UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Paulina Czechowicz

„Wpływ peptydów przeciwdrobnoustrojowych na szczepy *Candida* spp.”

Rozprawa doktorska

Katedra i Zakład Mikrobiologii
Uniwersytet Medyczny we Wrocławiu

Promotor:
prof. dr hab. Grażyna Gościniak

Promotor pomocniczy:
dr n. med. Joanna Nowicka

Wrocław, 2023 r.

Pani profesor dr hab. Grażynie Gościniak
za pomoc merytoryczną, opiekę w toku prowadzenia prac, pozwolenie na wdrażanie nowych pomysłów i zawsze otwarte dla mnie drzwi gabinetu.

Doktor Joannie Nowickiej
za nieustające wsparcie w nawet najbardziej śmiałych pomysłach badawczych omawianych nawet o najdziwniejszych porach dnia i nocy oraz za nieocenioną pomoc w organizacji i wykonywaniu zadań badawczych.

Mojej rodzinie, a w szczególności zawsze wierzącej we mnie Mamie i Siostrze, bez których zafascynowanego spojrzenia i otuchy nie udałoby mi się ukończyć żadnych ze swoich planów naukowych.

Wszystkim pracownikom Katedry Mikrobiologii
ze szczególnym uwzględnieniem doktor Kasi Jermakow i doktor Ady Janczury, za nieskończone pokłady wiary w moje możliwości i pomoc w pokonywaniu meandrów pracy naukowej i dydaktycznej.

Moim przyjaciołom
zawsze dbającym o moje dobre samopoczucie i niekończące się pokłady energii.

SPIS TREŚCI

1. WYKAZ PUBLIKACJI NAUKOWYCH WCHODZĄCYCH W SKŁAD CYKLU

1) **Paulina Czechowicz,** Joanna Nowicka, Grażyna Gościniak
Virulence factors of *Candida* spp. and host immune response important in the pathogenesis of vulvovaginal candidiasis.
International Journal of Molecular Sciences. 2022; 23(11): 5895
DOI: 10.3390/ijms23115895
IF: 6.208
MEiN: 140.00

2) **Paulina Czechowicz,** Damian Neubauer, Joanna Nowicka, Wojciech Kamysz, Grażyna Gościniak
Antifungal activity of linear and disulfide-cyclized ultrashort cationic lipopeptides alone and in combination with fluconazole against vulvovaginal *Candida* spp.
Pharmaceutics. 2021; 13(10): 1589
DOI: 10.3390/pharmaceutics13101589
IF: 6.525
MEiN: 100.00

3) **Paulina Czechowicz,** Joanna Nowicka, Damian Neubauer, Grzegorz Chodaczek, Paweł Krzyżek, Grażyna Gościniak
Activity of novel ultrashort cyclic lipopeptides against biofilm of *Candida albicans* isolated from VVC in the *ex vivo* animal vaginal model and BioFlux biofilm model - a pilot study.
International Journal of Molecular Sciences. 2022; 23(22): 14453
DOI: 10.3390/ijms232214453
IF: 6.208
MEiN: 140.00

Sumaryczny impact factor (IF): 18.941
Sumaryczna liczba punktów MEiN: 380.00

2. WSTĘP

Kandydoza pochwy i sromu (ang. *Vulvovaginal candidiasis,* VVC) jest drugim co do częstości zakażeniem ginekologicznym [1,2]. Od wielu lat niezmiennie szacuje się, że na całym świecie minimum 75% kobiet w wieku rozrodczym przynajmniej raz w życiu doświadczy objawowego epizodu VVC, a co dziesiąta pacjentka cierpieć będzie z powodu 4 lub więcej nawrotów infekcji rocznie (ang. *Recurrent vulvovaginal candidiasis,* RVVC) [3,4]. Typowymi symptomami zakażenia są swędzenie okolic pochwy i sromu, ból, uczucie pieczenia oraz obecność upławów [5]. Z powodu tak dużej powszechności tego zakażenia oraz stosunkowo łagodnych objawów, kandydoza pochwy pozostaje infekcją zbyt często bagatelizowaną zarówno przez lekarzy, jak i same pacjentki. Niejednokrotnie leczenie VVC jest empiryczne i opiera się na wywiadzie klinicznym i badaniu przedmiotowym, bez izolacji czynnika etiologicznego oraz oznaczania jego lekowrażliwości [6,7]. Badania epidemiologiczne wskazują, że choć nadal głównym patogenem odpowiedzialnym za VVC pozostaje *Candida albicans*, obserwuje się znacząco rosnący odsetek zakażeń powodowanych przez grzyby z grupy NCAC (ang. *Non-Candida albicans Candida*), jak przykładowo *Candida glabrata, Candida parapsilosis* czy *Candida lusitaniae* [8,9]. Jest to o tyle istotne, że drobnoustroje NCAC mają odmienny profil lekowrażliwości od *C. albicans* i są najczęściej oporne na klasycznie stosowane w ginekologii leki przeciwgrzybicze [8,10]. Dodatkowe utrudnienie kliniczne stanowi fakt, że około 1/5 kobiet jest bezobjawowymi nosicielami grzybów z rodzaju *Candida* na powierzchni błony śluzowej pochwy, gdzie drobnoustroje te klasyfikuje się jako komensale, a nie patogeny [10,11]. Problem odróżniania *Candida* stanowiących florę kolonizującą pochwę od tych, odpowiedzialnych za rozwój zakażenia VVC w ostatnich latach cieszy się coraz większym zainteresowaniem badaczy. Głównym zagadnieniem pozostaje nadal nieznany szczegółowy patomechanizm powierzchniowych infekcji śluzówkowo-skórnych o etiologii *Candida*, w tym właśnie kandydozy pochwy [12,13]. Badania ostatnich dwóch dekad dostarczyły dowodów naukowych przemawiających za hipotezą uwzględniającą dwukierunkowe podłoże rozwoju VVC. Z jednej strony, za niewątpliwie kluczowe uznaje się zweryfikowanie czynników związanych z układem immunologicznym gospodarza – w tym przypadku komponentami miejscowej odpowiedzi odporności nieswoistej – odpowiedzialnymi za rozpoznawanie grzybów drożdżopodobnych i ich kategoryzowanie jako komensale/patogeny. Choć dotychczas wszystko wskazuje, że najistotniejszą rolę pełnią tutaj komórki epitelium pochwy (ang. *Vaginal epithelium cells,* VECs), mechanizmy zaangażowane w opisywane zjawisko również pozostają obecnie

