE), phosphtidylglycerol (PG) and cardiolipin (CL) were determined. Each of these phospholipids showed a distinctive and characteristic fatty acid pattern (Table 1).

In the PE class, saturated, branched (mainly i16:0 (22%) and a15:0 (19%)) acids and a smaller amount of a17:0 (7%) acid constituted approximately half of total FAs. This fraction was also characterized by a high content of cyclopropyl 17:0 (18%) and straight-chain 16:0 (17%) acid. The FA composition of the dMePE class included considerable quantities of octadecanoic and hexadecanoic acids, which, together, accounted for 65%. This phospholipid also contained a substantial amount of cyclopropyl 17:0 acid (19%) and small quantities of unsaturated 18:1Δ^9, as well as long-chain FAs (22:0, 1.5%; 24:0, 2%).

The PC fraction was predominantly composed of cyclopropyl 17:0 acid, which accounted for 22% of the total FAs of this phospholipid. Both branched (*iso* and *anteiso*) and unbranched FAs were determined in this class. Hexadecanoic (18%) and octadecanoic (16%) acids with straight-chain saturated FAs were the most abundant. Branched FAs accounted for 30% of all acids, with a dominance of i16:0 (12%) and a15:0 (8%).

The PG class was characterized by high content of methyl-branched FAs (a15:0, 17%; i16:0, 19%; a17:0, 9%).

Compared to other phospholipid classes, the CL class contained the largest amount of unsaturated (16:1Δ^9, 6%; 18:1Δ^9, 5%) acids, as well as long-chain FAs with the number of carbon atoms ranging from 20 to 24.

Table 1. Fatty acid composition of individual classes of *L. gormanii* phospholipids identified by FAME analysis via GC/MS. Fatty acids were liberated by saponification (0.8 M NaOH/50% methanol, 1 h, 80 °C) and esterified by 1 M HCl/methanol (1.5 h, 85 °C).

		Relative Content [%]				
Retention Time	**Fatty Acid**	**PE + mMePE**	**dMePE**	**PC**	**PG**	**CL**
9.67	*i*14:0	0.5 ± 0.2	0	2.5 ± 0.2	0	tr
10.49	*n*14:0	tr	1± 0.2	0.5 ± 0.2	1 ± 0.4	1 ± 0.2
11.81	*i*15:0	tr	0	tr	0.5 ± 0.2	tr
11.97	*a*15:0	**19 ± 2**	1 ± 0.3	**8 ± 2**	**17 ± 2**	**10 ± 0.5**
12.33	9 *-15:1	0	0	0	0	0.6 ± 0.2
12.62	*n*15:0	3 ± 0.3	1 ± 0.5	2 ± 0.2	2 ± 0.4	2 ± 0.4
13.90	*i*16:0	**22 ± 0.5**	1 ± 0.2	**12 ± 1**	**19 ± 1**	**15 ± 4**
14.25	*cis* 9 *-16:1	1 ± 0.2	1 ± 0.3	0.7 ± 0.1	1 ± 0.1	6 ± 0.7
14.43	*trans* 9 *-16:1	2 ± 0.4	0	1 ± 0.2	1 ± 0.6	0.5 ± 0.2
14.66	*n*16:0	**17 ± 0.4**	**25 ± 3**	**18 ± 2**	**17 ± 1**	**14 ± 2**
15.92	*i*17:0	1 ± 0.1	0	1± 0.2	2 ± 0.4	1 ± 0.2
16.09	*a*17:0	**7 ± 0.5**	**3 ± 0.5**	**5.5 ± 0.8**	**9 ± 0.1**	**7 ± 0.6**
16.467	*c*17:0	**18 ± 1**	**19 ± 2**	**22 ± 2**	**10 ± 1**	**15 ± 1**
16.673	*n*17:0	3 ± 0.3	2 ± 0.2	4 ± 0.5	3 ± 0.4	3 ± 0.2
18.531	*cis* 9 *-18:1	0	2 ± 0.5	0	tr	5 ± 0.6
18.810	*n*18:0	**6 ± 1**	**40 ± 1.5**	**16 ± 1.5**	**13 ± 0.3**	**14 ± 3**
19.690	*i*19:0	0	0	2 ± 0	1 ± 0.3	0.5 ± 0.1
20.362	*n*19:0	0.5 ± 0.1	0.5 ± 0.2	2 ± 0.4	2 ± 0.2	1 ± 0.2
20.584	*i*20:0	0	0	0	0	1 ± 0.3
21.675	20:1	0	0	0	0	1 ± 0.1
22.100	*n*20:0	tr	0	0.8 ± 0.2	0.7 ± 0.4	0.9 ± 0.2

Table 1. *Cont.*

Retention Time	Fatty Acid	Relative Content [%]				
		PE + mMePE	dMePE	PC	PG	CL
23.740	21:0	tr	0	2 ± 0.8	0.8 ± 0.1	1 ± 0.2
25.346	22:0	0	1.5 ± 0.1	0	0	tr
28.303	24:0	0	2 ± 0.2	0	0	0.5 ± 0.1

a, methyl branch at the anteiso carbon atom; *i*, methyl branch at the iso carbon atom; *n*, unbranched acid; *c*, cyclopropane ring structure, tr—trace; * position of double bond. PE, phosphatidylethanolamine; mMePE, monomethylphosphatidylethanolamine; dMePE, dimethylphosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphtidylglycerol; CL, cardiolipin.

2.2. Separation of the Inner (IM) and Outer (OM) Membranes

To determine the lipid profile in the membranes of *L. gormanii* cultured on the medium with and without exogenous choline, the bacteria were separated into IM and OM fractions by sucrose density gradient ultracentrifugation. Biochemical analyses confirmed the effectiveness of membrane separation. Measurement of the protein concentration in each fraction showed two main peaks, one corresponding to fractions 13–17 and 10–16 for bacteria grown with and without the addition of exogenous choline, respectively, and the second peak corresponding to fractions 20–28 and 22–28 for bacteria cultured with and without choline supplementation, respectively (Figure 1). NADH oxidase activity was concentrated in pooled fractions (12–16 for the choline-supplemented bacteria and 10–15 for the choline non-supplemented bacteria) corresponding to the IM. The activity of NADH oxidase was 171 µmol min^{-1} mL^{-1} and 239 µmol min^{-1} mL^{-1} for bacteria cultured with exogenous choline and on the standard medium, respectively.

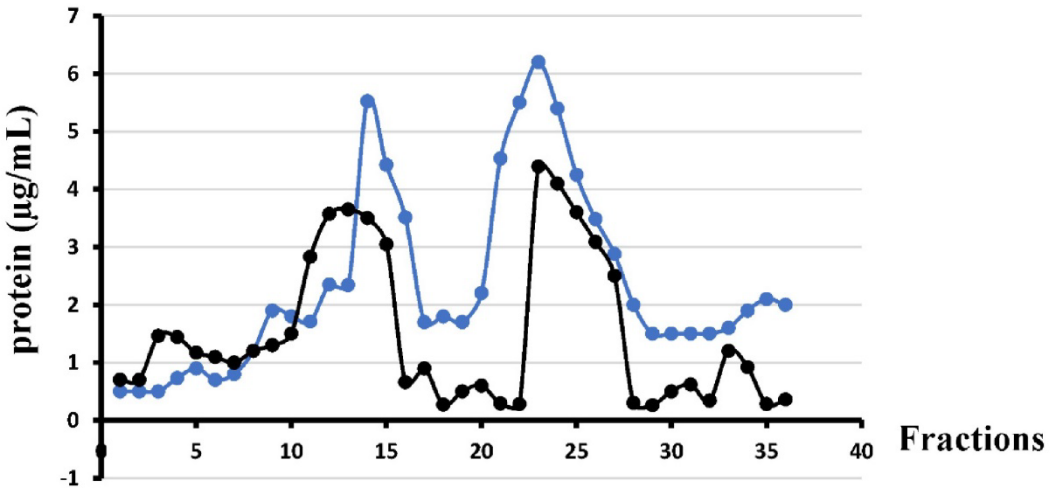


Figure 1. Separation of the *L. gormanii* inner (IM) and outer membrane (OM) by sucrose density gradient centrifugation. The 1 mL fractions were collected from the top of the gradient and assayed for the presence of protein (µg/mL). Black line: bacteria cultured on the exogenous choline non-supplemented medium; blue line: bacteria cultured on the exogenous choline-supplemented medium.

2.3. Lipidomic Analysis

Lipidomic analyses based on the UHP LC-MS/MS method allowed detection and relative quantification of individual molecular species of a wide range of *L. gormanii* membrane lipids contained in the OM and IM. The lipid profile comprised glycerolipids (triglyceride, TG; diglyceride, DG), phospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylglycerol, PG; cardiolipin, CL), and sphingolipids (ceramides, Cer, hexosylceramides, Hex1Cer). The most abundant lipid fraction in the IM and OM were phospholipids (Figure 2).

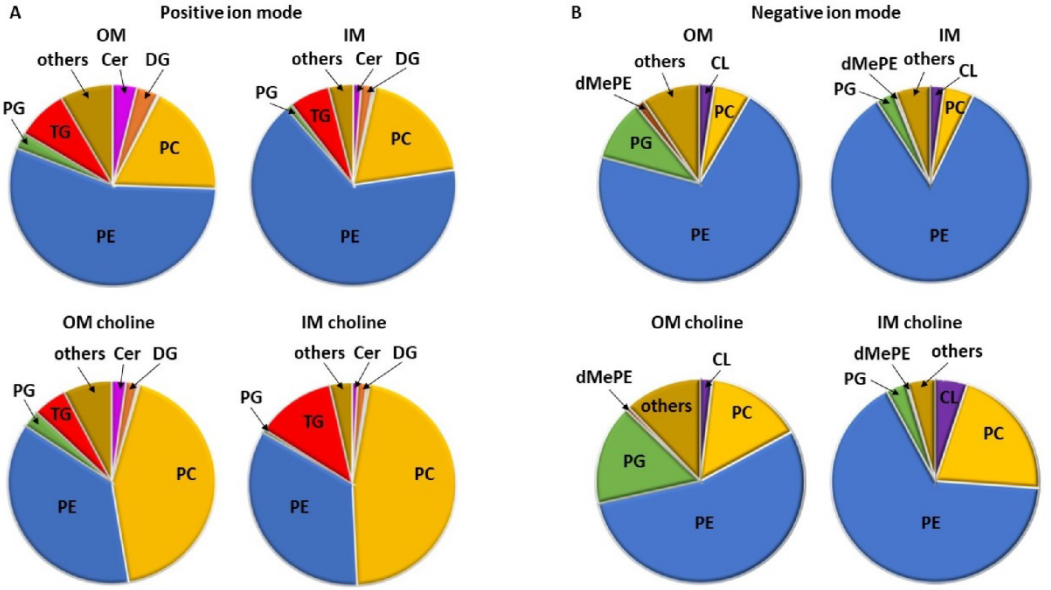


Figure 2. Lipid classes in the OM and IM of *L. gormanii* grown on a medium with and without the addition of exogenous choline. The lipid classes were analyzed by LC-MS in the positive (**A**) and negative ion mode (**B**).

2.4. Phospholipid Profile

Among polar lipids, PE was the dominant class in the OM and the IM. The analysis of the molecular profile of PE showed the presence of 35 PE species. The main species were shown in Figure 3A. The most abundant species were PE15:0_15:0 (16%) in the OM and IM (18%), PE15:0_16:0 (13% and 23% in the OM and IM, respectively), and PEcyclopropyl17:0_16:0 (9%) in the OM and (11%) IM (Figure 3A). Spearman's rank correlation coefficient for PE species content between the membranes was positive but moderate at 0.67 indicating that the PE compositions are characteristic for the IM and OM.

Methylated derivatives of PE were identified as well. The profile of dMePEs showed that the most abundant species were dMePE cyclopropyl17:0_17:0 (77%), dMePE16:0/16:1 (11%) in the OM and dMePE16:0_16:1 (44%), dMePE cyclopropyl17:0_16:1 (39%) in the IM (Figure 3B).

The second largest PL class found in *L. gormanii* membranes was PC. The major molecular PC species were as follows: PC15:0_15:0 (22% in the OM and 20% in the IM), PC15:0_16:0 (19% in the OM, 22% in the IM); PCcyclopropyl17:0_16:0 (15% in the OM, 20% in the IM), and PC cyclopropyl 17:0/15:0 (10% in the OM and 9% in the IM). PC

cyclopropyl17:0_15:0 was characteristic for this class (Figure 3C). The distribution of PC species in the membranes was very similar as indicated by the high value of Spearman's rank correlation coefficient ($\rho \geq 0.90$).

The PC and PE content in *L. gormanii* membranes changed significantly when the bacteria were cultured on a medium with the addition of exogenous choline. The PC content in the OM and IM increased by 25% and 28%, respectively, compared to the bacteria cultured without choline supplementation (Figure 2). The amount of PE contained in the OM was by 18% lower than in *L. gormanii* cultured without exogenous choline. The content of PE in the IM was 32% lower than in the bacteria grown without choline addition (Figure 2). The comparison of the molecular profile of PC species contained in the membrane lipids of *L. gormanii* cultured on the medium with and without choline showed quantitative differences in the PC pool (Figure 3C). The content of PC cyclopropyl17:0_15:0 in the IM lipids isolated from bacteria grown on the medium with exogenous choline was approx. twice as high as in the IM lipids from bacteria grown without choline. Also, PC cyclopropyl17:0_16:0 was present in the IM in higher quantities compared to the IM lipids isolated from *L. gormanii* cultured without choline. The OM lipids from the bacteria grown on the choline-supplemented medium contained more PC15:0_15:0 than the bacteria grown on the non-supplemented medium. Quantitative differences in PE species between bacterial membranes from different growth conditions were found (Figure 3A). The content of PE15:0_15:0, PE15:0_16:0, and PE16:0_16:1 was higher in IM lipids extracted from the bacteria cultured with choline addition. There were no significant differences in PE species present in the OM lipids from both conditions. The bacteria cultured on the medium with exogenous choline contained mainly dMePE cyclopropyl17:0_16:1, dMePE16:1_18:1, and dMePE cyclopropyl17:0_16:0 in the OM and dMePE cyclopropyl17:0_16:1 and dMePE16:1_18:1 in the IM. Compared to the bacteria grown on the medium without the addition of choline, they contained less dMePE cyclopropyl17:0_17:0, dMePE16:0_16:1 and more dMePEcyclopropyl17:0_16:1 in the OM (Figure 3B). There were clear differences in the content of dMePE in the OM of bacteria grown on the medium with and without the addition of choline, as indicated by the negative Spearman correlation coefficient (−0.49).

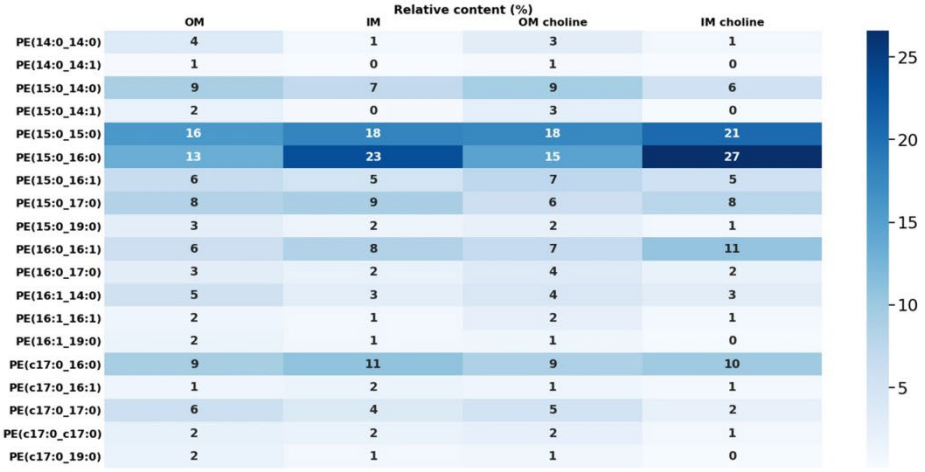
L. gormanii lipids contained also PG, the amount of which in the OM was higher than in the IM and did not change depending on the growth conditions. Twenty-two PG species were identified in both membranes. The major molecular species were composed of 15:0 and 16:0, similar to the PE and PC classes (Figure 3D). However, unlike the PE and PC classes, PG16:1_16:1 was abundant in the OM lipids. The OM lipids contained more PG15:0_15:0, PG16:1_14:0, and PG15:0_14:0 than in the IM, while the IM lipids were characterized by higher amounts of PG15:0_16:0, PG15:0_17:0, and PG16:1_17:0 compared to the OM.

The profile of CLs showed that CL66:1 and CL64:2 were the most abundant species. These species were found in both membranes. CL64:2 in the IM of the bacteria grown on the standard medium represented about 8%, while the IM of the bacteria grown on the choline-supplemented medium accounted for 45%. The content of this cardiolipin in the IM was about 14%, regardless of the growth conditions. CL66:1 was dominant in the IM of the bacteria cultured on the standard medium and accounted for 87% of all cardiolipins. In the OM, it represented 57%. The bacteria grown with the addition of exogenous choline synthesized lower amounts of this cardiolipin in both the OM and IM (44% and 23%, respectively).