nieznane [12,14–16]. Z drugiej jednak strony, nie bez znaczenia są także czynniki wirulencji samych grzybów z rodzaju *Candida* i ich działanie na powierzchni błony śluzowej. Podstawowe aspekty patogenności tych drobnoustrojów są stosunkowo dobrze poznane i szeroko opisywane w literaturze. Za najważniejsze czynniki zjadliwości uznaje się przede wszystkim białka istotne w adhezji blastospor do VECs (m. in. białka rodziny Als, Hwp1 czy Epa), enzymy hydrolityczne niezbędne przy zjawisku przełączania fenotypowego i formowania filamentów oraz inwazji tkanek (przykładowo Sap i japsyny), jak również zdolność do przeżywania w skrajnych warunkach mikrośrodowiska. Największą uwagę przyciąga jednak zjawisko formowania biofilmu – struktury, którą uznaje się za preferowaną formę funkcjonowania wielu drobnoustrojów, w tym *Candida* [7,10,17]. Również w przypadku VVC upatruje się ważnej roli biofilmu w patogenności i rozwoju objawowego zakażenia, ponieważ do jego utworzenia niezbędne są właściwie wszystkie wymieniane czynniki wirulencji *Candida*. Jego dojrzała struktura cechuje się heterogennością i wyspecjalizowaniem poszczególnych obszarów i elementów do pełnienia kluczowych dla przeżywania mikroorganizmów funkcji. Ponadto posiada ona wysoki stopień oporności na wpływ warunków środowiskowych i działanie środków przeciwdrobnoustrojowych oraz duże możliwości dalszej ekspansji na sąsiednie tkanki i narządy oraz ich inwazję i degradację [9,18,19]. Wszystkie wspomniane powyżej aspekty stanowią wyzwanie kliniczne i sprawiają, że poszukiwanie nowych opcji terapeutycznych użytecznych w przypadku VVC skupia się przede wszystkim na wielokierunkowym działaniu potencjalnych nowych antymikotyków, w tym możliwości skutecznego zwalczania biofilmu *Candida*.

Jedną z uznanych obiecujących klas stosunkowo nowych związków o działaniu przeciwbakteryjnym, przeciwgrzybiczym i przeciwbiofilmowym są peptydy przeciwdrobnoustrojowe (ang. *Antimicrobial peptides,* AMPs). W ostatnich dwudziestu latach zyskały one duże zainteresowanie, a niektóre z ich przedstawicieli są już na dalszych etapach badań klinicznych pod kątem terapii pewnych rodzajów zakażeń (np. Omiganan jako środek terapeutyczny skuteczny w trądziku różowatym, atopowym zapaleniu skóry czy śródbłonkowej neoplazji sromu) [3,20]. Jedną z grup AMPs intensywnie badanych w ostatnich latach są lipopeptydy, w tym ich podklasa - ultrakrótkie kationowe lipopeptydy (ang. *Ultrashort cationic lipopeptides,* USCLs). Są to krótkie peptydy składające się z maksymalnie siedmiu grup aminokwasowych, połączone z resztą kwasu tłuszczowego i posiadające wypadkowy ładunek dodatni. Dzięki takiej budowie mają one charakter amfipatyczny i są zdolne do skutecznego atakowania ujemnie naładowanych błon drobnoustrojów – w przypadku *Candida* reagują one z resztami kwasu sjalowego oraz fosfatydyloinozytolu obecnymi na powierzchni błon

komórkowych blastospor. Ich sposób działania opiera się przede wszystkim na permeabilizacji atakowanej dwuwarstwy błony, co końcowo prowadzi do śmierci komórki [20,21]. Wśród USCLs dotychczas najbardziej intensywnie przebadanym przykładem jest związek zbudowany z kwasu palmitynowego (o długości 16 atomów węgla, C_{16}) połączonego z czterema resztami L-lizyny (oznaczanej symbolem K) o uznanych właściwościach przeciwgrzybiczych – C_{16}-KKKK-NH_2 [22]. Ostatnie badania nad możliwościami poprawy działania przeciwdrobnoustrojowego lipopeptydów opierają się na testowaniu substytucji poszczególnych reszt L-lizyny na przykład resztami L-argininy (oznaczanej symbolem R) oraz cyklizacją USCLs za pomocą mostków disiarczkowych (oznaczanych jako C z uwagi na dołączenie reszt cysteiny). Ma to na celu przede wszystkim poprawę aktywności oraz zwiększenie selektywności tych związków wobec mikroorganizmów. Wśród szeregu analogów modyfikowanych w opisywany sposób za najbardziej obiecujące pod kątem działania wobec *Candida* w formie planktonowej i biofilmowej uznano ostatnio pary USCLs o strukturze liniowej i ich cykliczne odpowiedniki w postaci związków: C_{16}-KKKK-NH_2 i C_{16}-CKKKKC-NH_2 oraz C_{16}-KRKK-NH_2 i C_{16}-CKRKKC-NH_2 [21].