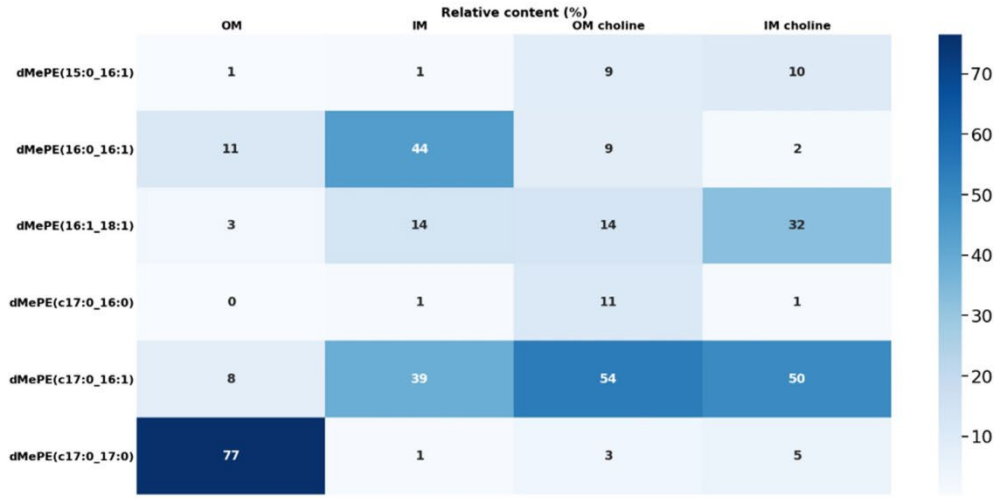
2.5. Analysis of Glycerolipids

Glycerolipids (TG and DG) were the second most abundant fraction of lipids from *L. gormanii* membranes. TG constituted the most diverse class of lipids including 125 species dominated by TG18:0_16:0_16:0. This lipid comprised approx. 50% of total TG species and was mainly localized in the OM. The other TG species present in significantly smaller amounts

were as follows: TG16:0_16:1_18:1, TG15:0_16:0_16:0, TG16:0_16:0_16:1, TG15:0_16:0_18:1, and TG18:0_18:1_18:1. The content of TG18:0_18:0_18:0 was 4% in the OM, and 13% in the IM (Figure 4A). Fatty acids with a hydrocarbon chain length of 16 and 18 were the main acids building of the lipid class. There were no significant differences in the content of TG species between the different growth conditions except for TG18:0_16:0_16:0, which was more than 3 times higher in the IM than the IM choline. However, the amount of TG 18:0_18:0_18:0 was higher in IM compared to IM choline.

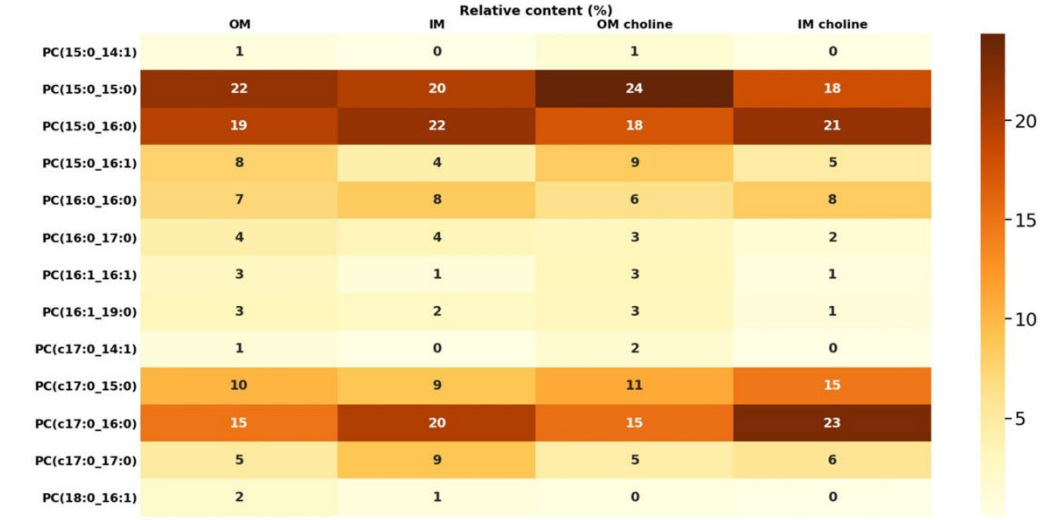


(**A**)



(**B**)

Figure 3. *Cont.*



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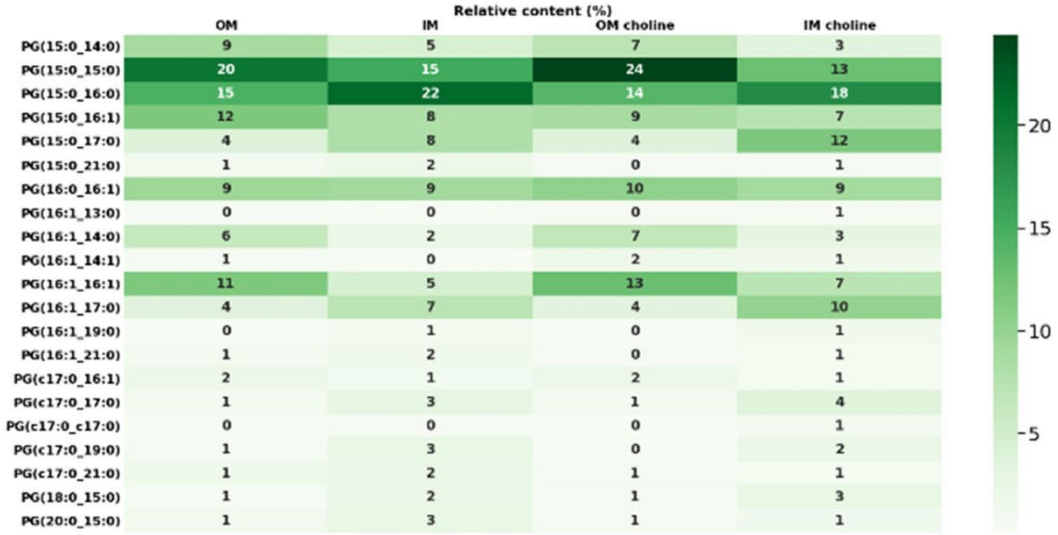
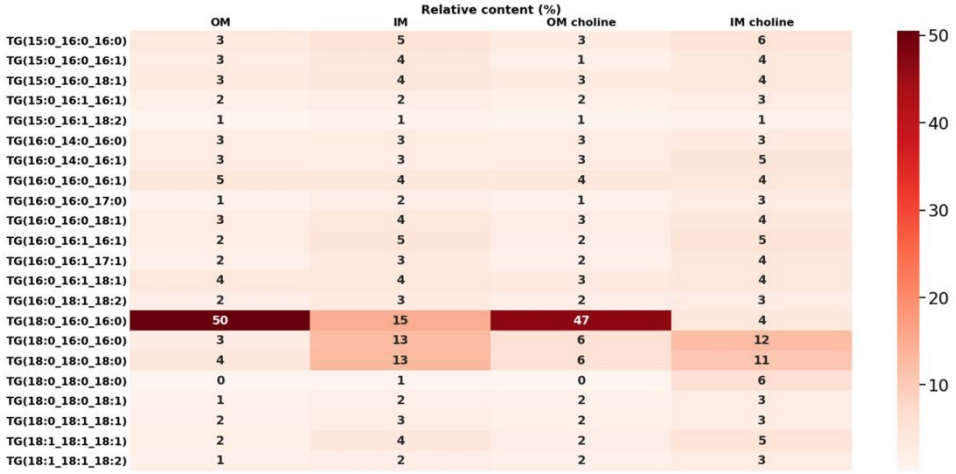


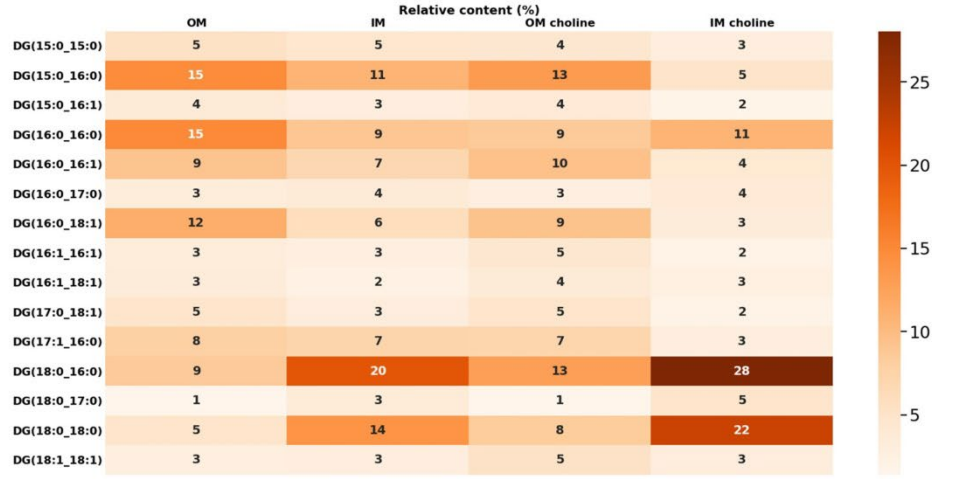
(**D**)

Figure 3. Heatmap displaying the relative abundances of PE (**A**), dMePE (**B**), PC (**C**), and PG (**D**) molecular species in total lipid extracts of the OM and IM of *L. gormanii* cultured on the standard medium and with exogenous choline. PE, PC and PG were analyzed by LC-MS(/MS) in the positive ion mode. The phospholipid class dMePE was analysed by LC-MS(/MS) in the negative ion mode.

In total, 27 DG molecular species were identified in the lipids of the *L. gormanii* membranes (Figure 4B). They were characterized by lower heterogeneity than TGs. Two DG species were dominant in the IM: DG18:0_16:0 (20%) and DG18:0_18:0 (14%). In turn, DG15:0_16:0 (15%) and DG16:0_16:0 (15%) were the most abundant in the OM. The analysis of the molecular profile of DGs showed that many of these species contained 16 or 18 carbon acid residues, similar to TG species. *L. gormanii* grown on a medium with the addition of exogenous choline synthesized more DG18:0_16: 0 (28%) and DG18: 0_18:0 (22%) in the IM, while less DG16:0_16:0 (9%) in the OM compared to the bacteria cultured without exogenous choline.



(**A**)

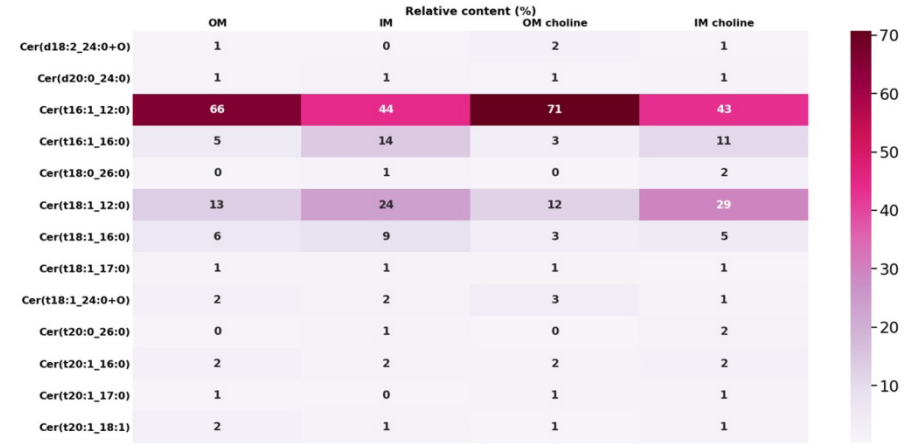


(**B**)

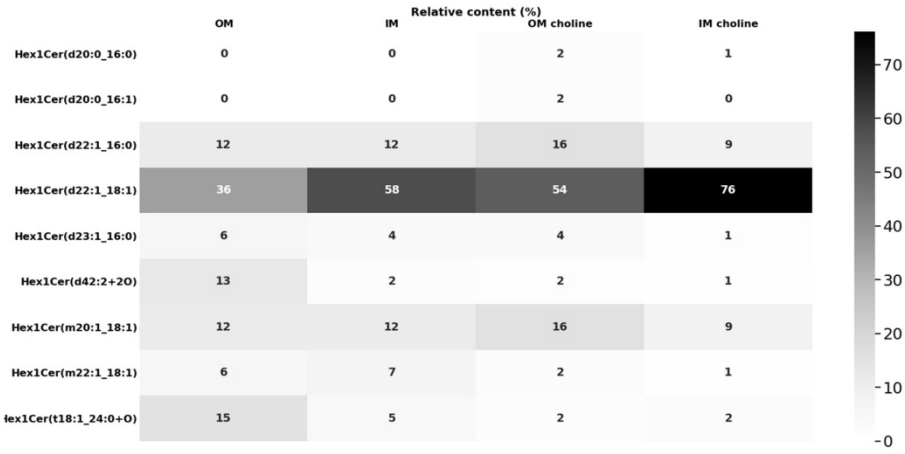
Figure 4. Profile of TG (**A**) and DG (**B**) of the OM and IM of *L. gormanii* grown on the medium without and with exogenous choline. Lipids were identified via LC-MS(/MS) in the positive ionization mode.

2.6. Characteristic of Ceramides

Ceramides are a subtype of sphingolipids characterized by a long chain amino alcohol sphingoid backbone (in contrast to glycerolipids which have a glycerol backbone) with amide bound fatty acyl chains. The structural diversity of these lipids is related to differences in the length, degree of saturation, and methylation of fatty acids, as well as the variation in the lipid headgroup. *L. gormanii* synthesizes 23 various ceramide species with saturated and one-unsaturated FAs with a 12 to 24, as well as oxidized ceramides and more complex hexosylceramides (Hex1Cer) containing sugar moieties (Figure 5A,B).



(**A**)



(**B**)

Figure 5. Ceramides (**A**) and hexosylceramides (**B**) in total lipid extracts of the OM and IM of *L. gormanii* cultured on the choline-supplemented and non-supplemented medium. Ceramides were analyzed by LC-MS(/MS) in the positive ionization mode.

In Figure 6A,B, the fragmentation mass spectra of the most dominant ceramide and hexosylceramide of *L. gormanii* are shown.

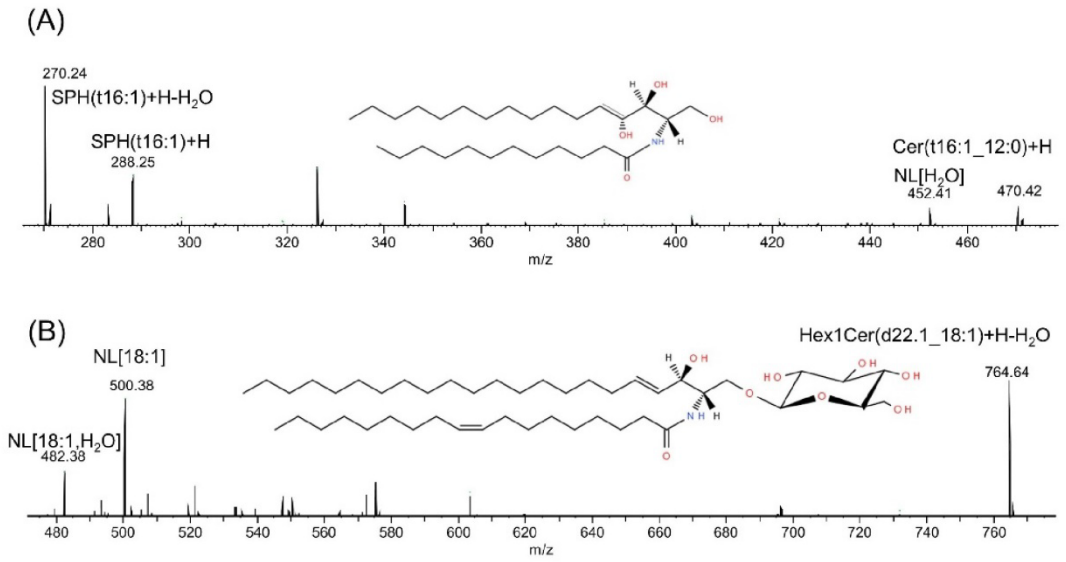


Figure 6. Fragmentation mass spectra in the positive ion mode of Cer(t16:1_12:0) (**A**) and Hex1Cer(d22:1_18:1) (**B**) Abbreviations: NL neutral loss, SPH sphingosine. The position of double bond was not identified in 16:1, 22:1 and 18:1 fatty acids.

All ceramides were distributed in the OM and IM, but there were clear quantitative differences for some species between the membranes. Among the total 14 ceramides and oxidized ceramides, one species, cer(16:1_12:0), represented approx. 66% of ceramides in the OM and approx. 44% in the IM in the case of the bacteria cultured without exogenous choline. The content of the cer(t16:1_12:0) (71%) species was higher in the OM, but similar in the IM of bacteria cultured with the addition of choline. Cer(t18:1_12:0) was the second most abundant species, which was localized mainly in the IM (24% or 29% IM choline), while its content in the OM was approx. half, regardless of the bacterial culture conditions (Figure 5A).

L. gormanii synthesizes three main hexosylceramides. These neutral glycosphingolipids, commonly called cerebrosides, usually contain glucose or galactose, with β-glycosidic linkages to the primary alcohol of an N-acyl sphingoid base. The dominant Hex1Cer22:1/18:1, accounting for 76% of total hexosylceramide species, was contained in the lipids isolated from the IM of the bacteria growing on the choline-supplemented medium. The content of this lipid in the OM was lower and amounted to 54%. The bacteria grown on a medium without choline supplementation compared to those supplemented with choline had a lower content of this lipid, i.e., 58% and 36% in the IM and OM, respectively (Figure 5B).

2.7. Bioinformatic Search for Ceramide Synthesis Pathways in the Genome of L. gormanii

To predict functional enzymes responsible for the synthesis of ceramides in *L. gormanii*, the genome of the strain ATCC 33279 was analyzed using the KEGG pipeline tools [13]. The reference point was a map describing the sphingolipid metabolism pathway (KEGG map00600), which includes the synthesis of ceramides. Enzymes with KO (KEGG Orthology) identifiers, found in the genome of *L. gormanii* ATCC 33279, were mapped into the discussed pathway (Figure 7A). According to the current knowledge, the first step in the synthesis of bacterial sphingolipids is the condensation of L-serine and palmitoyl-CoA to produce 3-ketodihydrosphingosine (KDS), a reaction catalyzed by the PLP-dependent enzyme serine palmitoyltransferase (SPT; EC 2.3.1.50, K00654) [14]. The coding sequences for SPT were not found in the genome, based on the standard KO number mapping pipeline. Additional searches with the use of PSI-BLAST and the sequence of the reference SPT from *Sphingomonas paucimobilis* (UniProt: Q93UV0_SPHPI) as a query allowed us to find two CDSs (GenBank locus tag: A8O30_RS00250 and A8O30_RS04185) in the genome that encode hypothetical proteins with the identity level of 31 and 25% to *S. paucimobilis* SPT respectively (Figure 7B). For a more complete picture, the annotation from NCBI (PGAP) [15] and PATRIC (RASTtk) [16] for these CDSs was checked and showed that CDS A8O30_RS00250 was annotated as "aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme" and "2-amino-3-ketobutyrate coenzyme A ligase (EC:2.3.1.29)", while A8O30_RS04185 was annotated as "glycine C-acetyltransferase" and "8-amino-7-oxononanoate synthase (EC:2.3.1.47)". Both 2-amino-3-ketobutyrate CoA ligase and 8-amino-7-oxononanoate synthase belong to the same protein family as SPT, the α-oxoamine synthase (AOS) subfamily of the larger group of pyridoxal-5′-phosphate (PLP)-dependent enzymes [17]. MSA of protein sequences of *S. paucimobilis* SPT and predicted proteins of *L. gormanii* A8O30_RS00250 and A8O30_RS04185 clearly shows conserved regions within the analyzed proteins (Figure 7B).

Ceramides can be synthesized de novo from serine and palmitate or produced by the cleavage of sphingomyelins by sphingomyelinases: SMase Cs (SMPD1, EC 3.1.4.12, K12350), which hydrolyze the ester bond between Cer and phosphorylcholine, and SMase Ds (EC3.1.4.41), which hydrolyze the phosphodiester bond between Cer-1-phosphate and choline [17]. Standard KO mapping gave negative results concerning the presence of the SMPD1 coding sequence in the *L. gormanii* genome (Figure 7A). However, in contrast to SPT, genes encoding for SMPD1 have already been found in other *Legionella* species. In particular, the lpp2641 gene in *L. pneumophila* strain Paris and the llo2622, llo1999, and llo1141 genes in *L. longbeachae* strain NSW150 were postulated to code for SMPD1 enzyme [17]. Using the lpp2641 translated sequence as a query in the blastp search against the *L. gormanii* genome, we found CDS with 61% identity, which can be regarded as coding for sphingomyelinase (A8O30_RS11520). To support this hypothesis, a comparison of the genomic regions surrounding the gene encoding SMDP1 in *L. pneumophila* str. Paris and *L. gormanii* was performed. In the analysis carried out done with the use of the clinker tool [18], a small synteny block is visible covering four genes for ParB partition protein, D-alanyl-D-alanine carboxypeptidase, sphingomyelinase and SGNH/GDSL hydrolase family protein, respectively (Figure 7C). This suggests a common evolutionary history of this gene block and makes the assumptions about the aforementioned CDS function plausible.

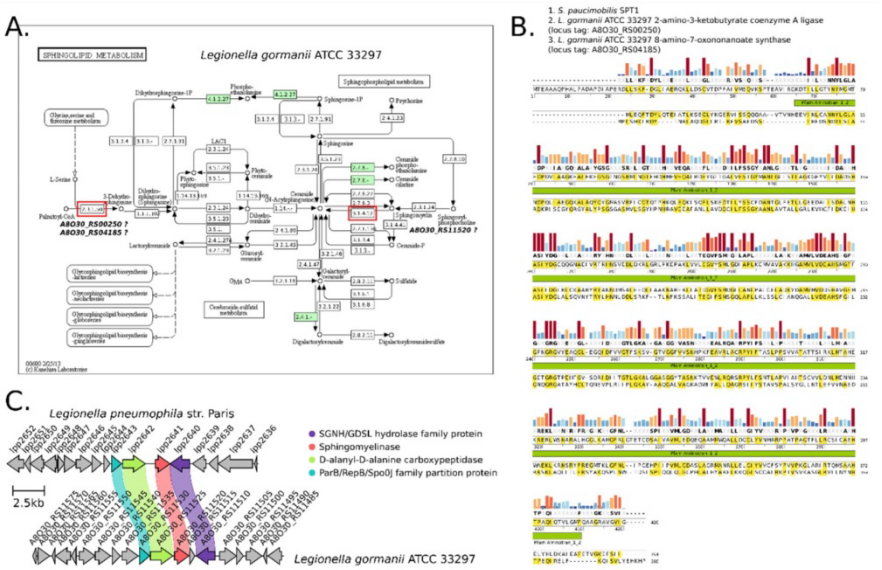


Figure 7. Searching for pathways of sphingolipid metabolism in the genome of *L. gormanii* ATCC 33297. (**A**) Mapping the *L. gormanii* ATCC 33297 genome annotation to the KEGG sphingolipid metabolism pathway map (map00600). Chemical compounds are represented as circles, and gene products are represented as rectangles. The two important entry enzymes in the pathway: serine palmitoyltransferase (SPT, EC 2.3.1.50, K00654) and sphingomyelin phosphodiesterase (SMPD1, 3.1.4.12, K12350) are outlined in red. Green rectangles represent enzymes found in the genome of *L. gormanii* ATCC 33297 via KofamKOALA and with assigned KO identifiers. For the first step of sphingolipid synthesis, i.e., condensation between serine and the acyl-CoA thioester, no SPT enzyme was found in the *L. gormanii* ATCC 33297 genome, based on the standard thresholds for KO identification. However, there are sequences encoding SPT protein homologues (8-amino-7-oxononanoate synthase, locus tag: A8O30_RS00250; 2-amino-3-oxobutyrate coenzyme A ligase, locus tag: A8O30_RS04185) present, belonging to the bacterial α-oxoamine synthases family. In the case of SMPD1, which catalyzes the breakdown of sphingomyelin into ceramide and phosphatidylcholine, although KO identification failed to assign this function, the homologue of predicted sphingomyelinase encoded by the lpp2641 *L. pneumophila* str. Paris gene with a level of identity of 61% was found in the *L. gormanii* ATCC 33297 genome. (**B**) Multiple protein sequence alignment of reference bacterial SPT from *Sphingomonas paucimobilis* (Q93UV0_SPHPI) and SPT homologues present in the genome of *L. gormanii* ATCC 33297. The color-coded bar at the top indicates the conservation degree. Below, there is the consensus sequence. In addition, the recognized Pfam domain (Pfam_Aminotran_1_2) is marked with a green rectangle, which indicates the presence of certain mechanistic features, such as the covalent binding of the pyridoxal-phosphate group to a lysine residue. The level of identity between *Sphingomonas paucimobilis* SPT and *L. gormanii* ATCC 33297 homologues is at the level of 30%. The alignment was generated using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm in SnapGene (GSL Biotech). (**C**) Comparison of genomic regions encoding SMPD1 between in *L. pneumophila* str. Paris and *L. gormanii* ATCC33297. The individual coding sequences are marked with arrows according to their orientation. Above is the locus tag for the individual coding sequence in the GenBank database. The color lines joining the two regions indicate the level of identity above the designated threshold (0.22) for specific proteins. There are four CDSs with significant similarity within the analyzed regions coding for SGNH/GDSL hydrolase (violet), sphingomyelinase (red), D-alanyl-D-alanine carboxypeptidase (green), and ParB partition protein (green). This may indicate a common evolutionary origin of SMPD1 in these species. The comparison and visualization were made using the clinker tool.

3. Discussion

Although bacteria and eukaryotic cells have evolved the ability to produce membranes with different compositions, *L. gormanii* synthesizes phosphatidylcholine and ceramides, i.e., typical components of eukaryotic membranes. Phospholipids were the predominant lipids in the OM and IM of *L. gormanii* cells. PE was the major class phospholipid in the *L. gormanii* membranes. The distribution of PE species in the membranes was similar except for PE15:0/16:0, where content in the IM was twice as high as in the OM, and PE cyclopropyl 17:0/17:0 with an amount in the OM was twice as high as in the IM.

The presence of methylated PE derivatives in the *L. gormanii* membranes indicates that one of the ways of PC synthesis in these bacteria is a pathway based on the triple methylation of PE. The pathway is catalysed by one or more phospholipid *N*-methyltransferases (PmtA) in various organisms [19]. The *Legionella* PmtA enzymes, encoded by *pmtA* genes, exhibit homology to the *Rhodobacter* Pmt-type enzyme, which in turn is homologous to UbiE (ubiquinone/menaquinone biosynthesis methyltransferase). However, in the previous studies, it was not possible to obtain a sequence of the *L. gormanii* pmtA gene using several degenerative primer pairs homologous to *Legionella*, and *Rhodobacter* bacteria [10]. Similar results were obtained by mapping the complete genome sequence of *L. gormanii* ATCC 33297 to the glycerophospholipid metabolism pathway (KEGG map 00564). Neither phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17) nor phosphatidyl-N-methylethanolamine N-methyltransferase (EC 2.1.1.71) coding sequences have been identified in the genome of this strain. The lack of the *pmtA* homologue in *L. gormanii* genome, like *L. dumoffii*, may indicate the presence of a new type of enzyme with phospholipid *N*-methyltransferase activity in these blue–white fluorescent *Legionella* [10]. PC is the second most abundant *L. gormanii* phospholipid distributed in a similar amount in the OM and the IM. The molecular PC profile in both membranes was similar, but there were quantitative differences in the PC species. The IM contained higher content of PCcyclopropyl17:0/17:0 and PCcyclopropyl17:0/16:0, and lower amounts of PC15:0/16:1 than the OM. *L. gormanii* synthesizes PC also in a one-step process of direct choline condensation with CDP-diacylglyceride [10]. The bacteria grown with the addition of exogenous choline synthesized by 10% and 20% higher levels of PC in the OM and the IM, respectively, than the bacteria grown on the standard medium. The comparison of the pattern of PC species synthesized in the PmtA and Pcs pathways showed no differences in the PC pool originating from both pathways. However, *L. gormanii* grown on the choline-supplemented medium synthesized more PC species with cyclopropane acid (e.g., cyclopropyl17:0/15:0, cyclopropyl17:0/16:0) than the bacteria grown without the addition of choline. A characteristic feature of all classes of *L. gormanii* PLs was the high content of cyclopropyl17: 0 fatty acid. The presence of the acid with a cyclopropane ring, which is more stable than the double bond may increase the stability of *L. gormanii* membranes and thus be essential for the survival of the bacteria in the environment. Further analyses are needed to conclude its significance in environmental persistence.

The presence of ceramides is an unusual feature of *L. gormanii* membrane lipids. Sphingolipids (SphL) are important components of the plasma membrane of mammalian cells, where they are typically localized in the outer leaflet [20]. They are also membrane components of a narrow group of bacteria mainly associated with a eukaryotic host. Most of the sphingolipid producing bacteria belonging to the Bacteroidetes phylum (e.g., such genera as *Flectobacillus*, *Porphyromonas*, *Prevotella*, *Parabacteroides*, *Bacteroides*) and the Chlorobi phylum (e.g., *Chlorobium*). Bacteria that synthesize sphingolipids also represent α-Proteobacteria (*Acetobacter*, *Sphingomonas*, *Novosphingobium*) and Δ-Proteobacteria (*Myxococcus*, *Bdellovibrio*) [20]. In bacteria, SphL seem to be localized in the outer leaflet of the outer membrane [21]. The ceramides of *L. gormanii* were found in both the OM and IM, but one species, i.e., cer(16:1_12:0), accounted for 50% of the ceramides present in the OM. The *N*-acyl groups of *L. gormanii* ceramides were even-chain length, long-chain saturated or monoenoic fatty acids with 16C, 18C and 20 to 24C. The structure of *L. gormanii* ceramides is similar to that of mammalian ceramides, whose acyl chain lengths range from 14 to

26 carbon atoms (or greater), with palmitic (C16:0) and stearic (C18:0) acids as the most common fatty acids. In contrast, fatty acids attached to the sphingoid backbones of bacterial sphingolipids are often odd-chain length, methylated, or hydroxylated [21]. *L. gormanii* also produces two species of oxidized ceramides and the ceramide with one hexosyl group. The synthesis of *L. gormanii* ceramides and their role, besides the structural function, remain to be clarified. The bioinformatic search for genes encoding proteins involved in the synthesis of sphingolipids showed that *L. gormanii* encodes hypothetical proteins with the level of identity of 31 and 25% to *S. paucimobilis* SPT respectively.

Some pathogenic bacteria are able to degrade sphingolipids of the host cell and thus counteract the host cell's response and promote intracellular replication. *L. pneumophila* encodes three enzymes: a sphingomyelinase, a sphingosine kinase and a sphingosine-1-phosphate lyase [22,23]. The activity of a sphingosine-1-phosphate lyase (LpSpl) prevents an increase in the level of sphingosine in macrophages infected with *L. pneumophila* and thus inhibits the process of autophagy [24]. *L. gormanii* encodes a hypothetical sphingomyelinase with a similarity of 61% to *L. pneumophila*.

4. Materials and Methods

4.1. Strain and Growth Condition

Legionella gormanii (ATCC 33297) strain was cultured at 37 °C for 96 h on buffered charcoal yeast extract (BCYE) agar plates (Oxoid, UK) or on this medium enriched with 100 µg/mL choline chloride (Sigma-Aldrich, St. Louis, MO, USA). Bacterial cells, harvested by centrifugation were washed twice in 0.5 M NaCl, once in distilled water, and lyophilised. The dry bacterial mass underwent the lipid extraction procedure.

4.2. Isolation of Phospholipids and Thin-Layer Chromatography

The Bligh and Dyer method (1959) with a chloroform–methanol–water mixture was used to extraction of lipids from 200 mg of the bacteria cultured on the medium without and with exogenous choline [25]. Two-dimensional thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Tokyo, Japan, 20 × 20 cm) was used to separate PLs into individual classes. The plates were washed twice with chloroform:methanol (1:1, *v*/*v*) and activated at 180 °C before use. PLs were spotted in one corner of the plate and subsequently separated using a solvent system consisting of chloroform, methanol and water (14:6:1, *v*/*v*/*v*) in the first dimension and chloroform, methanol and acetic acid (13:5:2, *v*/*v*/*v*) in the second dimension. Visualization of PLs was achieved using a charring plate with a solution of 5% sulfuric acid in methanol followed by heating to 180 °C for approximately 1 min. Identification of individual PLs was made by comparing R_f coefficients of individual components for both systems of solvents in comparison with R_f values for standard lipids. Synthetic phosphatidylcholine, phosphatidylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine, phosphatidylglycerol, and cardiolipin from the bovine heart (Sigma-Aldrich, St. Louis, MO, USA) were used as standards.

PLs (1 mg) were applied about 1 cm from the bottom of the silica plate as a narrow band and separated with chloroform:methanol:acetic acid (13:5:2, *v*/*v*/*v*). The bands containing individual PLs were visualized with iodine vapour and next scraped off, transferred to screw-capped tubes, and extracted from silica gel with a mixture of chloroform:methanol (1:1, *v*/*v*). These separations were carried out in triplicate and the obtained fractions of individual phospholipids were intended for the analysis of the fatty acid composition.

4.3. Preparation of Fatty Acid Methyl Esters

Individual PL fractions were saponified in the presence of 1 mL of 0.8 M sodium hydroxide in 50% methanol on a heating block at 80 °C for 1 h. After cooling to room temperature, the samples were acidified with 6N HCl to pH 2 and then evaporated to dryness with an evaporator at 40 °C (Büchi, Rotavapor R-100, Postfach, Switzerland). Extraction of released fatty acids was carried out by 1.5 mL of a mixture of chloroform:water (1:2 *v*/*v*) and mixed by vortex for 2 min. The mixture was centrifuged at 5500× *g* at 10 min

(Sigma, 6-KS). The upper aquatic phase was withdrawn with the aid of the Pasteur pipette and a new portion of water was added to the chloroform layer. After being shaken vigorously, the sample was centrifugated as above, and the organic phase was dried with anhydrous sodium sulphate and the chloroform was removed in the nitrogen stream. Next, 1 mL of 1M HCl/methanol (prepared from acetyl chloride) was added and the mixture was heated at 85 °C for 1.5 h to methylate free fatty acids. After evaporated to dryness with an evaporator, the fatty acid methyl esters (FAMEs) were extracted using 1 mL of a mixture of chloroform and water (1:2 v/v), centrifugated as above and the upper aquatic layer was discarded. The chloroform layer was additionally washed twice with 1mL of MQ water. Then the chloroform solution of FAMEs was dried with anhydrous sodium sulphate and the chloroform was removed in the nitrogen stream. FAMEs were resuspended in 50 µL chloroform before chromatographic analysis.