Z uwagi na nie do końca poznany patomechanizm VVC oraz najbardziej prawdopodobne wielokierunkowe działanie *Candida* na powierzchni epitelium pochwy, za zasadne uznaje się poszukiwanie także możliwości terapii skojarzonej w przypadku kandydozy pochwy [23]. Jednoczesne stosowanie dwóch lub więcej środków przeciwdrobnoustrojowych jest obecnie często rekomendowane, a działanie takie przejawia wiele zalet [23-25]. Przede wszystkim dzięki użyciu kombinacji różnych związków uzyskuje się poszerzenie spektrum działania i wzmocnienie efektu bójczego poprzez dobieranie w pary środków o różnych mechanizmach działania wobec mikroorganizmów. Skutkuje to także znaczącym zmniejszeniem ryzyka nabywania oporności przez zwalczane drobnoustroje w toku prowadzenia leczenia. Wiele badań nad terapią skojarzoną wskazuje także na możliwości zastosowania łączonych ze sobą związków w stężeniach dużo niższych niż w przypadku ich stosowania osobno – co może potencjalnie rozwiązywać problem toksyczności analizowanych substancji wobec komórek gospodarza. Dodatkowo, w przypadku poszukiwania nowych środków przeciwdrobnoustrojowych warto analizować możliwość ich kombinacji z lekami znanymi i stosowanymi już dotychczas w zwalczaniu danych infekcji [23–25]. Dzięki temu w sposób znaczący poprawiane może być bezpieczeństwo takiej terapii, jak również chociażby koszt jej prowadzenia. W przypadku kandydoz, głównie typu układowego, istnieją już doniesienia o pozytywnych efektach jednoczesnego stosowania nowych AMPs z konwencjonalnymi lekami przeciwgrzybiczymi, np. flukonazolem [24,25]. Choć dotychczas nie przeprowadzono

analogicznych badań w przypadku związków USCLs oraz kandydozy pochwy, istnieją przesłanki, że flukonazol o działaniu mykostatycznym oddziaływujący z demetylazą 14α-lanosterolu *Candida* mógłby wzmagać działanie permeabilizujące takich związków jak lipopeptydy [26]. Z drugiej strony, USCLs są potencjalnie zdolne do pewnego rodzaju uwrażliwiania komórek grzybiczych na działanie wymienianego azolu dzięki dysrupcji błony komórkowej [20,21]. Flukonazol jest także środkiem o stosunkowo niskiej toksyczności wobec komórek eukariotycznych i jest klasyfikowany jako jedna z bezpieczniejszych opcji terapeutycznych, obarczona niewielką liczbą potencjalnych działań ubocznych. Jest to szczególnie istotne w sytuacji, gdy bójcze działanie USCLs także wobec komórek gospodarza jest często podkreślane i stanowi kluczowy problem w badaniach nad potencjałem przeciwdrobnoustrojowym tych związków [21]. Tak więc badanie konkretnych ultrakrótkich kationowych lipopeptydów jako alternatywnych środków skutecznych wobec grzybów izolowanych w VVC powinno być także poszerzone o analizowanie możliwości ich zastosowania w połączeniu właśnie z przedstawicielem triazoli, flukonazolem.

Na temat sposobów oznaczania lekowrażliwości różnych drobnoustrojów obecnie istnieją niemal w każdym przypadku szczegółowe opracowania wraz z uznanymi rekomendacjami postępowania w praktyce laboratoryjnej. Standardy europejskie opierają się na dokumentach prezentowanych i uaktualnianych rokrocznie przez EUCAST (ang. *The European Committee on Antimicrobial Susceptibility Testing*). Amerykańskim odpowiednikiem tego towarzystwa również wydającym swoje zalecenia jest CLSI (ang. *The Clinical and Laboratory Standards Insititute*) [27,28]. W przypadku badania grzybów drożdżopodobnych z rodzaju *Candida* istnieją pewne niuanse i trudności, których rozwiązywanie bywa w odmienny sposób rekomendowane przez oba wymienione stowarzyszenia. Sprawia to, że niejednokrotnie użytkownicy stoją przed koniecznością wyboru metody postępowania i liczenia się z możliwością zaistnienia problemów z interpretacją uzyskiwanych rezultatów i trudnościami w porównywaniu wyników uzyskiwanych w różnych laboratoriach i przez różnych badaczy [29,30]. Dodatkowo, wszystkie wspomniane rekomendacje oznaczania lekowrażliwości dotyczą drobnoustrojów występujących w formie planktonowej i opierają się na wyznaczaniu wartości MIC (ang. *Minimum inhibitory concentration*). Choć istnieją metody najczęściej używane i uznawane przez grupy naukowców za pewnego rodzaju standardy postępowania, w przypadku badania lekowrażliwości biofilmów (zwłaszcza grzybiczych) nie opracowano dotychczas ujednoliconych rekomendacji wyznaczania stężeń hamujących i eradykujących tę strukturę (ang. *Minimum biofilm eradication concentration*, MBEC). Opisywane aspekty sprawiają, że w przypadku badania wpływu przeciwgrzybiczego i przeciwbiofilmowego

nowych związków oraz ich kombinacji istnieje konieczność opracowywania metod badawczych umożliwiających uzyskiwanie wiarygodnych, jak najlepiej odzwierciedlających procesy zachodzące w miejscu zakażenia rezultatów. W takiej sytuacji za najbardziej zasadne wydaje się postępowanie badawcze polegające na analizowaniu działania różnych substancji wobec *Candida* w formie planktonowej oraz biofilmu różnymi metodami i porównywaniu uzyskiwanych wyników. Dodatkowe uwiarygodnienie rezultatów będą także stanowiły próby ich weryfikacji w modelach potencjalnie odzwierciedlających zachowanie się drobnoustrojów na powierzchni błony śluzowej pochwy w toku rozwoju VVC – bardziej zaawansowane modele do badania biofilmów czy wykorzystanie tkanek zwierzęcych i/lub linii komórkowych [31,32]. Eksperymenty takie mogłyby również potencjalnie dostarczyć dodatkowych informacji na temat procesów zachodzących w mikrośrodowisku pochwy podczas inwazji grzybów drożdżopodobnych oraz zidentyfikować mechanizmy odpowiedzialne za obserwowane niepowodzenia terapeutyczne i nawroty VVC.