4.4. Gas-Liquid Chromatography and Mass Spectrometry

The analysis of FAMEs was carried out using a gas chromatograph (Agilent Technologies, instrument 7890A) connected to a mass selective detector (Agilent Technologies MSD 5975C, inert XL EI/CI) (GLC-MS), using helium as a carrier gas. The chromatograph was equipped with an HP-5MS column (30 m × 0.25 mm). The temperature program was as follows: 150 °C for 5 min. raised to 310 °C (5 °C min^{-1}), and the final temperature was maintained for 10 min.

FAMEs were identified by an analysis of their mass spectra and fragmentation patterns. Each fatty acid was quantified by calculating its peak area relative to the total peak area. The positions of the branching methyl group and the double bonds were determined by an analysis of mass spectra of fatty acid pyrrolidides [26]. The *cis* and *trans* isomers of 16:1 and 18:1 FAs were identified based on their retention times.

4.5. Separation of IM and OM in a Sucrose Density Gradient

L. gormanii cells harvested from 6 BCYE agar plates with or without exogenous choline were washed twice in cold 10 mM HEPES (N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid; Sigma-Aldrich, Steinheim, Germany) buffer (pH 7.4) and centrifuged at 8000× g, 15 min at 4 °C (Sigma, 6-16KS). The cell pellets were suspended in 15 mL of 10 mM HEPES buffer containing 20% sucrose (w/v) and incubated with DNase (0.3 mg) (Sigma-Aldrich, Steinheim, Germany) and RNase (0.3 mg) (Sigma-Aldrich, Steinheim, Germany) at 37 °C for 30 min. The cells were lysed through a French Press (SLM-Amico Instruments, Thermo Spectronic, Rochester, NY, USA) three times at 18,000 psi and these lysates were centrifuged at 1000× g for 20 min at 4 °C. Total membrane fractions collected by centrifugation for 60 min at 100,000× g at 4 °C (SW 32Ti rotor, Beckman Coulter, Brea, CA, USA) and were washed twice in cold 10 mM HEPES buffer. The pelleted membranes were suspended in 2.5 mL of 10 mM HEPES buffer and layered onto a seven-step sucrose gradient consisting of 6 mL 70%, 9 mL 64%, 8 mL 58%, 5 mL 52%, 4 mL 48%, 3 mL 42%, and 3 mL 36% (w/v) in 10 mM HEPES buffer at pH 7.5. The samples were centrifuged at 114,000× g, 20 h, at 4 °C (SW 32Ti rotor, Beckman Coulter, Brea, CA, USA). The fractions were manually collected from the top of the gradient in 1-mL steps and were used to identify the IM via nicotinamide adenine dinucleotide (NADH) oxidase activity assay as described [27], and the OM by esterase activity as described previously [28]. Fractions containing the IM and the OM were combined separately. Next, the IM and OM were suspended in 10 mM HEPES buffer, centrifuged at 54,000× g for 1 h (MLA 80, Optima MAX-XP, Beckman Coulter, Brea, CA, USA), and washed twice in cold deionized water. The samples were suspended in MQ water and lyophilized. The concentration of protein in the fractions was determined using the Bradford method and bovine serum albumin as a standard [29]. Lipids isolated from the IM and OM according to the procedure described in Section 2 were subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

4.6. Ultra-High Performance Liquid Chromatography/Mass Spectrometry (UHP LC-MS/MS)

Ultrapure water, NH_4formiate, isopropanol, chloroform, acetonitrile, and formic acid were obtained from Merck (Darmstadt, Germany) in LC MS grade purity.

The samples were transferred to glass vials and dried in a SpeedVac concentrator. Dried samples were reconstituted in 900 µL 20% of mobile phase B and 100 µL chloroform and centrifuged. A total of 3 µL was injected into the UHPLC/MS system.

Lipids were then directly analyzed using a Vanquish UPLC-System (Thermo Scientific, Waltham, MA, USA) with a heated electrospray ionization (HESI) QExactive plus Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). Chromatographic separation took place on a reversed-phase column (Accucore Polar Premium 100 × 2.1 mm (2.6 µ) with guard column: Accucore Polar Premium 10 × 2.1 mm (2.6 µ)) from Thermo Scientific (Waltham, MA, USA). Autosampler temperature was 10 °C throughout the whole measurements.

Mobile phase A consisted of 60% acetonitrile, 10 mM NH_4formiate and 0.1% HCOOH in ultrapure water. Mobile phase B was 90% isopropanol, 10 mM NH_4formiate and 0.1% HCOOH in ultrapure water. Separation was performed with an increasing gradient of B (20–100% from 0.5 to 8.5 min and 20% from 12.5 to 15 min) over a total time of 15 min. Flow rate employed was 0.4 mL/min and the column temperature was 55 °C. The analytes were detected using a Thermo Orbitrap mass spectrometer equipped with a HESI source operated in the positive and negative ion mode. MS data were acquired over a scan range of 250–1200 m/z with full MS resolution of 70,000 and data-dependent MS^2 resolution of 17,500.

Identification and quantification of individual lipid species were performed by LipidSearch Software from Thermo Scientific (Waltham, MA, USA) on product level (MS/MS fragmentation) (Supplementary Materials).

4.7. Bioinformatic Analysis

4.7.1. Sequence Retrieval and Gene Annotation

L. pneumophila str. Paris (GCA_000048645.1) and *L. gormanii* ATCC33297 (GCA_001648685.1) genomes were downloaded from National Center for Biotechnology Information (NCBI) as .gbff files, containing PGAP annotation [15]. For comparison and complement, the annotation obtained using RASTtk [16] from the PATRIC [30] website for both genomes were also downloaded. Protein sequence of SPT enzyme from *Sphingomonas paucimobilis* was downloaded from UniProtKB [31] with ID Q93UV0_SPHPI.

4.7.2. BLASTp and PSI-BLAST Searches

Homologs of SPT were searched using PSI-BLAST [32] with iterations and default thresholds against *L. gormanii* ATCC33297 genome with Q93UV0_SPHPI sequence as a query. Homologs of lpp2641 gene were searched by BLASTp against *L. gormanii* ATCC33297 genome also with default thresholds.

4.7.3. K Number Assignment and KEGG Mapping

All predicted protein coding sequences of *L. gormanii* ATCC33297 genome were subjected to K number assignment with the use of BlastKOALA and KofamKOALA tools [13,33]. After assignment, all proteins were linked to the KEGG pathways and EC numbers [33].

4.7.4. Gene Cluster Comparisons

Genomic regions spanning approximately 10 kbp upstream and downstream from loci: lpp2641 in *L. pneumophila* str. Paris and A8O30_RS11520 in *L. gormanii* ATCC33297 were manually extracted using SnapGene software (GSL Biotech) and submitted to comparison using clinker tool with identity threshold set to 0.22 [18].

4.7.5. Statistical Analysis

Results were statistically evaluated using Spearman's rank correlation coefficient.

5. Conclusions

The in-depth analyses of the lipid components of the OM and IM in the *L. gormanii* cell wall showed that this bacterium contains components that are unique to prokaryotes but characteristic for eukaryotes. PC was the second most abundant PL after PE distributed in the OM and IM in a similar amount. The bacteria grown on a medium with exogenous choline increased the PC content in the OM and IM by 25 and 28%, respectively, and reduced the PE content in the OM and IM by 19 and 29%, respectively. The comparison of the molecular profile of PC species contained in the lipids of *L. gormanii* membranes cultured on the medium with and without exogenous choline showed only quantitative differences in the PC pool. *L. gormanii* synthesized ceramides with only an even number of carbon atoms in the acyl chains, which makes them more similar to mammalian than bacterial ceramides. These compounds were found in both the OM and IM but one species, i.e., cer(16:1_12:0), accounted for 65% of the ceramides present in the OM. The addition of exogenous choline to the growth medium increased the content of cer(16:1_12:0) in the OM to 70%.

As shown by the bioinformatic analyses of the *L. gormanii* genome, this bacterium encodes putative enzymes that may be involved in both de novo ceramide synthesis and sphingolipid degradation.

The presence of such macromolecules as PC and ceramides in *L. gormanii* membranes may be an example of the molecular mimicry used by these bacteria to infect a eukaryotic host cell effectively. Investigation of the phenotypic plasticity of the surface structures of *Legionella* bacteria is essential for understanding the pathogenesis mechanisms behind the evolutionary success of these bacteria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/metabo12050418/s1, Table S1: All lipid identifications showing accurate mass measurements at the MS and MS/MS level (identified lipid ions and corresponding product ions in the positive ionization mode). Table S2: All lipid identifications showing accurate mass measurements at the MS and MS/MS level (identified lipid ions and corresponding productions in the negative ionization mode). Grade A: Class and all fatty acid chains are completely identified. Grade B: Both class specific ion and fatty-acid-derived product ions are detected. Grade C: Either class specific ion or fatty-acid-derived productions are detected. Grade D: Unable to identify the lipid structure, such as a dehydrated ion.

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Publikacja 4

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Physicochemical characteristics of model membranes composed of *Legionella gormanii* lipids

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Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids

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Abstract: *Legionella gormanii* is one of the species belonging to the genus *Legionella*, which causes atypical community-acquired pneumonia. The most important virulence factors that enable the bacteria to colonize the host organism are associated with the cell surface. Lipids building the cell envelope are crucial not only for the membrane integrity of *L. gormanii* but also by virtue of being a dynamic site of interactions between the pathogen and the metabolites supplied by its host. The utilization of exogenous choline by the *Legionella* species results in changes in the lipids' composition, which influences the physicochemical properties of the cell surface. The aim of this study was to characterize the interfacial properties of the phospholipids extracted from *L. gormanii* cultured with (PL+choline) and without exogenous choline (PL−choline). The Langmuir monolayer technique coupled with the surface potential (SPOT) sensor and the Brewster angle microscope (BAM) made it possible to prepare the lipid monomolecular films (model membranes) and study their properties at the liquid/air interface at 20 °C and 37 °C. The results indicate the effect of the choline addition to the bacterial medium on the properties of the *L. gormanii* phospholipid membranes. The differences were revealed in the organization of monolayers, their molecular packing and ordering, degree of condensation and changes in the components' miscibility. These findings are the basis for further research on the mechanisms of adaptation of this pathogen, which by changing the native composition and properties of lipids, bypasses the action of antimicrobial compounds and avoids the host immune attack.

Keywords: *Legionella gormanii*; phospholipids; Langmuir monolayer technique

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1. Introduction

The cell envelope is a complex multilayered structure that, in most bacteria, is essential for the maintenance of cell shape and structural integrity. The Gram-negative bacterium cell envelope consists of the outer (OM) and inner (IM) membranes. The periplasm with the peptidoglycan cell wall and unique proteins separate these membranes. The OM is an unusual lipid bilayer that contains phospholipids in the inner leaflet and glycolipids, principally lipopolysaccharides (LPS), in the outer leaflet [1]. This asymmetric nature and the unique biochemistry of LPS contribute to the ability of OM to function as a molecular permeability barrier that protects the bacterium, confining the access of noxious compounds present in the environment. The macromolecules of the cell envelope play a central role in the properties and capabilities of the cell. This holds particularly true for the pathogenic bacteria whose highly specific interactions with the human organism depend on surface structures to a large extent. *Legionella* are Gram-negative bacteria that are ubiquitous in

freshwater reservoirs, soils, and water-based engineered structures. Human infection leads to legionellosis, which can be present in its non-pneumonic (Pontiac fever) and acute pneumonic (Legionnaires disease) forms. The ability of *Legionella* to overcome the killing mechanisms of phagocytes depends on many factors, both the specialized systems of protein secretion and the components related to the unique structure of their cell envelope.

L. gormanii is one of the species belonging to the genus *Legionella*, which causes atypical community-acquired pneumonia in immunocompromised and immunocompetent individuals [2,3]. Rare cases of *L. gormanii*-induced pneumonia were diagnosed in children with immunodeficiency [4]. The pathogenicity of this bacterium is determined, among others, by the high efficiency of pulmonary surfactant degradation comparable to the activity of *L. pneumophila*. *L. gormanii* bacteria produce phospholipase B, for which the substrate is phosphatidylcholine (PC), accounting for 80% of the lung surfactant [5].

This bacterium does not reproduce in *Acanthamoeba* spp., but it has the ability to adhere, invade and replicate in human macrophages of the THP-1 line [6]. The proteins secreted by the T4 secretion system play a key role in the formation of a distinct membrane-bound replicative niche by *L. pneumophila* inside the macrophages. The bioinformatic analyses of the *L. gormanii* genome revealed the presence of a gene cluster encoding the T4SS components [7]. The activity of membrane proteins, their folding, stability or localization are controlled by the protein–lipid interactions regulated by phospholipids [8].

The *L. gormanii* membranes were characterized by the unique bacterial lipid composition. The lipid profile of the OM and the IM *L. gormanii* comprised glycerolipids (triglycerides, diglycerides), sphingolipids (ceramides, hexosylceramides) and the most abundant, phospholipids (phosphatidylethanolamine, PE; phosphatidylcholine, PC; cardiolipin, CL; phosphatidylglycerol, PG). The presence of PC and ceramides—the lipids commonly occurring in the eukaryotic cells and rarely found in the prokaryotic organisms—is a unique feature of *L. gormanii* membranes. This bacterium can synthesize PC via two independent pathways, namely the phospholipid N-methylation (Pmt) pathway and the phosphatidylcholine synthase (Pcs) pathway. The bacteria condense choline with CDP-DAG by the Pcs synthase in the Pcs pathway. Since choline is not a biosynthetic product of bacteria, its source for *L. gormanii* are metabolites derived from the eukaryotic cells. In this way, these bacteria come into close contact with the host cell in which they replicate. A rapid PC synthesis (in a one-step reaction) may be required for the *L. gormanii* bacteria to adjust their membrane physiology rapidly to new environmental conditions. *L. gormanii* cultured on the medium with exogenous choline was synthesized by 21% more PC and less PE and CL (12% and 9%, respectively) compared with the bacteria grown on the medium without the addition of choline [9]. Our previous lipidomic analyses showed quantitative differences in the molecular profile of the various phospholipids contained in the OM and IM of *L. gormanii* grown on the choline-supplemented and non-supplemented media. The content of PE15:0_15:0, PE15:0_16:0 and PE16:0_16:1 was higher in the IM lipids extracted from the bacteria cultured with the choline addition. The amount of PC cyclopropyl17:0_15:0 and PC cyclopropyl17:0_16:0 in the IM lipids isolated from the bacteria grown on the medium with exogenous choline was higher compared to the IM lipids isolated from *L. gormanii* cultured without choline. The OM lipids from the bacteria grown on the choline-supplemented medium contained more PC15:0_15:0 than the bacteria grown on the medium without exogenous choline [10]. The results indicate that the lipid membranes are a dynamic site of interactions between the bacterial pathogen and the choline delivered into the growth medium.

The structure of lipids, mainly the length and degree of saturation of the phospholipids' hydrocarbon tails, influence the physicochemical properties of the membrane that determine bacterial survival and the adaptation to the living environment. One of the simple and at the same time the most precise methods for monitoring the physicochemical properties of membranes is the Langmuir technique. This technique enables the formation of high-quality, ordered monolayers that can be used as a model for bacterial membranes. The use of the Langmuir technique in combination with the Brewster angle microscope

allows for the precise imaging of the monolayer morphology upon the film compression. This study aimed at determining how the changes in the structure of phospholipids isolated from the bacteria cultured with and without the exogenous choline affect the biophysical properties of *L. gormanii* membranes using the Langmuir monolayers. In addition, the use of the Brewster angle microscope allowed for a more detailed analysis of the film architecture and visualization of the interfacial organization of PL constituents of the monolayer. These studies will provide the basis for further analysis of the effectiveness of interactions of various antimicrobial compounds with the biological systems obtained from *L. gormanii* grown under different culture conditions.

2. Materials and Methods

2.1. Bacterial Strain and Media

Legionella gormanii (ATCC 33297) was cultured on the buffered charcoal yeast extract (BCYE) agar plates (Oxoid, Basingstoke, UK) or on this medium enriched with 100 µg/mL choline chloride (Sigma-Aldrich, St. Louis, MO, USA; referred to as choline in this paper) at 37 °C and 5% CO_2 in a humid atmosphere for 3 days. Biomass was harvested by centrifugation at 8000 rpm, 20 min, washed with 0.5 M NaCl twice, distilled water once and then freeze-dried.

2.2. Extraction and Separation of Phospholipid by TLC Plates

Lipids were extracted from 500 mg of freeze-dried bacterial cells according to the procedure of Bligh and Dyer [11]. The extracts were concentrated in a vacuum evaporator until complete dryness and resolved in 100 µL of chloroform/methanol (1:2; v/v). The organic layer contained lipids and pigments, mainly legioliulin, which is responsible for the blue-white autofluorescence under the long-wavelength UV light [12]. The phospholipids (PLs) were purified from the pigment using one-dimensional thin-layer chromatography (TLC) on the silica gel 60 F254 plates of the size 10 cm × 10 cm (Merck, Darmstadt, Germany). Before the application of the organic phase, the plates were washed with chloroform/methanol (1:1, v/v) to remove all contaminants and dried at room temperature. The organic fraction (about 2 mg) was applied on the silica gel as a narrow band and developed with the solvent system chloroform/methanol/acetic acid (98:2:1, $v/v/v$). PLs were visualized with iodine vapor, and legioliulin was detected under the long-wavelength UV (Transiluminator UV-953). The PL-containing band was scraped off, transferred to the screw-capped tubes and extracted from the silica gel with a mixture of chloroform/methanol (1:1, v/v). PLs purified from the pigment were intended for testing in the Langmuir trough and separation into individual classes.