3. CEL I ZAŁOŻENIA PRACY

Głównym celem pracy było określenie przeciwdrobnoustrojowego i przeciwbiofilmowego wpływu czterech przedstawicieli nowo syntezowanych ultrakrótkich kationowych lipopeptydów wobec grzybów drożdżopodobnych z rodzaju *Candida* izolowanych z kandydozy pochwy i sromu. W toku pracy przeprowadzono także badania dotyczące możliwości zastosowania badanych związków w terapii skojarzonej z konwencjonalnie stosowanym w kandydozie pochwy flukonazolem. Ponadto dokonano weryfikacji rezultatów uzyskanych klasycznymi metodami laboratoryjnymi *in vitro* za pomocą bardziej zaawansowanych metod i modeli badania biofilmu mikroorganizmów.

Cele te zostały zrealizowane poprzez:

1) Wyznaczenie minimalnych stężeń hamujących (ang. *Minimum inhibitory concentration*, MIC) czterech ultrakrótkich kationowych lipopeptydów o najbardziej obiecujących właściwościach przeciwgrzybiczych oraz flukonazolu wobec różnych gatunków *Candida* izolowanych z zakażeń pochwy,
2) Wyznaczenie minimalnych stężeń eradykujących biofilm (ang. *Minimum biofilm eradication concentration*, MBEC) wszystkich badanych substancji wobec dojrzałej struktury utworzonej przez izolowane szczepy *Candida* spp. *in vitro* na płytkach polistyrenowych,
3) Ocenę działania synergistycznego *in vitro* lipopeptydów w skojarzeniu z flukonazolem wobec *Candida*,
4) Opracowanie i zastosowanie modelu tkankowego *ex vivo* wykorzystującego fragmenty epitelium pochwy mysiej do weryfikacji skuteczności działania badanych substancji w stężeniach MBEC wyznaczonych *in vitro* oraz w skojarzeniu z flukonazolem wobec biofilmu wybranych szczepów *Candida*,
5) Wykorzystanie technologii BioFlux umożliwiającej badanie biofilmu *Candida* w warunkach mikroprzepływowych do określenia wpływu analizowanych lipopeptydów w stężeniach MBEC wyznaczonych klasycznymi metodami.

4. MATERIAŁY I METODY

4.1. Szczepy *Candida* spp.

Badania przeprowadzono na 62 szczepach klinicznych *Candida* izolowanych z kandydozy pochwy i zdeponowanych w Kolekcji Katedry i Zakładu Mikrobiologii Uniwersytetu Medycznego we Wrocławiu. Do eksperymentów dołączono także dwa szczepy referencyjne: *Candida albicans* ATCC 90028 oraz *Candida glabrata* ATCC 15126 (uzyskane z PAN, Wrocław). Większość drobnoustrojów została zidentyfikowana jako *C. albicans* (52), a pozostałe 10 szczepów należało do grupy NCAC: *C. glabrata* (5), *C. lusitaniae* (2), *C. kefyr* (2) oraz *C. parapsilosis* (1). Na przeprowadzenie opisywanych badań w toku przewodu doktorskiego uzyskano zgodę Komisji Bioetycznej Nr 774/2018, z dnia 27 grudnia 2018.

4.2. Wyznaczanie wartości MIC

Minimalne stężenia hamujące badanych lipopeptydów: C_{16}-KKKK-NH_2, C_{16}-CKKKKC-NH_2 oraz C_{16}-KRKK-NH_2 i C_{16}-CKRKKC-NH_2, jak również flukonazolu zostały wyznaczone metodą mikrorozcieńczeń zgodnie z rekomendacjami CLSI [33]. Do tego celu wykorzystano 96-dołkowe płytki polistyrenowe oraz podłoże płynne RPMI 1640. Za wartości odpowiadające MIC dla USCLs uznawano stężenia badanych substancji, przy których nie obserwowano wzrostu drobnoustrojów [21]. Dla mykostatycznego flukonazolu MIC stanowiło stężenie hamujące przynajmniej 50% wzrostu grzybów *Candida*, co wyliczano na podstawie wartości zmierzonych spektrofotometrycznie przy długości fali 530 nm w aparacie BiochromAsys UVM 340 Microplate Spectrophotometer (Biochrom Ltd., Holliston, USA). Wszystkie eksperymenty wykonywane były w trzykrotnym powtórzeniu.