Phospholipids were separated into different classes using chloroform/methanol/acetic acid (13:5:2, $v/v/v$) as the solvent system. Four bands were detected after the iodide vapor exposure; they were scraped and extracted with chloroform/methanol (1:1, v/v). The identification of individual PLs was made comparing the retention factor (Rf) coefficients of individual components with the Rf values for standard phospholipids ($Rf_{,PC}$ = 0.09, $Rf_{,PE}$ = 0.37, $Rf_{,PG}$ = 0.56, $Rf_{,CL}$ = 0.79). The Rf value of a PL was equal to the distance traveled by the compound divided by the distance traveled by the solvent front. Separations of PLs were made in six replications. Lipids from all fractions were collected, evaporated to dryness under nitrogen, weighed and stored at −20 °C before the analysis of fatty acids. To assess the reproducibility of the results, three independent culturing experiments for each condition with and without the choline supplementation were analyzed.

2.3. Preparation of Fatty Acid Methyl Esters (FAMEs)

A phospholipid sample (1 mg) in the Teflon-capped Pyrex tube was hydrolyzed with 1 mL of 0.8 M NaOH in 50% methanol at 80 °C for 1 h. After cooling to room temperature, the reaction mixture was acidified with 200 µL of 6 M HCl, evaporating to dryness under a nitrogen stream at 40 °C. The released free fatty acids (FFAs) were extracted with 1 mL of chloroform. The chloroform solution of FFAs was dried with anhydrous sodium sulfate and

the chloroform was removed under the nitrogen stream. The FFAs were methylated with 300 µL of 0.02 M trimethylsilyl diazomethane (TMSD, Sigma-Aldrich) in 20% methanol in acetone at room temperature for 30 min. The excess solvent and diazomethane were removed under a gentle stream of nitrogen. A total of 2 mL of water and 1 mL of chloroform were added to the sample. After thorough mixing, the sample was centrifuged at 5000× *g*, for 10 min, and the FAMEs dissolved in the chloroform layer were analyzed by gas–liquid chromatography and mass spectrometry (GLC/MS).

2.4. Gas-Liquid Chromatography and Mass Spectrometry

FAMEs were analyzed using a gas chromatograph (Agilent Technologies, Santa Clara, CA, USA, instrument 7890A) connected to a mass selective detector (Agilent Technologies MSD 5975C, inert XL EI/CI) (GLC-MS), using helium as a carrier gas. The chromatograph was equipped with an HP-5MS column (30 m × 0.25 mm). The temperature program was as follows: 150 °C for 5 min raised to 310 °C (5 °C/min), and the final temperature was maintained for 10 min. FAMEs were identified by an analysis of their mass spectra and fragmentation patterns. The relative content (%) of each fatty acid was calculated from the ratio of the area of its peak to the total area of all peaks. The positions of the branching methyl group, cyclopropane ring and the double bonds were determined by an analysis of the mass spectra of fatty acid pyrrolidines.

On the basis of the relative content of identified fatty acids, the molecular weight of the particular phospholipid classes, and then the total value for the mixture was calculated according to the following equation (Equation (1)):

$$\overline{M} = M_1x_1 + M_2x_2 + \ldots + M_nx_n \quad (1)$$

where $\overline{M}$ is a mean molecular weight, M is a molecular weight of a given compound and x is a molar fraction of a given compound in the mixture.

The obtained results are presented in Table 1. They were used in the Langmuir monolayer studies.

Table 1. Molecular weight of phospholipid classes and their mixtures.

Phospholipid Class	Molecular Weight (g/mol)
PC−choline	730.6
PC+choline	729.4
PE−choline	683.5
PE+choline	680.9
CL−choline	1389.7
CL+choline	1385.9
PG−choline	715.8
PG+choline	714.0
Phospholipid Mixture	**Molecular Weight (g/mol)**
PL−choline	845.0
PL+choline	789.3

2.5. Langmuir Monolayer Studies

The Langmuir (KSV NIMA) and Langmuir–Blodgett (KSV 2000 Standard) troughs equipped with symmetrical barriers and a Wilhelmy plate were used for the surface pressure-mean molecular area ($\pi - A$) isotherm determination. The accuracy of the pressure sensor was 0.1 mN/m. For all the experiments, the trough was filled with 0.01% acetic acid solution prepared by diluting the concentrate one (99.7%) with Milli-Q water purified by the Milli-Q Plus system (resistivity 18.2 MΩ cm). The temperature was kept constant (20 or 37 °C) by the external water circulating system (Lauda). Phospholipids (PLs) isolated from *L. gormanii* cultured with or without choline were prepared in chloroform/methanol

(4:1, v/v) to obtain the concentration of 1 mg/mL. The solvents of high purity (≥99%) were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). The determined molecular weights (Table 1) were used to estimate the number of molecules to be deposited on the subphase. To achieve this, the correct volume of a phospholipid solution with known concentration (usually 1 mg/mL) was dropped carefully onto the subphase (0.01% acetic acid) with the glass-made Hamilton microsyringe and left for 10 min to ensure full evaporation of the volatile solvents. Then, the monolayer was continuously compressed at the rate of 10 mm/min to its collapse. Each experiment was performed at least three times and the obtained $\pi - A$ isotherms were found to be reproducible with the average error of ± 2 Å^2/molecule. Simultaneously, the surface potential-mean molecular area ($\Delta V - A$) isotherms were recorded by means of vibrating plate surface potential sensor (SPOT, Biolin Scientific, Gothenburg, Sweden) with the accuracy of 1 mV.

The monolayer morphology was directly visualized with the help of Brewster angle microscope (nanofilm_ultrabam, Accurion, Göttingen, Germany). The use of 50 mW internal solid-state laser emitting p-polarized light with the wavelength of 658 nm enabled the recording of BAM images with the lateral resolution of 2 µm and the field of view of 720 × 400 µm^2. The angle of incident was fixed to the Brewster angle (53.2°).

2.6. Statistical Analysis

The values were expressed as the mean ± SD for three independent experiments. The results were statistically evaluated using the one-sided asymptotic Mann–Whitney U test with the continuity correction (implemented in Python's 1.10.0 version of the Scipy package). The significance level was set to 5%.

3. Results

3.1. Fatty Acid Composition of Phospholipids

The composition of fatty acids, which are the main components of lipids, determines the physical, chemical and physiological properties of each class of phospholipids. Our previous research proved that the *L. gormanii* phospholipid classes have characteristic FA distributions [10]. In the present studies, it is shown that adding choline to the growth medium changes the content of unsaturated, saturated and long-chain fatty acids in *L. gormanii* phospholipids (Figure 1 and Table 2). The bacteria grown on the medium with choline on the average synthesized 2.4 times more long-chain (from C19 to C21) fatty acids, less saturated fatty acids and fatty acids with the acyl chain length from C14 to C18 found in the PC class. On the contrary, *L. gormanii* grown on the medium with choline synthesized less saturated and long-chain fatty acids, and more unsaturated fatty acids in the PG class. A similar pattern of fatty acids was found in the PE class of the bacteria grown on the choline-supplemented and non-supplemented medium. In the CL class, the content of saturated and unsaturated fatty acids was similar regardless of the culture conditions. However, the addition of choline to the growth medium caused these bacteria to synthesize 3% less long-chain fatty acids and 3% more fatty acids with the acyl chain length of C14 to C18. Additionally, there were minor differences in the share of saturated fatty acids between the culture conditions, but none was significant.

3.2. $\pi - A$ Isotherms and Compression Modulus

Phospholipids extracted from the *L. gormanii* bacteria, both supplemented and non-supplemented with choline, form Langmuir monolayers at the air–liquid interface at 20 °C and 37 °C. The obtained $\pi - A$ isotherms are presented in Figure 2a.

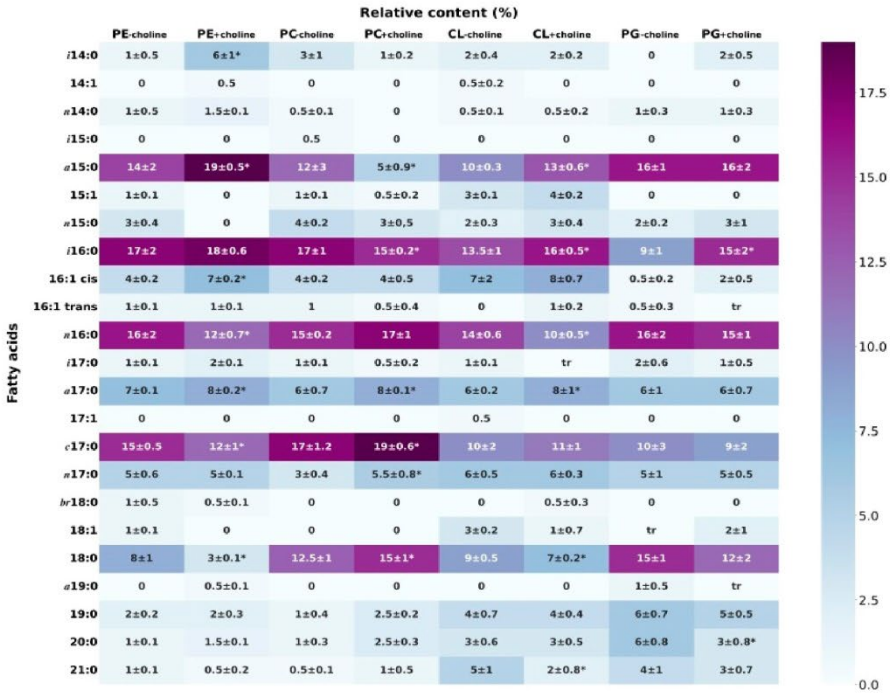

Figure 1. Relative content (%) of fatty acids in the PL classes isolated from the bacteria cultured on the medium without and with choline. *a*, methyl branch at the anteiso carbon atom; *i*, methyl branch at the iso carbon atom; *n*, unbranched acid; *c*, cyclopropane ring structure; ± standard deviation; * indicates a significant difference in the MW test for choline vs. the lack of choline comparison in each PL. The test was carried out for fatty acids with at least 5% relative content.

Table 2. Fatty acid (FA) composition (relative content, %) of individual phospholipids (this study). The percentage of individual classes in the phospholipid mixture isolated from *L. gormanii* cultured on the medium without and with choline (previous study [9]); * indicates a significant difference in the MW test for choline vs. the lack of choline comparison in each PL.

	Sum of Saturated FA	Sum of Unsaturated FA	Sum of FA 14–18	Sum of FA 19–21	Percentage of Phospholipid Class in PL Mixture [9]
PC−choline	77.0 ± 0.6	23.0 ± 0.5	97.5 ± 0.7	2.5 ± 0.3	26.0 ± 2.0
PC+choline	75.0 ± 0.5	24.0 ± 0.4	94.0 ± 0.5 *	6.0 ± 0.3 *	47.0 ± 0.0
PE−choline	79.0 ± 0.7	21.0 ± 0.2	96.0 ± 0.7	4.0 ± 0.1	50.0 ± 1.4
PE+choline	79.5 ± 0.3	20.5 ± 0.3	95.5 ± 0.4	4.5 ± 0.2	38.0 ± 0.6
CL−choline	76.0 ± 0.5	24.0 ± 0.8	88.0 ± 0.6	12.0 ± 0.8	21.0 ± 1.4
CL+choline	75.0 ± 0.4	25.0 ± 0.6	91.0 ± 0.5 *	9.0 ± 0.6 *	12.0 ± 0.6
PG−choline	89.0 ± 0.9	11.0 ± 1.0	83.0 ± 1.0	17.0 ± 0.6	3.0 ± 0.7
PG+choline	87.0 ± 1.0	13.0 ± 1.0	89.0 ± 1.0 *	11.0 ± 0.7 *	3.0 ± 0.6

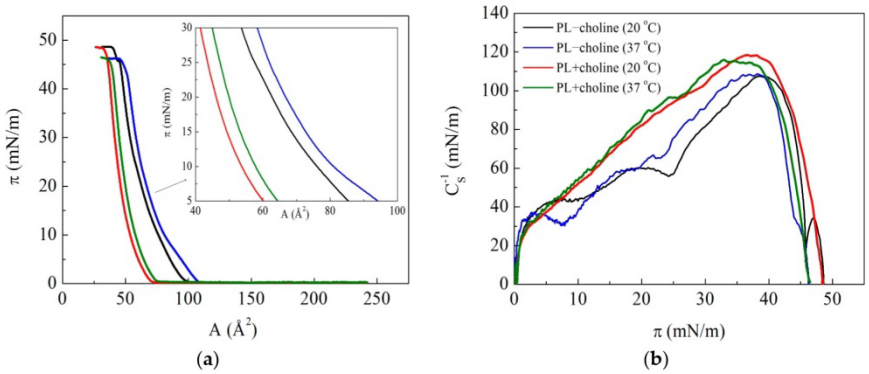

Figure 2. Surface pressure-area per molecule ($\pi - A$) isotherms registered at 20 °C and 37 °C (**a**) and determined the compression modulus-surface pressure ($C_S^{-1} - \pi$) dependencies based on them (**b**) for the monolayers of phospholipids (PL) extracted from *L. gormanii* bacteria supplemented (PL+choline) or not (PL−choline) with choline.

Based on them (Figure 2a), the lift-off point (A_0) and the collapse pressure (π_c) were determined. The lift-off point (A_0) is the area per molecule at which the surface pressure can be detected (≈0.5 mN/m). It is ascribed to the monolayer transition from the gas to liquid-expanded phase. The collapse pressure (π_c), at which a sudden change in the slope of the $\pi - A$ isotherm is observed, corresponds to breakdown of the two-dimensional monolayer structure and formation of three-dimensional aggregates (collapsed domains) and/or loss of molecules from the monolayer by their dissolution in the subphase. The values of the above-mentioned parameters ($A_0 \pm 2$ Å^2/molecule, $\pi_c \pm 0.1$ mN/m) are summarized in Table 3 for measurements conducted at 20 °C and 37 °C.

Table 3. Lift-off point (A_0), collapse pressure (π_c) and maximal ($C_{s,max}^{-1}$) compression modulus along with the corresponding surface pressure and area per molecule ($\pi_{C_{S,max}^{-1}}$, $A_{C_{S,max}^{-1}}$) for the indicated monolayers.

Monolayer	A_0 (Å^2)	π_c (mN/m)	$C_{S,max}^{-1}$ (mN/m)	$\pi_{C_{S,max}^{-1}}$ (mN/m)	$A_{C_{S,max}^{-1}}$ (Å^2)
PL−choline (20 °C)	99	49	108	39	49
PL−choline (37 °C)	108	46	109	38	54
PL+choline (20 °C)	71	48	119	37	39
PL+choline (37 °C)	76	46	116	33	44

A difference between the PL−choline and the PL+choline monolayers concerns the course of $\pi - A$ isotherms, both their shape and area per molecule at a given surface pressure and temperature. As regards to the shape, the $\pi - A$ isotherm of the PL+choline monolayer displays a moderate increase in the surface pressure with compression, without any visible discontinuities, while that of the PL−choline monolayer displays subtle inflections as changes in the curve slope at the surface pressure of about 8 mN/m (at 37 °C) or 10 mN/m (at 20 °C) and 25 mN/m (at both). These can be indicative of a change in the arrangement and packing of molecules in the PL−choline monolayer which does not occur for PL+choline. At 37 °C, the $\pi - A$ isotherms are slightly shifted toward larger molecular areas (to the right side of the x axis) and the film collapse pressure is lower than at 20 °C. Moreover, the lift-off point and the area per molecule in the full range of the surface pressures are lower for PL+choline than for PL−choline, suggesting a more condensed character of the former monolayers (Table 3). This fact correlates with a larger

content of cardiolipin in PL−choline (21%) and can be a result of the anionic character of this phospholipid as well as its large headgroup, which is discussed in the following part of this paper.

To confirm the above assumption, the compression modulus (C_S^{-1}, Figure 2b) was calculated directly from the $\pi - A$ isotherm data based on the following formula [13]:

$$C_S^{-1} = -A\left(\frac{d\pi}{dA}\right)_{T,p} \quad (2)$$

The compression modulus is a typical parameter providing information about packing and ordering of the monolayer at constant temperature (T) and external pressure (p). The determined values of C_S^{-1} as a function of π are presented in Figure 2. Moreover, the maximal C_S^{-1} values along with the surface pressure and mean molecular area at which they occur are listed in Table 3.

The maximal values of C_S^{-1} for both PL−choline and PL+choline monolayers are within the range assigned to the liquid-condensed (LC) state according to the Davies and Rideal criterion, i.e., 100 mN/m < C_S^{-1} < 250 mN/m [13]. However, up to the collapse pressure, the C_S^{-1} values for PL+choline are greater than for PL−choline at both temperatures, thus revealing a tighter packing of molecules. It should be emphasized that the course of the $C_S^{-1} = f(\pi)$ function determined for PL−choline shows the presence of two distinct minima (inflections) at about 8–10 mN/m and 25 mN/m, which corresponds to the discontinuities on the $\pi - A$ isotherms (Figure 2). The C_S^{-1} values of the peaks rise from the range characteristic of the LE (40 mN/m) through the LE-LC (60 mN/m) to the LC (108 mN/m) phase for the monolayer at 20 °C. As a contiguous LE monolayer is created, the $\pi - A$ isotherm takes off, giving rise to a positive surface pressure. Then, the hydrophobic tails of the molecules come into contact with each other by being largely disordered and fluid [14]. Since there is the change in the physical state of the monolayers from the LE to the LC phase, the first inflection therefore proves that the LE–LC phase transition takes place. However, it is not typical of a first-order phase transition represented by a horizontal *plateau* in the $\pi - A$ isotherm and nearly zero compression modulus [15]. It is blurred due to the multicomponent composition of the PL−choline monolayer whose molecules contain different headgroups and chains. The second inflection indicates further alterations in the film organization, but it is difficult to unequivocally determine their origin. A few options can be considered: (1) a second-order phase transition related to the changes in alkyl chain tilting; (2) partial miscibility and/or removal of some molecules into the subphase as molecules can be forced out of the interface; (3) some nucleation process [15].

In the case of PL+choline monolayers, the $\pi - A$ isotherms demonstrate no visible inflections, and even after differentiation, no discontinuities are observed on the compression modulus. This indicates that, during the compression, the PL+choline film is more tightly packed than PL−choline, while remaining in the same physical state.

These findings show that the choline addition to the bacteria medium increases the condensation of the PL+choline monolayers as manifested by larger values of the compressibility modulus than those of PL−choline. The reason for that can be stronger Lifshitz-van der Waals forces between the molecules possessing longer acyl chains. Moreover, the hydrophilic headgroup sizes are of great importance, which is discussed below.

3.3. Surface Potential

The surface potential changes (ΔV) reveal the alteration of orientation and conformation of molecules in the monolayers upon compression. Measured ΔV defines the difference between the surface potential of pure subphase and the surface with the monolayer [16]. The alterations of the normal component of the dipole moment density with respect to the surface, caused by phospholipid film compression, result in proportional surface potential change, which allows one to analyze molecular behavior [17]. As can be seen in Figure 3, the ΔV values of all monolayers are positive upon compression. Variations in the slope

of $\Delta V - A$ isotherms reflect molecular orientation and/or conformational changes in the monolayer since ΔV is proportional to the magnitude of the electrostatic field gradient perpendicular to the subphase surface [17,18]. During the compression, the hydrocarbon chains orient more vertically to the surface, changing the orientation of the polar groups, which contributes to an increase in the surface potential. Figure 3 shows a systematic increase in the ΔV values with the decreasing area per molecule of PL films in the range from 0 to 0.3 V.

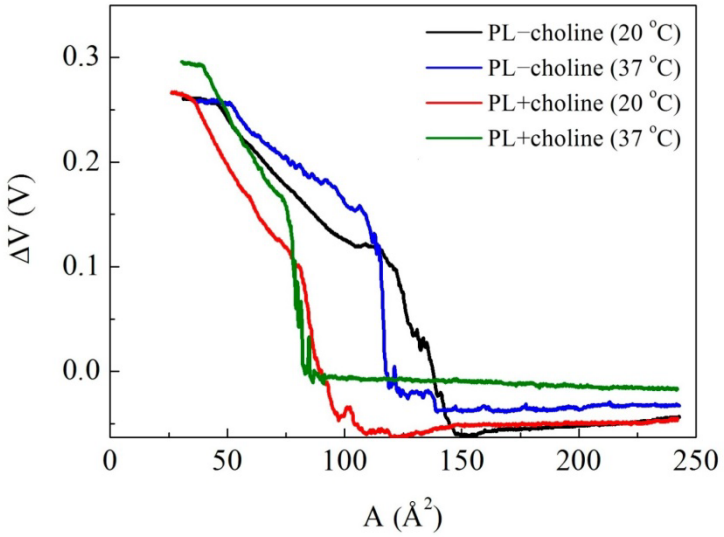

Figure 3. Surface potential changes ΔV vs. the mean molecular area (A) for the PL−choline and PL+choline monolayers at 20 °C and 37 °C.

At 37 °C, the ΔV changes are adequate, but the values of this parameter are greater than at 20 °C. It is also important to note that the sharp increase in the surface potential corresponds to the extensive surface coverage with the condensed domains, as revealed by the BAM images (see Section 3.4). When the PL−choline films are compressed up to the close-packed state, their ΔV values reach ~0.26–0.27 V, while for PL+choline it is 0.27–0.30 V.

For better visualization, the correlation between the determined parameters, i.e., surface pressure (π), compressibillity modulus (C_S^{-1}) and surface potential changes (ΔV) as a function of area per molecule (A), is presented in Figure 4. A sharp increase in the ΔV values is found at larger areas than the lift-off point for the $\pi - A$ isotherm. At certain area values, the ΔV increases more slowly. It is important to note that this inflection correlates with the $\pi - A$ isotherm lift-off area. This suggests a change in the molecule orientation with respect to the subphase surface, related to the formation of more ordered structures. Moreover, the maximal values of the surface pressure usually correspond to the closest packing of molecules in the monolayer [18,19], which in the presented research can be observed as a strict correlation between the maximal values of ΔV and C_S^{-1} as they occur at nearly the same area per molecule in all analyzed monolayers (Figure 4).

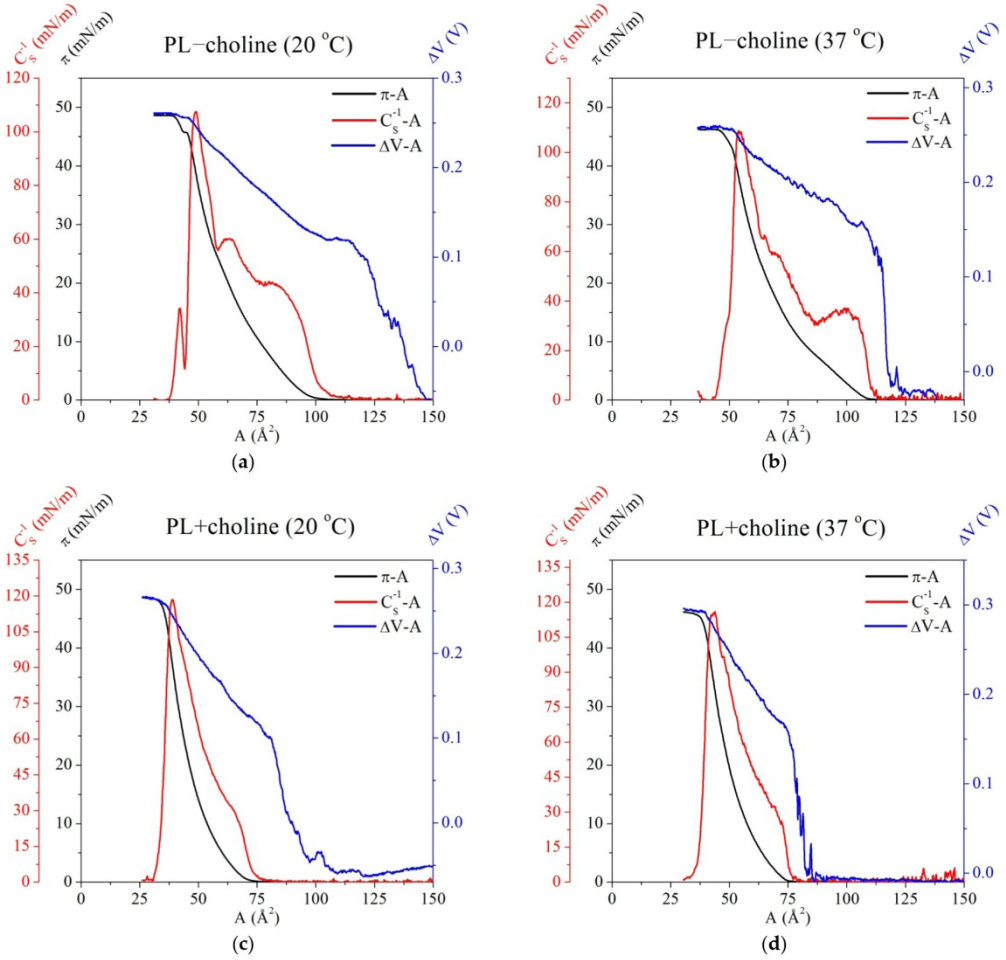

Figure 4. Surface pressure (π), compressibility modulus (C_S^{-1}) and surface potential changes (ΔV), vs. the mean molecular area (A) for the obtained monolayers: PL−choline at 20 °C (**a**), PL−choline at 37 °C (**b**), PL+choline at 20 °C (**c**) and PL+choline at 37 °C (**d**).

3.4. Morphology

The above observations can be evidenced by the BAM images presented in Figure 5. They depict that, during the compression of the PL−choline monolayer at 20 °C, some inhomogeneity appears at the surface pressure around 10 mN/m, after the reorganization indicated by the $\pi - A$ isotherm. Namely, the coexistence of bright areas of more condensed domains (irregular oblong structures) surrounded by darker regions of the less condensed phase (patches) is observed. At about 25 mN/m the condensed domains are being restructured to larger ones, which is demonstrated by a small reduction in the compression modulus (Figure 2b, second inflection). At 37 °C, the domains are smaller in size and with the compression form regular circular structures densely distributed over the subphase. They appear in greater quantities at smaller values of surface pressure as compared to the analysis conducted at a lower temperature, and their reorganization at

25 mN/m is less pronounced at a temperature higher than 20 °C, which is also reflected in a smaller decrease in the compression modulus values (Figure 2b). Simultaneously, the changes in the monolayer organization correlate with the above-mentioned inflections of the $\pi - A$ isotherms (Figure 2a).

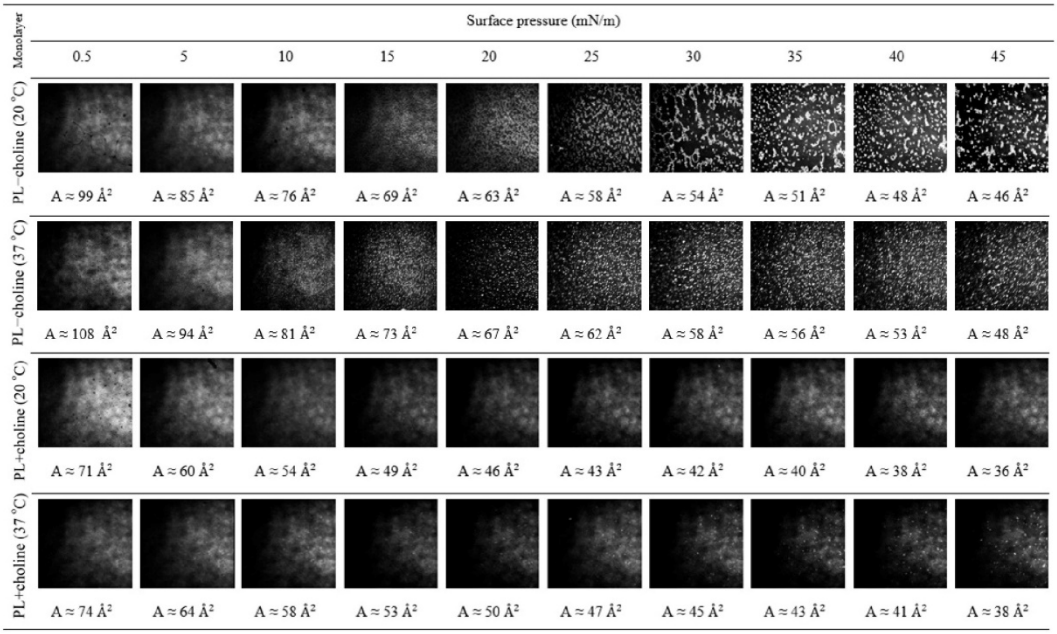

Figure 5. BAM images taken for the PL−choline and PL+choline monolayers at 20 °C and 37 °C at given values of surface pressure and mean molecular area.

Considering the morphology of the PL+choline monolayers, one can see much smaller and fewer circular domains, causing the film to be more homogeneous. At a lower temperature, more condensed structures can be clearly observed at about 25 mN/m, while at 37 °C a few domains can be noticed around 10 mN/m. However, their presence is not manifested either in the $\pi - A$ isotherm inflections or in the discrete course of the $C_S^{-1} - \pi$ functions. Thus, the BAM images confirm the greater homogeneity of the PL+choline monolayers. This correlates with the greater condensation of the monolayers indicated by the higher C_S^{-1} values.

4. Discussion

In this contribution, we studied the properties of model membranes (Langmuir monolayers) composed of lipids isolated from the *L. gormanii* cells grown on the medium with and without choline. The presence of choline affects the fatty acid composition (Figure 1) and proportions of the individual classes of phospholipids produced by bacteria (Table 2) and thus, the properties of the monolayers formed from them at the liquid–gas interface as well. The relative content of individual lipid classes shows that the zwitterionic phospholipids PE and PC dominate, the total amount of which is 76% for *L. gormanii* (−choline) and 85% for *L. gormanii* (+choline), while the negatively charged lipids (PG and CL) account for 24% and 15%, respectively [9]. These differences are of great importance for the model membrane behavior and intermolecular interactions in the phospholipid mixture, as can be seen on the $\pi - A$ isotherms (Figure 2a).

Comparing the $\pi - A$ isotherms registered for the monolayers of PL−choline and PL+choline mixtures at 20 °C, it can be found that, in principle, the curves obtained for PL+choline are located at smaller areas per molecule than the $\pi - A$ isotherms acquired for PL−choline (Figure 2a). This suggests that the distance between the PL+choline molecules is much smaller than between the PL−choline molecules. This fact correlates well with the higher values of the compressibility modulus for the PL+choline than for PL−choline monolayers, clearly indicating that the PL+choline monolayer is more packed at both temperatures (Figure 2b). A tighter packing of molecules contributes to the increased ordering of the acyl chains. This can be associated with the synergistic effect of Lifshitz-van der Waals (mainly dispersion) interactions that are larger for longer alkyl chains, C19–C21 (Table 2), fostering an easier ordering of molecules in the PL+choline monolayer under the compression. Such behavior is confirmed by the morphology images showing more homogeneous phase (in comparison to PL−choline, Figure 5), due to the increased packing density and miscibility of the compounds. Instead, in the PL−choline monolayers, the decisive factor for packing seems to be the presence of shorter C14–C18 unsaturated chains, which provokes a fluidizing disorder in the hydrophobic region, as well as higher CL percentage (21%). Furthermore, headgroup type and size are of great importance, as they determine the shape of the molecule. Small headgroup or large hydrophobic region result in a more conical shape [20], causing steric effects and affecting intermolecular interactions (Figure 6).

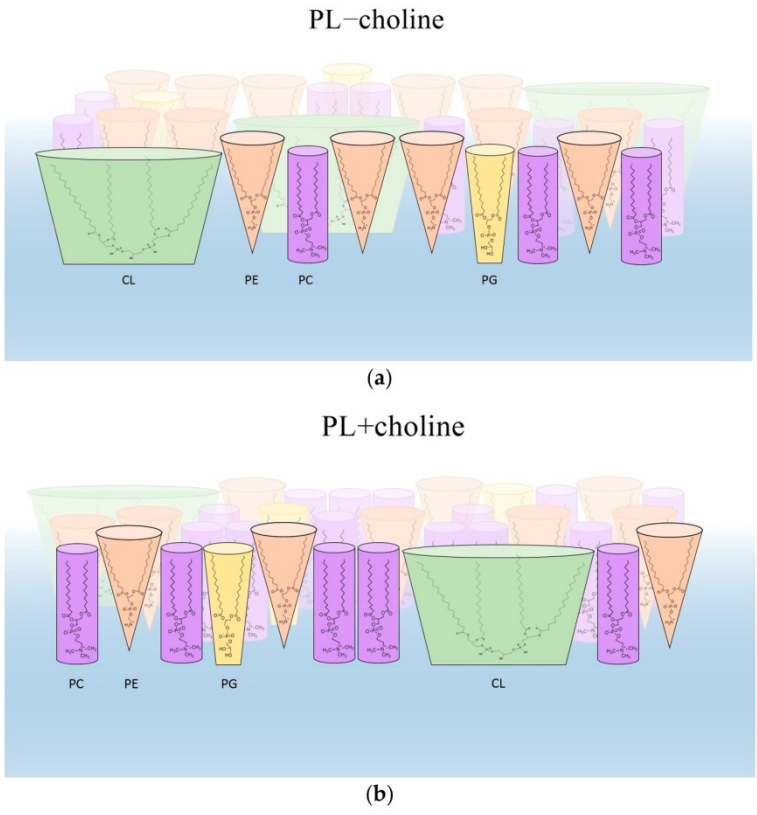

Figure 6. The effect of molecule shape on packing of the mixed monolayers, composed of phospholipids isolated from *L. gormanii* bacteria cultured without (**a**) and with (**b**) choline.

In PL+choline high percentage of PC, cylindrical-shaped phospholipid, results in a greater condensation of the molecules in the monolayer [21]. On the contrary, as mentioned, the PL−choline mixture contains more CL and PE (Table 2). Due to head-tail mismatch in these compounds, greater steric effects occur, and therefore a lower degree of packing is observed (Figure 6). The increased expansion in the headgroup region of this monolayer is revealed by the smaller values of C_S^{-1} with respect to PL+choline (Figure 2b). Another cause of the decreased elasticity can be the reorganization of the monolayer detected by the domains' formation.