4.3. Wyznaczanie wartości MBEC

Do wyznaczania wartości minimalnych stężeń eradykujących biofilm wykorzystano metodę z użyciem soli tetrazoliowej MTT jako wskaźnika (3-(4,5-dimetylo-2-tiazolylo)-2,5-difenylo-2H-tetrazoliowy bromek) [34]. Dojrzałą strukturę biofilmu *Candida* utworzoną w ciągu 24 godzin na 96-dołkowych płytkach polistyrenowych w podłożu RPMI 1640 traktowano badanymi lipopeptydami oraz flukonazolem przez kolejną dobę. Po tym czasie dodawano odpowiednio przygotowanego roztworu MTT i inkubowano 3 godziny w 37°C bez dostępu światła. Żółty roztwór MTT przez żywe komórki biofilmu rozkładany jest do fioletowo-niebieskich związków formazanu. Za stężenia odpowiadające MBEC uznawano

najniższe wartości, przy których nie obserwowano zmiany zabarwienia, świadczącej o braku obecności aktywnych metabolicznie komórek grzybiczych. Wszystkie eksperymenty zostały wykonane w trzykrotnym powtórzeniu.

4.4. Ocena działania synergistycznego badanych związków

Do oceny wzajemnych interakcji pomiędzy badanymi lipopeptydami a flukonazolem wykorzystano metodę szachownicy [35]. W 96-dołkowych płytkach polistyrenowych przygotowano szeregi rozcieńczeń USCLs oraz azolu w taki sposób, aby w każdym dołku znajdowała się inna kombinacja stężeń badanych substancji. Następnie dodawano zawiesiny szczepów *Candida* oraz inkubowano 24 godziny w 37°C. Przygotowanie szczepów, warunki inkubacji oraz odczytywanie uzyskanych wyników przeprowadzono analogicznie jak w metodzie wyznaczania MIC. Następnie obliczano współczynnik FIC index (ang. *Fractional inhibitory concentration index*, FICi), stosując standardowo wykorzystywany wzór [35]. Wyniki interpretowano zgodnie z rekomendacjami EUCAST: za interakcje synergistyczne (SYN) uznawano wartości FICi ≤0.5, za addytywne (ADD) wartości zawierające się pomiędzy 0.5 – 1.0, indyferentne (IND) – między 1.0 a 2.0, a wszystkie wartości powyżej 2.0 uznawano za świadczące o antagonizmie (ANT) [36].

4.5. Model tkankowy *ex vivo*

Do badań wykorzystano fragmenty tkanek nabłonka pochwy mysiej pozyskane od samic myszy C57BL/6 w wieku 8–12 tygodni (Jackson Laboratory, Bar Harbor, ME, USA), opracowywanych w Polski Ośrodek Rozwoju Technologii, PORT Wrocław (Sieć Badawcza Łukasiewicz). Przygotowywanie tkanek opierało się o założenia pracy Harriott *et al.* z 2010 r. [18]. Gotowe fragmenty epitelium umieszczano stroną błonową ku górze w 6-dołkowych płytkach polistyrenowych i zanurzano w 500 µl roztworu PBS z antybiotykami (penicylina (100 U/mL) i streptomycyna (100 µg/mL), AppliChem GmBH, Darmstadt, Niemcy). Następnie dodawano wybrane szczepy *C. albicans* przygotowane analogicznie, jak w metodzie wyznaczania MBEC i pozostawiano do inkubacji (24h, 37°C, CO_2) w celu utworzenia biofilmu na powierzchni błony śluzowej. Dojrzały biofilm płukano oraz dodawano badane związki w stężeniach odpowiadających MBEC oraz dodatkowo w stężeniach równych ½ MBEC i FIC (dla kombinacji lipopeptyd-flukonazol), ponownie pozostawiając próbki na 24 godziny w 37°C i w obecności CO_2. Kontrolę eradykacji biofilmu stanowiły wysokie stężenia amfoterycyny B (AMB, 50 µg/mL). Wszystkie próby wykonywano podwójnie. Wizualizacji otrzymanych

rezultatów eradykacji dokonywano na dwa sposoby. Połowę tkanek poddawano homogenizacji, a następnie wysiewano w sposób ilościowy na podłoże stałe Sabouraud Dextrose Agar z chloramfenikolem (100 mg/L), inkubując następnie 24 godziny w 37°C. Po tym czasie wyrosłe kolonie zliczano i dokonywano obliczeń wartości CFU/mL oraz CFU/g tkanki (ang. *Colony forming unit*, jednostki tworzące kolonię). Drugą część tkanek wybarwiano barwnikiem Calcofluor White (1 mg/mL, Fluka) przez 20 minut w temperaturze pokojowej, a następnie umieszczano na szkiełkach mikroskopowych i oglądano w mikroskopie konfokalnym (Leica SP8, Leica Microsystems, Wetzlar, Niemcy) w obiektywie 10 ×. Barwnik ten przy długości fali 405 nm pozwala na obserwowanie blastospor oraz strzępek *Candida* na tle wybarwionej za pomocą składnika Evans blue tkanki epitelium (długość fali 638 nm). Obrazowano całość powierzchni badanej tkanki pochwy w celu zminimalizowania ryzyka subiektywnej oceny poszczególnych jej fragmentów w mikroskopie. Całość doświadczenia wykonana została w czterech powtórzeniach.

4.6. Model biofilmu w warunkach przepływowych z wykorzystaniem BioFlux

Dzięki wykorzystaniu aparatury BioFlux 1000Z (Fluxion Biosciences, San Francisco, CA, USA) połączonej z odwróconym mikroskopem fluorescencyjnym (GmbH, Jena, Niemcy) wygenerowano warunki mikroprzepływowe do badania formowania się biofilmu *Candida* oraz stopnia jego eradykacji [32]. W specjalnie przeznaczonych do badań z użyciem BioFlux 48-dołkowych płytkach do mikroprzepływu przygotowano zawiesiny wybranych szczepów *C. albicans* w RPMI1640 sposób analogiczny, jak w badaniu MBEC. W celu umożliwienia adhezji grzybów drożdżopodobnych do powierzchni mikrokanałów BioFlux ustalono przepływ zawiesiny o dużej szybkości przez 5 sekund (jednostka 5 dyne/cm^2), a następnie pozostawiono na 1 godzinę. Po tym czasie dodano podłoża RPMI 1640 i ustalono stałe warunki przepływu przez badane kanały na poziomie 0.5 dyne/cm^2 przez 24 godziny. Tak przygotowany dojrzały biofilm *Candida* w mikrokanałach traktowano następnie dwoma USCLs (wykazującymi najkorzystniejszy efekt eradykujący w poprzednich etapach badań) w stężeniach równych MBEC, również przez dobę przy ustalonej prędkości przepływu 0.5 dyne/cm^2. W ciągu całego eksperymentu (48 godzin) wykonywane były zdjęcia mikroskopowe w odstępach jednogodzinnych celem obserwowania zmian zachowania się komórek grzybiczych w czasie rzeczywistym. Następnie dokonywano analizy uzyskanych rezultatów dzięki oprogramowaniu BioFlux Montage software, wykorzystując wykonane serie zdjęć i obliczając stopień przyrostu oraz eradykacji biomasy biofilmu.

4.7. Analiza statystyczna

Dane uzyskane w toku eksperymentów z zastosowaniem modelu zwierzęcego *ex vivo* oraz modelu biofilmu w warunkach przepływowych w BioFlux poddano analizie statystycznej z użyciem GraphPad Prism version 9 (GraphPad Co., San Diego, CA, USA). Do potwierdzenia normalności rozkładu uzyskanych danych zastosowano test Shapiro-Wilka, a następnie analizy dokonywano za pomocą testu ANOVA. Istotność statystyczną uznawano przy $p < 0.05$.

5. PODSUMOWANIE WYNIKÓW

5.1. Artykuł nr 1: Virulence factors of *Candida* spp. and host immune response important in the pathogenesis of vulvovaginal candidiasis

Publikacja stanowi przegląd literatury i obecnego stanu wiedzy na temat grzybów drożdżopodobnych z rodzaju *Candida* spp. w kontekście czynników zależnych od mikroorganizmów oraz zależnych od układu immunologicznego gospodarza, o potencjalnym znaczeniu w rozwoju objawowej kandydozy pochwy i sromu. Podsumowano informacje na temat znanych aspektów patogenności omawianych grzybów w postaci m. in. adhezji i czynników regulujących ten proces, enzymów hydrolitycznych, sposobów adaptacji do warunków mikrośrodowiska, w tym istnienia dimorfizmu i zjawiska przełączania fenotypowego oraz tworzenia struktury biofilmu. Opisano także istniejące doniesienia na temat badań nad regulacją lokalnej odpowiedzi immunologicznej na błonie śluzowej pochwy i procesów zaangażowanych w rozpoznawanie i zwalczanie mikroorganizmów w postaci *Candida* podczas rozwoju infekcji.

Przedstawiono uznawaną obecnie za najbardziej prawdopodobną trzyczęściową hipotezę na temat procesów potencjalnie inicjujących zakażenie w postaci VVC. Postuluje się, że (z powodów obecnie niewyjaśnionych) populację kobiet można podzielić na „niewrażliwe" i „wrażliwe" na kandydozę pochwy. W pierwszym przypadku receptory (ang. *Pattern recognition receptors,* PRRs) znajdujące się na powierzchni komórek VECs rozpoznają komponenty blastospor (głównie Pra1p) i klasyfikują *Candida* jako organizmy komensalne, nie wzbudzając żadnych dalszych procesów immunologicznych. U kobiet wrażliwych na zakażenie, ten sam proces zależny od PRRs i białka Pra1p skutkuje zainicjowaniem procesów zapalnych wraz z uwolnieniem prozapalnych cytokin, chemokin i alarmin. W dalszej kolejności dochodzi do infiltracji VECs przez polimorfonukleary (ang. *Polimorphonuclear cells*, PMNs), co w bezpośrednim przełożeniu związane jest z wystąpieniem standardowych objawów klinicznych VVC. PMNs jednak nie są zdolne do skutecznego zabijania komórek *Candida* ani nie powodują zniszczenia tkanki pochwy. W obu przypadkach szczegóły opisywanych zjawisk pozostają niewyjaśnione. Trzecia część omawianej hipotezy dotyczy przyczyn leżących u podstaw procesów uszkodzenia i/lub zniszczenia błony śluzowej w toku kandydozy pochwy. Dzięki przełączaniu fenotypowemu i formowaniu filamentów przez *Candida* zachodzi możliwość produkowania i uwalniania przez komórki strzępek stosunkowo nowo odkrytego związku o nazwie candidalizyna (ang. *candidalysin*). Udowodniono, że białko