Since PE and PC quantitatively constitute the largest fraction of the studied PL, it is important to analyze intermolecular interactions between them and changes in films caused by the altered amounts of these compounds in the mixed phospholipid monolayers. In the mixtures, the electroneutral PC and PE molecules separate the PG and CL molecules, thus reducing the repulsion between them [22–24]. The PC group is of particular importance in this study, as choline is added to the medium for the bacteria culture. The polar phosphocholine (PC) group consists of negatively ($-OPO_3^-$) and positively ($-N^+(CH_3)_3$) charged moieties separated by two $-CH_2-$ groups. Such a separation allows for the formation of two ionic forms. The first one involves the maximal distance of charges, whereas the other one involves the reduced distance due to the internal salt linkage between the ionic charges in the PC head [25]. In the pH range of 2–8, the PC-containing monolayers do not bear an electric charge, which means that the phosphate and trimethylammonium groups neutralize each other, and this neutralization depends on the intermolecular spacing in the monolayers [25]. The molecular simulation study of the PC/PE mixtures [26] showed that most of the PC headgroups point toward the aqueous phase, while the majority of the PE headgroups point toward the hydrophobic core. Thus, the PE headgroups are probably localized closer to the fatty acid chain region, resulting in the closer packing of PL molecules. Therefore, the PC groups more exposed to the aqueous phase are more flexible.

When the PL+choline molecules become closer to each other during the compression, the intermolecular spacing decreases. The simulations conducted by Leekumjorn and Sum showed that the PC group becomes more aligned with the normal membrane due to the close packing of the PL molecules (smaller area/headgroup) [26]. This contributes to the ionic repulsion between similar charges of the polar groups and/or H-bonds formation, weakening the internal salt linkage. In contrast to PC, the PE groups can form inter- and intramolecular hydrogen bonds where the amine group interacts favorably with the phosphate/carbonyl moieties or water. Presumably, the PL monolayer compression provokes a change in the orientation of the headgroup from horizontal to more vertical. This is particularly observed for the PL+choline monolayer where, in consequence, a smaller area per molecule than in the PL−choline monolayer was obtained (Figure 2a). The heads become more aligned with the normal to membrane due to the close packing of the PL molecules. In contrast, it is likely that the heads in PL−choline monolayers are more horizontally oriented, so the distance between the phosphate groups is greater. If the area per headgroup is larger than the minimum cross-sectional area of two hydrocarbon chains measured perpendicularly to the tail direction, the chains must be tilted to some extent to compensate for the head-tail mismatch and thus maximize the contact in order to form a stable monolayer at the liquid-air interface [27,28]. Probably, such an inclination takes place in PL−choline molecules which thereby occupy larger areas, and the corresponding $\pi - A$ isotherm is shifted to the right (Figure 2a). Furthermore, the ionic repulsions between the polar groups are reduced, whereas the intermolecular hydrogen bonds are weaker (or their total number decreases) and/or the internal salt linkage is strengthened.

A higher content of negatively charged molecules in the PL−choline film (24%) likely contributes to the limited miscibility of the components and greater expansion of the monolayer than that of PL+choline, where the PG and CL contents are much smaller (15%). This effect can be attributed specifically to the increased CL content in the PL−choline mixture as the PG content is the same in both cases. As already mentioned, cardiolipin has a specific, dimeric structure and the molecule is rather large in comparison to the other

analyzed phospholipids, which causes the sterical effects [29]. It has been noted that it also causes less condensation of the films [23], so it is justified to assume that in addition to the FA composition (the presence of short chains), it is partially responsible for smaller compressibility modulus values for PL−choline than for PL+choline (Figure 2b). Moreover, this weakens the interactions between the other components of the monolayer, such as PE and PG [29]. Beyond the CL percentage, the reorganization of the monolayer revealed in the formation of condensed domains and/or the dissolution of some molecules into the subphase may contribute to the increased expansion and the differences between the analyzed films.

It is worth mentioning that the domains occur naturally in the bacterial membranes to minimize the system energy [30]. A key role in their formation is ascribed to the phospholipid composition and stronger interactions between particular components, which allow for sequestering preferentially into distinct regions of the cell [31]. As a result, the domains enriched in some of the components are segregated from the rest of the membrane depleted in these phospholipids which, beyond composition, also differ in the degree of order. The appearance of such inhomogeneity is manifested in the BAM images (Figure 5). Thus, the presence of domains demonstrates the partial miscibility of the compounds in the monolayers and the formation of regions with greater or lesser condensation (phase coexistence) depending on the monolayer composition. In the case of monolayers of phospholipids with the same headgroups in the molecules, the presence of domains results from the variation in the length and proportion of the saturated and unsaturated FA chains, which also has an effect on the mixed monolayer structure. Contrary to the phospholipids with unsaturated fatty acids, the phospholipids with saturated FAs tend to be close to each other, forming more condensed microdomains of relatively high molecular ordering [32], which are visible as brighter regions in the BAM images. This corresponds well to the condensed areas present in the PL−choline films (Figure 5), in contrast to PL+choline. As mentioned above, the saturated and long chains form favorably more condensed domains due to stronger Lifshitz-van der Waals interactions between fatty acid chains [18]. Moreover, the important factor in the domain formation seems to be the presence of phospholipids with negative curvature tendency, such as PE and CL [30]. Due to the shape of the molecules (Figure 6), the compounds can be separated to the concave-shaped areas of the model membrane [30], which differentiates the surface morphology in these areas. As PL−choline contains 21% of CL and 50% of PE, it is justified to assume that large, condensed regions are a consequence of curvature properties of the mentioned phospholipid classes. Smaller contents of CL (9% less) and PE (12% less) in PL+choline, in comparison to PL−choline, contribute to the better miscibility (Figure 5) of the phospholipid molecules as well as a higher level of condensation (Figures 2b and 6). As the temperature increases, the breaking of Lifshitz-van der Waals forces between the hydrocarbon chains converts them into a much more fluid and disordered state with the increased cross-sectional area per lipid [33]. Moreover, the dipole-dipole interactions within the headgroup region can result in the weakening of in-plane polar attractions and contribute to lower homogeneity occurring at 37 °C (Figure 5).

Molecular reorganization and domain formation can be accompanied by the compensation of the dipole moments (monolayer depolarization) [17,34] as evidenced by the slope of $\Delta V - A$ isotherm changes (Figure 3). It is worth highlighting that the polarization of carbonyl groups $> C = O$ within the PL heads makes a positive contribution to the ΔV values. The other groups are of less importance because they are submerged in the aqueous solution, so they are highly shielded due to the high dielectric constant and the conductivity of the subphase. Thus, the $> C = O$ groups located in the hydrophobic chain region are the potential determining groups. Fluctuations in the surface potential at large areas or nearly zero surface pressure are likely due to the formation of small domains within the mostly gas phase monolayer (Figure 3). At very low surface pressures ($\pi \approx 0$ mN/m), the *plateau* in $\pi - A$ isotherm corresponds to the G-LE phase transition, where LE domains can be seen in the BAM images (not shown). By coalescing the domains to the expanded

films (as the transition from gas (G) to the liquid-expanded (LE) state occurs), ΔV increases (Figure 3) abruptly, which is associated with a change in the slope of molecules from the horizontal to more vertical position with respect to the subphase plane [35]. The monolayer begins to form a more organized structure due to a larger amount of molecules in the close proximity being able to interact with one another, which can be observed as the sharp ΔV increase. This potential rise corresponds to the so-called critical area, which indicates the point when the hydrogen bonds with the water molecules are broken and the monolayer becomes structured [19,36,37]. This entails changes in the orientation of the intrinsic molecular dipole of the molecules in the film and of the water molecules near the monolayer interface [17]. With the structuring of the monolayer at the critical area, water is probably removed from the headgroup/subphase interface since the H-bonded network is broken. Therefore, the dielectric constant of such an interface is decreased sharply. This causes the increase in the surface potential [36,37]. However, a change in the surface pressure is not observed at the critical area as the accuracy of the surface pressure measurements is not enough to detect such a change [36]. Moreover, the critical area is larger for the PL−choline monolayer as presumably its molecules are involved in the H-bonds with water to a greater extent than those of PL+choline. The reason for that can be the increased amount of PE in PL−choline (Table 2), which exhibits a particular ability to interact strongly through inter- and intramolecular hydrogen bonds with both the other phospholipids and water [27]. A similar interpretation of the critical area increase by involvement in the hydrogen bonding with water molecules was applied by Brekiesz et al. for perfluorodecyldecane and its derivatives [18].

As mentioned above, as molecules (FA chains and headgroups) change their orientation to a more vertical one, they force the variation in the > C=O groups' positioning, causing the surface potential increase (Figure 3). It is also important to mention that the surface potential values are higher at 37 °C compared to 20 °C. The reason for this phenomenon is the greater thermal motion of the molecules. As the temperature rises, phospholipid molecules have a larger kinetic energy, resulting in a lower ordering of molecules [38].

In summary, the PL+choline monolayer is found to be more densely packed and ordered owing to favorable interactions via the H-bonding and Lifshitz-van der Waals forces between the headgroups and long fatty acid chains C19–C21, respectively, as hydrocarbon chains are more perpendicular to the liquid-air interface. The intermolecular forces between the molecules in the PL−choline monolayers can be less attractive, due to large CL and short-chain FA contents, leading to lower miscibility and domain formation.

Analyzing the changes in monolayer behavior caused by external factors allows for a better understanding of the physiology of *L. gormanii* bacteria and therefore possibly leads to the development of new infection treatments in the future. The ordering and packing of monolayers, which are a model of bacterial membranes, can determine the way they interact with drugs and antimicrobial peptides, such as the LL-37 peptide [39,40].

5. Conclusions

The aim of this study was to determine how the ability of using extracellular choline for the PC synthesis in the Pcs pathway affects the physicochemical characteristics of model membranes composed of *L. gormanii* phospholipids by means of the Langmuir monolayer technique coupled with the surface potential sensor and Brewster angle microscope. The following conclusions can be drawn:

1. External conditions, such as temperature or presence of choline, have an impact on the bacterial membrane composition and in consequence, the physicochemical properties of the cell.
2. The obtained results exhibited differences in the content of fatty acids present in individual phospholipids extracted from bacteria cultured on a medium with and without choline. These differences made it possible to explain the changes in the degree of packing and ordering of monolayers.

3. The physical state of phospholipid monolayers can determine the interactions with external factors acting on bacteria. The denser packing and ordering of *L. gormanii* membranes composed of PLs extracted from bacteria grown on the medium with choline can suggest that such bacteria will be more resistant to the bactericidal action of agents inside the nutrient-rich but hostile environment of the host.
4. Domain formation of different condensation and composition can be of great importance for *L. gormanii* membrane functioning, including proper activity of proteins, and developing the interaction mechanisms with the host cell and antibacterial substances.

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PRAKTYKI ZAWODOWE:

2008 r.

Ogólnopedagogiczna praktyka asystencka w II Liceum Ogólnokształcącym w Chełmie

2010 r.

Praktyka specjalizacyjna w Ośrodku Diagnostyki i Zwalczania Zagrożeń Biologicznych Wojskowego Instytutu Higieny i Epidemiologii w Puławach

2011 r.

Praktyka pedagogiczna w XXIII Liceum Ogólnokształcącym w Lublinie

2011 r.

Praktyka pedagogiczna w Lubelskim Społecznym Gimnazjum im. Jana III Sobieskiego w Lublinie

2016 - 2020 r.

Praca na stanowisku nauczyciel biologii w gimnazjum i liceum w VII Liceum Ogólnokształcącym im. Marii Konopnickiej (do 19.06.2019 Zespół Szkół nr 11) w Lublinie

2020 – obecnie

Praca na stanowisku nauczyciel biologii, wychowawca w Szkole Podstawowej nr 27 im. Marii Montessori w Lublinie

PUBLIKACJE NAUKOWE:

1. **Chmiel, E**., Palusińska-Szysz, M., Zdybicka-Barabas, A., Cytryńska, M., Mak, P. (2014). The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. *Acta Biochim. Pol.*, 61(1); 123-127.
2. Palusińska-Szysz, M., Szuster-Ciesielska, A., Kania, M., Janczarek, M., **Chmiel, E**., Danikiewicz, W. (2014). *Legionella dumoffii* utilizes exogenous choline for phosphatidylcholine synthesis. *Int. J. Mol. Sci*., *15*(5), 8256-79; doi:10.3390/ijms15058256.
3. Palusińska-Szysz M., Zdybicka-Barabas A., Cytryńska M., Wdowiak-Wróbel S., **Chmiel E**., Gruszecki W. I.**,** Analysis of cell surface alterations in *Legionella pneumophila* cells treated with human apolipoprotein E. *Pathog Dis*. 73(2):1-8. doi: 10.1111/2049-632X.12214.
4. Kutkowska, J., Michalska-Szymaszek, M., Matuszewska, R., **Chmiel, E**., Urbanik-Sypniewska, T. (2015); Antygeny powierzchniowe i czynniki wirulencji *Escherichia coli* O157. *Postep. Mikrobiol.* 54(1): 53–64; http://www.pm.microbiology.pl
5. Kowalczyk, B., **Chmiel, E.,** Palusińska-Szysz, M. (2021). The role of lipids in *Legionella*-host interaction. *Int. J. Mol. Sci.*, 2;*22*(3), 1487. doi: 10.3390/ijms22031487.
6. **Chmiel, E.,** Galuska, C.E., Koper, P., Kowalczyk, B., Urbanik-Sypniewska, T., Palusińska-Szysz, M., Fuchs, B. (2022). Unusual lipid components of *Legionella gormanii* membranes. *Metabolites,* 6;12(5), 418. doi: 10.3390/metabo12050418.

7. Pastuszak, K., **Chmiel, E.**, Kowalczyk, B., Tarasiuk, J., Jurak, M., Palusińska-Szysz, M. (2023). Physicochemical characteristics of model membranes composed of *Legionella gormanii* lipids. *Membranes*, *13*(3), 356. doi. 10.3390/membranes13030356.

UDZIAŁ W KONFERENCJACH:

1. Palusińska-Szysz M., Janczarek M., Dadas A., **Wiśniewska E**., Urbanik-Sypniewska T. **(2010)**; Identyfikacja genów *pmtA* oraz *pcs* zaangażowanych w syntezę fosfatydylocholiny – ważnego składnika osłon komórkowych. III Kongres Genetyki, Lublin, (POSTER - str. 261)
2. Palusińska-Szysz M., Turska-Szewczuk A., **Wiśniewska E**., Gęca M., Urbanik-Sypniewska T. **(2011)**; Characteristic of polar membrane lipids of *Legionella dumoffii*. 54. Zjazd PTChem i SiTPChem, Lublin, (POSTER - str. 400)
3. Palusińska-Szysz M., Turska-Szewczuk A., **Wiśniewska E**., Urbanik-Sypniewska T. **(2011)**; Molecular characterystic of *Legionella micdadei* phospholipids. 54. Zjazd PTChem i SiTPChem, Lublin, (POSTER - str. 401)
4. Palusińska-Szysz M., Rogulska D., **Wiśniewska E**., Urbanik-Sypniewska T. **(2012)**; Wpływ temperatury na skład długołańcuchowych, wielonienasyconych kwasów tłuszczowych *Acanthamoeba castellanii.* XXVII Zjazd Polskiego Towarzystwa Mikrobiologów, Lublin, (POSTER - P-IV-134)
5. **Wiśniewska E**., Palusińska-Szysz M., Kania M., Danikiewicz W., Urbanik-Sypniewska T. **(2012)**; Profil fosfolipidowy *Legionella gormanii*. XXVII Zjazd Polskiego Towarzystwa Mikrobiologów, Lublin, (POSTER - P-IV-332)
6. Palusińska-Szysz M., Kania M., **Wiśniewska E**., Danikiewicz W., Urbanik-Sypniewska T. **(2012)**; *Legionella dumoffii* utilizes extracellular ethanolamine to synthesis of phosphatidylethanolamine and phosphatidylcholine. 1st Polish-German Biochemical Societies Joint Meeting, Poznań, (POSTER - str. 55)
7. **Chmiel E**. **(2012)**; Mikroskopia elektronowa i jej zastosowania w badaniach materiałów, Lublin, (UDZIAŁ BIERNY)
8. **Chmiel E**. **(2012)**; IV Konferencja Naukowo-Szkoleniowa „Antybiotyki 2012”, Lublin, (UDZIAŁ BIERNY)