to jest zdolne do aktywacji tzw. inflamasomu NLRP3, co skutkuje jego oligomeryzacją. Proces ten również inicjuje miejscową odpowiedź zapalną z wytworzeniem interleukiny 1β i alarminy S100A8, a następnie dalszej kaskady cytokin prozapalnych, chemokin i alarmin – jednak z pominięciem roli PRRs na powierzchni VECs. Również w tym przypadku dochodzi do infiltracji błony śluzowej przez PMNs i etap ten związany jest z wystąpieniem objawów klinicznych, podobnie jak w opisywanym wyżej przypadku kobiet wrażliwych na zakażenie. Jednak w omawianym wariancie dochodzi następnie do śmierci komórek PMNs i uwolnienia zawartości ich ziarnistości i toksycznego działania wobec VECs. Dodatkowo okazało się, że candidalizyna jest także czynnikiem bezpośrednio działającym niszcząco na komórki epitelialne pochwy. W toku dalszych badań wykazano również, dlaczego najprawdopodobniej dochodzi do dalszej ekspansji grzybów i dlaczego układ immunologiczny nie reaguje w sposób prawidłowy na istniejące lokalnie uszkodzenie komórek błony śluzowej. Candidalizyna jest także jednym z wielu czynników, od których zależy penetracja strzępek w głąb atakowanej tkanki, co także działa toksycznie wobec VECs. Zaobserwowano, że w takiej sytuacji dochodzi do uwolnienia m. in. związku siarczanu heparanu (oznaczanego jako HS). HS jest inhibitorem reakcji rozpoznawania wspominanego już Pra1p (znajdującego się zarówno na blastosporach, jak i strzępkach *Candida*) przez immunologiczny kompleks MAC1 na powierzchni PMNs – powoduje to tzw. anergię neutrofilów, czyli ich niezdolność do prawidłowej reakcji i w efekcie finalnie hamuje to dalszą odpowiedź immunologiczną. Z jednej więc strony objawy kliniczne VVC związane są bezpośrednio z istniejącą reakcją odpornościową zachodzącą na błonie śluzowej pochwy w odpowiedzi na obecność *Candida*. Z drugiej zaś, obserwowana odpowiedź immunologiczna jest niewystarczająca i hamowana przez grzyby drożdżopodobne, skutecznie uniemożliwiając komórkom i tkankom gospodarza zwalczanie tego drobnoustroju i zakażenie postępuje.

Wiadomo także, że przedstawiane aspekty rozwoju VVC nie są jedynymi, które z pewnością zachodzą na powierzchni błony śluzowej pochwy podczas infekcji grzybiczej. Więcej nadal pozostaje także do wyjaśnienia w kontekście obserwowanych procesów i przyczyn ich występowania.

5.2. Artykuł nr 2: Antifungal activity of linear and disulfide-cyclized ultrashort cationic lipopeptides alone and in combination with fluconazole against vulvovaginal *Candida* spp.

Celem publikacji było określenie wpływu czterech USCLs o najkorzystniejszych właściwościach przeciwgrzybiczych (określonych w badaniach wstępnych na szczepach referencyjnych) wobec izolowanych z VVC różnych gatunków *Candida* w formie planktonowej i biofilmu. Dodatkowo zbadano lekowrażliwość tych szczepów na flukonazol i określono wzajemne interakcje tego azolu z testowanymi lipopeptydami.

Wszystkie badanie izolaty *C. albicans* (52), *C. kefyr* (2), *C. parapsilosis* (1) oraz jeden szczep *C. lusitaniae* okazały się wrażliwe na flukonazol (MIC<4 μg/mL). Zgodnie ze stanem wiedzy na temat wrażliwości gatunków NCAC, dla każdego z pięciu szczepów *C. glabrata* wartość MIC wynosiła 4 μg/mL. Jedynym drobnoustrojem wysoce opornym na badany azol okazał się drugi izolat gatunku *C. lusitaniae* (MIC 64 μg/mL). W przypadku USCLs zakres uzyskiwanych stężeń hamujących był nieco szerszy. Dla obu par analogów liniowy-cykliczny (C_{16}-KKKK-NH_2 (L1) i C_{16}-CKKKKCNH_2 (C1) oraz C_{16}-KRKK-NH_2 (L2) i C_{16}-CKRKKC-NH_2 (C2)) znacząco niższe wartości obserwowano w przypadku związków cyklicznych. Dla C1 i C2 średnie geometryczne MIC były zbliżone i wynosiły odpowiednio 4.89 i 3.83 μg/mL. W przypadku lipopeptydów liniowych L1 i L2 wartości te były wyższe i bardziej zróżnicowane – 26.46 oraz 15.47 μg/mL. Uzyskane wyniki są zgodne z wstępnymi obserwacjami. Cyklizacja okazywała się wzmagać działanie bójcze wobec *Candida* spp. w sposób dotychczas niewyjaśniony. Podobnie, wskazywano już wcześniej na możliwość poprawy aktywności przeciwgrzybiczej USCLs za pomocą substytucji L-lizyny resztą L-argininy – choć także w tym przypadku brakuje udokumentowanego wyjaśnienia tych obserwacji. Uzyskane średnie geometryczne posłużyły także do wyliczenia współczynników selektywności (ang. *Selectivity indices*, SIs) badanych substancji wobec komórek *Candida* i eukariotycznych. Wysokie wartości dla analogów cyklicznych (5.50 dla C1 i 8.83 dla C2) wskazują, że niestety wraz ze zwiększeniem aktywności tych związków wobec grzybów, narasta także ich toksyczność. Dla związków liniowych ponownie korzystniejszą wartość uzyskano dla USCL zawierającego argininę, czyli L2 (0.28) niż dla L1 (0.89).

Analogiczną analizę przeprowadzono podczas określania minimalnych stężeń eradykujących biofilm (MBEC). Zgodnie z przewidywaniami, obserwowane wartości MBEC były kilkukrotnie wyższe niż określone uprzednio MIC. Wynika to z charakterystyki struktury

biofilmu, czyli skomplikowanej i heterogennej społeczności komórek wyspecjalizowanych do pełnienia różnych funkcji, otoczonych nieprzepuszczalną macierzą pozakomórkową (ang. *Extracellular matrix,* ECM). Flukonazol okazał się nieskuteczny w eradykacji biofilmu utworzonego przez badane szczepy *Candida*. Jest to o tyle istotne, że mogłoby potencjalnie tłumaczyć obserwacje kliniczne związane z niepowodzeniem terapii empirycznej oraz celowanej – z uwagi na badanie lekowrażliwości szczepów w formie planktonowej i najbardziej prawdopodobnym występowaniem *Candida* w formie biofilmu na błonie śluzowej pochwy. Wartości średnich geometrycznych MBEC lipopeptydów ponownie były niższe w przypadku związków cyklicznych i wynosiły 56.59 (C1) i 65.45 µg/mL (C2). Dla analogów liniowych stężenia te były znacząco wyższe i niejednokrotnie przekraczały badane zakresy; uzyskane średnie wynosiły 236.73 (L1) i 223.86 µg/mL (L2). Przewagi lipopeptydów zawierających mostki disiarczkowe oraz związku liniowego wzbogaconego argininą w przypadku wpływu wobec komórek biofilmu, (choć również obserwowanych wcześniej) dotychczas także nie wyjaśniono.