9. **Chmiel E**. **(2013)**; Międzynarodowa Studencko-Doktorancka Konferencja Naukowa- "Trzy Wymiary Informacji", Lublin, (UDZIAŁ BIERNY)
10. Pac M., **Chmiel E**. **(2013)**; Udział powierzchniowych polisacharydów w nawiązywaniu efektywnej symbiozy. III Kopernikańskie Sympozjum Studentów Nauk Przyrodniczych, Toruń, (POSTER - str. 215-216)
11. **Chmiel E**. **(2013)**; Konferencja Naukowo-Szkoleniowa „Skuteczna terapia raka płuc największym wyzwaniem współczesnej onkologii", Lublin, (UDZIAŁ BIERNY)
12. **Chmiel E**., Fiołka M., Kutkowska J., Mazur L., Janczarek M., Marcyniuk N., Pac M., Palusińska-Szysz M., Zalewska J. **(2013)**; Ocena aktywności przeciwdrobnoustrojowej płynu celomatycznego z aksenicznej hodowli *Dendrobaena veneta*. X Międzynarodowe Seminarium Studenckich Kół Naukowych nt. "Środowisko – Zwierzę – Produkt", Lublin, (POSTER - str. 83-84, WYRÓŻNIENIE)
13. Kutkowska J., Janczarek M., Marek-Kozaczuk M., Wielbo J., **Chmiel E**., Urbanik-Sypniewska T. **(2013)**; Wzorce antygenowe oraz cechy fenotypowe szczepów izolowanych z brodawek *Pisum arvense* i *P. sativum*. 47 Ogólnopolska Konferencja Naukowa „Mikroorganizmy-roślina-środowisko w warunkach zmieniającego się klimatu", Puławy, (POSTER)
14. Palusińska-Szysz M., Szuster-Ciesielska A., Kania M., **Chmiel E**., Danikiewicz W., Gęca M. **(2013)**; The role of *Legionella micdadei* phosphatidylcholine in TNF-α induction. 3rd Workshop on Microbiology in Health and Environmental Protection – MICROBIOT 2013, Łódź, (Konferencja międzynarodowa) (POSTER - I-P 18)
15. **Chmiel E**., Palusińska-Szysz M., Zdybicka Barabas A., Cytryńska M., Pawlikowska-Pawlęga B., Urbanik-Sypniewska T. **(2013)**; *Galleria mellonella* hemolymph peptides – a new weapon against *Legionella* bacteria. 3rd Workshop on Microbiology in Health and Environmental Protection – MICROBIOT 2013, Łódź, (Konferencja międzynarodowa) (WYSTĄPIENIE USTNE oraz POSTER - III-O)
16. Kutkowska J., Janczarek M., Fiołka M., Rachwał K., **Chmiel E**., Marczak M, Urbanik-Sypniewska T. **(2014)**; The susceptibility of Gram-negative pathogenic and soil bacteria to the coelomic fluid of earthworms *Dendrobaena veneta*. XV Zjazd Polskiego Towarzystwa Immunologii Doświadczalnej i Klinicznej, Wrocław, (POSTER)

SEMINARIA DOKTORSKIE:

2011

- Systemy sekrecji bakterii *Legionella*

2013

- Charakterystyka profilu fosfolipidowego *Legionella gormanii*
- Cellular envelope phospholipids from *Legionella gormanii*. Prezentacja wyników dotychczasowych badań podczas seminarium doktorskiego zorganizowanego z okazji wygłoszenia cyklu wykładów Prof. dr rer. nat. Otto Holsta, które odbyły się w ramach programu Erasmus

2014

- Wpływ peptydów przeciwbakteryjnych zawartych w hemolimfie *Galleria mellonella* na przeżywalność *Legionella gormanii*

DYDAKTYKA:

Prowadzenie ćwiczeń z przedmiotu: „mikrobiologia" dla studentów:

- II roku biologii (semestr I 2011/2012), (semestr I 2012/2013)
- I roku ochrony środowiska (semestr I 2011/2012)
- I roku biotechnologii (semestr I 2013/2014)

UDZIAŁ W GRANTACH NAUKOWYCH:

1. Udział w realizacji projektu grantowego „Genetyczne i biochemiczne badanie szlaków biosyntezy fosfatydylocholiny" MNiSW N303 822640 **(2011-2014)**.
2. „Badanie wpływu apolipoforyny wyizolowanej z hemolimfy *Galleria mellonella* na *Legionella gormanii*" Grant indywidualny z dotacji celowej na finansowanie działalności polegającej na prowadzeniu badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich **(2013)**.
3. „Analiza profilu fosfolipidowego *Legionella gormanii* oraz rola lipidów błonowych we wrażliwości bakterii na peptydy antybakteryjne" Grant indywidualny z dotacji

celowej na finansowanie działalności polegającej na prowadzeniu badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich **(2014-2015)**.

PATENTY:

Sposób hodowli bakterii *Legionella lytica* na sztucznym podłożu – P.396985 z dnia 2011.11.15

NAGRODY I WYRÓŻNIENIA:

Uzyskanie na drugim roku studiów doktoranckich stypendium projakościowego dla najlepszych doktorantów

PRACE NA RZECZ UCZELNI I KÓŁ NAUKOWYCH:

2011-2013

- Członek Studenckiego Kola Naukowego Mikrobiologów UMCS "Bakcyl".

2012-obecnie

- Członek Studenckiego Kola Fotografii Przyrodniczej UMCS - Pełniona funkcja: przewodnicząca / vice-przewodnicząca/ członek

2013

- Organizator konkursu fotograficznego pt. „Oblicza przyrody”
- Pomoc w organizacji imprez promujących Wydział i Uczelnie: tj. X Lubelski Festiwal Nauki „Człowiek – Nauka – Pasja”, Lubelska edycja Nocy Biologa, Drzwi otwarte.

DODATKOWA DZIAŁALNOŚĆ:

2016 - obecnie

- Prowadzenie zajęć w ramach projektu unijnego "Otwarci na sukces"

- Współorganizator VII i VIII Wojewódzkiego Konkursu Artystycznego 7 TALENT
- Opiekun szkolnego kółka fotograficznego
- Współorganizator drzwi otwartych i targów edukacyjnych
- Organizator konkursów szkolnych
    - Konkurs zoologiczny „Bezkręgowce”, „Cykl życiowy motyla”
    - I, II i III edycja konkursu fotograficznego „Tropem natury”
    - Konkurs na plakat promujący Światowy Dzień Ziemi

13. Oświadczenia

Lublin dn. 03.04.2023 r.

mgr Elżbieta Chmiel
ul. Sławinkowska 6i/1
20-810 Lublin

Oświadczenie

Oświadczam, że w pracach:

1. Elżbieta Chmiel, Marta Palusinska-Szysz, Agnieszka Zdybicka-Barabas, Małgorzata Cytryńska and Paweł Mak. The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. Acta Biochim. Pol. 2014, 61 (1):123–127

mój udział polegał na: namnożeniu bakterii *L. gormanii* na podłożu BCYE oraz BCYE z dodatkiem egzogennej choliny, wykonaniu testów przeżywalności bakterii po zadziałaniu ekstraktu hemolimfy i apolipoforyny III, przygotowaniu preparatów do pomiarów w AFM zmian powierzchniowych i wykonaniu analiz, interpretacji i dyskusji wyników, przygotowaniu wykresów i współredagowaniu tekstu manuskryptu.

2. Kowalczyk Bożena, **Chmiel Elżbieta**, Palusińska-Szysz Marta. The Role of Lipids in *Legionella*-Host Interaction. Int. J. Mol. Sci. 2021, 2(3):1487. https://doi: 10.3390/ijms22031487

mój udział polegał na: zebraniu literatury, opisaniu części dotyczącej budowy i znaczenia lipidów *Legionella.*

3. Chmiel E, Galuska CE, Koper P, Kowalczyk B, Urbanik-Sypniewska T, Palusińska-Szysz M, Fuchs B. Unusual lipid components of *Legionella gormanii* membranes. Metabolites. 2022, 6;12(5):418. https://doi: 10.3390/metabo12050418

mój udział polegał na: namnożeniu *L. gormanii* na podłożu z dodatkiem i bez dodatku egzogennej choliny, rozdziale błon komórkowych na błonę wewnętrzną i zewnętrzną, oznaczeniu zawartości białka oraz aktywności enzymów we frakcjach błonowych, izolacji lipidów z błon. Izolacji fosfolipidów z całych komórek bakterii i ich rozdziale na poszczególne klasy metodą 2D TLC, uzyskaniu estrów metylowych kwasów tłuszczowych z

poszczególnych klas fosfolipidów, analizie składu kwasów tłuszczowych, analizie struktury lipidów, graficznym przedstawieniu wyników, współredagowaniu tekstu manuskryptu.

4. Katarzyna Pastuszak, **Elżbieta Chmiel**, Bożena Kowalczyk, Jacek Tarasiuk, Małgorzata Jurak, and Marta Palusinska-Szysz. Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids. Membranes 2023, 13, 356. https://doi.org/10.3390/membranes13030356

mój udział polegał na: namnożeniu bakterii *L. gormanii* w różnych warunkach hodowli, izolacji lipidów, rozdziale lipidów na poszczególne klasy, przygotowanie próbek estrów metylowych kwasów tłuszczowych, identyfikacji kwasów tłuszczowych, określeniu zawartości poszczególnych kwasów, analizie i dyskusji wyników, przygotowaniu tabel, współredagowaniu tekstu manuskryptu.

Elżbieta Chmiel

Lublin dn. 29.03.2023 r.

Dr hab. Marta Palusińska-Szysz
Katedra Genetyki i Mikrobiologii
Instytut Nauk Biologicznych
Wydział Biologii i Biotechnologii
Uniwersytet Marii Curie-Skłodowskiej
ul. Akademicka 19
20-033 Lublin

Oświadczenie

Oświadczam, że w pracach:

1. Elżbieta Chmiel, **Marta Palusinska-Szysz**, Agnieszka Zdybicka-Barabas, Małgorzata Cytryńska and Paweł Mak. The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. Acta Biochim. Pol. 2014, 61 (1):123–127

mój udział polegał na: opracowaniu koncepcji i planu badań, analizie wyników, przygotowaniu manuskryptu.

2. Kowalczyk Bożena, Chmiel Elżbieta, **Palusińska-Szysz Marta**. The Role of Lipids in *Legionella*-Host Interaction. Int. J. Mol. Sci. 2021, 2(3):1487. https://doi: 10.3390/ijms22031487

mój udział polegał na: współredagowaniu tekstu manuskryptu, pełnieniu funkcji autora do korespondencji.

3. Chmiel E, Galuska CE, Koper P, Kowalczyk B, Urbanik-Sypniewska T, **Palusińska-Szysz M**, Fuchs B. Unusual lipid components of *Legionella gormanii* membranes. Metabolites. 2022, 6;12(5):418. https://doi: 10.3390/metabo12050418

mój udział polegał na: opracowaniu koncepcji, planu badań, analizie wyników i dyskusji.

4. Katarzyna Pastuszak, Elżbieta Chmiel, Bożena Kowalczyk, Jacek Tarasiuk, Małgorzata Jurak, and **Marta Palusinska-Szysz**. Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids. Membranes 2023, 13, 356. https://doi.org/ 10.3390/membranes13030356

mój udział polegał na: opracowaniu koncepcji, analizie uzyskanych wyników, redagowaniu manuskryptu.

M. Palusińska-Szysz

mgr Katarzyna Pastuszak
Katedra Zjawisk Międzyfazowych
Instytut Nauk Chemicznych
Wydział Chemii
Uniwersytet Marii Curie-Skłodowskiej
Pl. Marii Curie-Skłodowskiej 3
20-031 Lublin

Lublin dn. 29.03.2023 r.

Oświadczenie

Oświadczam, że w pracy:

Katarzyna Pastuszak, Elżbieta Chmiel, Bożena Kowalczyk, Jacek Tarasiuk, Małgorzata Jurak, and Marta Palusinska-Szysz. Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids. Membranes 2023, 13, 356. https://doi.org/10.3390/membranes13030356

mój wkład polegał na wykonaniu analiz monowarstw za pomocą wanny Langmuira sprzężonej z miernikiem potencjału powierzchniowego i mikroskopem kąta Brewstera, opracowaniu danych eksperymentalnych, współredagowaniu tekstu manuskryptu, przygotowaniu rysunków, współudziale w przygotowaniu odpowiedzi na uwagi recenzentów oraz wniesieniu stosowanych poprawek do manuskryptu po recenzji.

Katarzyna Pastuszak

Lublin dn. 27.03.2023 r.

mgr Bożena Kowalczyk
Katedra Genetyki i Mikrobiologii
Instytut Nauk Biologicznych
Wydział Biologii i Biotechnologii
Uniwersytet Marii Curie-Skłodowskiej
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20-033 Lublin

Oświadczenie

Oświadczam, że w pracach:

Kowalczyk Bożena, Chmiel Elżbieta, Palusińska-Szysz Marta. The Role of Lipids in *Legionella*-Host Interaction. Int. J. Mol. Sci. 2021, 2(3):1487. https://doi: 10.3390/ijms22031487

mój udział polegał na zebraniu literatury i napisaniu części dotyczącej struktury i funkcji lipopolisacharydu *Legionella*.

Chmiel E, Galuska CE, Koper P, Kowalczyk B, Urbanik-Sypniewska T, Palusińska-Szysz M, Fuchs B. Unusual lipid components of *Legionella gormanii* membranes. Metabolites. 2022, 6;12(5):418. https://doi: 10.3390/metabo12050418

mój udział polegał na rozdziale fosfolipidów metodą chromatografii cienkowarstwowej.

Katarzyna Pastuszak, Elżbieta Chmiel, Bożena Kowalczyk, Jacek Tarasiuk, Małgorzata Jurak, and Marta Palusinska-Szysz. Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids. Membranes 2023, 13, 356. https://doi.org/ 10.3390/membranes13030356

mój udział polegał na oczyszczeniu fosfolipidów od składników białkowych oraz barwników.

Bożena Kowalczyk

Lublin, 24.03.2023

Prof. dr hab. Teresa Urbanik-Sypniewska
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Katedra Genetyki i Mikrobiologii
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OŚWIADCZENIE

Oświadczam, że w pracy:

Elżbieta Chmiel, Christina E. Galuska, Piotr Koper, Bożena Kowalczyk, Teresa Urbanik-Sypniewska, Marta Palusińska-Szysz, Beate Fuchs. Unusual Lipid Components of *Legionella gormanii* Membranes. Metebolites, 2022, 12(5):418, DOI:10.3390/metabo12050418

mój udział polegał na uczestniczeniu w przygotowaniu tekstu manuskryptu.

Teresa Urbanik-Sypniewska

Teresa Urbanik-Sypniewska

Lublin dn. 27.03.2023 r.

Prof. dr hab. Małgorzata Cytryńska,
Katedra Immunobiologii,
Wydział Biologii i Biotechnologii
ul. Akademicka 19
20-033 Lublin

Oświadczenie

Oświadczam, że w pracy:

Elżbieta Chmiel, Marta Palusinska-Szysz, Agnieszka Zdybicka-Barabas, Małgorzata Cytryńska and Paweł Mak. The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. Acta Biochim. Pol. 2014, 61 (1):123–127

mój udział polegał na planowaniu badań, analizie i dyskusji wyników oraz uczestniczeniu w opracowaniu ostatecznej wersji pracy.

Lublin dn. 27.03.2023 r.

dr hab. Agnieszka Zdybicka-Barabas,
Katedra Immunobiologii,
Wydział Biologii i Biotechnologii
ul. Akademicka 19
20-033 Lublin

Oświadczenie

Oświadczam, że w pracy:

Elżbieta Chmiel, Marta Palusinska-Szysz, Agnieszka Zdybicka-Barabas, Małgorzata Cytryńska and Paweł Mak. The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. Acta Biochim. Pol. 2014, 61 (1):123–127

mój udział polegał na planowaniu badań i interpretacji wyników, immunizacji gąsienic *Galleria mellonella*, pobieraniu hemolimfy, otrzymywaniu metanolowych ekstraktów z hemolimfy, analizie statystycznej wyników oraz wykonaniu dokumentacji graficznej.

Agnieszka Zdybicka-Barabas

Lublin dn. 29.03.2023 r.

mgr Jacek Tarasiuk
Katedra Genetyki i Mikrobiologii
Instytut Nauk Biologicznych
Wydział Biologii i Biotechnologii
Uniwersytet Marii Curie-Skłodowskiej
ul. Akademicka 19
20-033 Lublin

Oświadczenie

Oświadczam, że w pracy:

Katarzyna Pastuszak, Elżbieta Chmiel, Bożena Kowalczyk, **Jacek Tarasiuk**, Małgorzata Jurak, and Marta Palusinska-Szysz. Physicochemical Characteristics of Model Membranes Composed of *Legionella gormanii* Lipids. Membranes 2023, 13, 356. https://doi.org/10.3390/membranes13030356

mój udział polegał na: rozdziale lipidów *Legionella gormanii* na poszczególne frakcje fosfolipidowe.

Jacek Tarasiuk

Kraków dn. 29.03.2023 r.

Dr hab. Paweł Mak, prof. UJ
Zakład Biochemii Analitycznej,
Wydział Biochemii Biofizyki i Biotechnologii UJ
ul. Gronostajowa 7
30-387 Kraków

Oświadczenie

Oświadczam, że w pracy:

Elżbieta Chmiel, Marta Palusinska-Szysz, Agnieszka Zdybicka-Barabas, Małgorzata Cytryńska and Paweł Mak. The effect of *Galleria mellonella* hemolymph polypeptides on *Legionella gormanii*. Acta Biochim. Pol. 2014, 61 (1):123–127

mój udział polegał na: oczyszczeniu i identyfikacji apolipoforyny III wyizolowanej z hemolimfy *Galleria mellonella.*

Paweł Mak

Elektronicznie
podpisany przez
Paweł Mak
Data: 2023.03.29
13:29:14 +02'00'

Lublin dn. 27.03.2023 r.

dr Piotr Koper,
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Oświadczenie

Oświadczam, że w pracy:

Elżbieta Chmiel, Christina E. Galuska, **Piotr Koper**, Bożena Kowalczyk, Teresa Urbanik-Sypniewska, Marta Palusińska-Szysz, Beate Fuchs. Unusual Lipid Components of *Legionella gormanii* Membranes. (2022), Metabolites 12, 418. https://doi.org/10.3390/metabo12050418, mój udział polegał na przeprowadzeniu analiz bioinformatycznych i ich wizualizacji oraz uczestniczeniu w opracowaniu ostatecznej wersji pracy.