Wstępne badania nad wzajemnymi interakcjami flukonazolu z każdym z badanych USCLs przeprowadzono na 24 wybranych szczepach (15 *C. albicans* i 9 NCAC). W żadnym przypadku FIC index nie wskazywał na działanie synergistyczne badanych par związków (FICi ≤0.5). Jednak w przypadku L1 i L2 przeważającym obserwowanym efektem kombinacji z flukonazolem okazała się addycja (1.0< FICi >0.5). Dla związków cyklicznych najczęściej dochodziło do efektu indyferentnego (FICi ≥1.0), czyli neutralnego. Bardziej szczegółowa analiza wskazała, że najkorzystniejsze oddziaływania wobec *Candida* w połączeniu z flukonazolem prezentuje związek L1 – dla którego efekt antagonistyczny wystąpił jedynie dla jednego szczepu *C. albicans* i jednego NCAC, a ponad połowa badanych przypadków zaklasyfikowana została jako addycja. Zaobserwowano także, że żaden ze związków C1 i C2 wraz z badanym azolem nie wpłynął w sposób pozytywny (synergizm lub addycja) na szczepy z grupy NCAC. Dalsze badania z udziałem pozostałej puli szczepów przeprowadzono więc przede wszystkim dla kombinacji L1-flukonazol. Dla weryfikacji i porównania aktywności związków liniowych i cyklicznych, w kolejnych eksperymentach uwzględniono także parę C1-flukonazol.

Analiza wpływu działania skojarzonego L1 z flukonazolem wobec wszystkich badanych szczepów potwierdziła obserwacje badań wstępnych. Dominującym efektem działania obu związków była addycja (84%); przy czym dla szczepów *C. albicans* odsetek ADD wynosił 91%, a dla NCAC – 55%. W przypadku kombinacji C1 z flukonazolem dla 64 szczepów w 53% dochodziło do addycji działania obu substancji – 60% w przypadku grupy *C. albicans*

oraz zaledwie 18% dla NCAC. Potencjalny efekt addytywny, zwłaszcza analogu liniowego z flukonazolem, mógłby być tłumaczony wzajemnym uzupełnianiem mechanizmów aktywności obu klas związków wobec *Candida*. Głównym celem działania USCLs jest dwuwarstwa błony drobnoustrojów i doprowadzenie do jej permeabilizacji – co prowadzi ostatecznie do śmierci komórki, ale także może ułatwić dostęp do celu działania takich związków, jak flukonazol i dzięki temu skuteczniejszy i/lub szybszy efekt bójczy wobec patogenu. W kolejnym etapie przeanalizowano skuteczność konkretnych par stężeń L1-flukonazol. Aż w 46 przypadkach udało się znaleźć takie konkretne kombinacje stężeń, żeby uzyskać działanie synergistyczne wobec różnych szczepów *Candida*. Przy czym stężenia te były przynajmniej 4 rzędy niższe niż odpowiadające im wartości MIC. Oznacza to, że pomimo stosunkowo wysokich stężeń hamujących lipopeptydu w kontekście toksyczności i selektywności, można by zastosować stężenia znacznie niższe (i mniej toksyczne) dzięki dołączeniu równie niskich stężeń flukonazolu. Obserwowane pozytywne efekty terapii skojarzonej dotyczyły jednak przede wszystkim analogów liniowych – dla których MIC i MBEC są istotnie wyższe, niż w przypadku odpowiadających im wartości dla związków cyklicznych. Oznaczać to może, że pomimo zdecydowanie korzystniejszego wpływu C1 i C2 wobec komórek w formie planktonowej i biofilmu *Candida*, obserwowane wysokie współczynniki selektywności świadczące o dużej toksyczności tych związków oraz stosunkowo niewielkie efekty pozytywne kombinacji z flukonazolem – ich użyteczność i znaczenie w opracowywaniu nowych strategii terapeutycznych w VVC może być dużo niższe, niż w przypadku pierwotnie syntezowanych związków liniowych.

5.3. Artykuł nr 3: Activity of novel ultrashort cyclic lipopeptides against biofilm of *Candida albicans* isolated from VVC in the *ex vivo* animal vaginal model and BioFlux biofilm model - a pilot study

Głównym celem publikacji była weryfikacja rezultatów uzyskanych w poprzedniej pracy. Porównano wyniki uzyskane uprzednio klasycznymi metodami laboratoryjnymi *in vitro* za pomocą dwóch rodzajów modeli biofilmu – modelu *ex vivo* wykorzystującego tkanki pochwy mysiej oraz modelu biofilmu w warunkach mikroprzepływowych przy wykorzystaniu technologii BioFlux.

Do badań wybrano dwa szczepy *C. albicans* spośród tych, dla których wyznaczone poprzednio wartości MIC, MBEC i FIC były najniższe. W modelu tkankowym dojrzałą strukturę biofilmu grzybiczego uzyskiwano bezpośrednio na powierzchni błony śluzowej

fragmentów pochwy mysiej, co zweryfikowano za pomocą mikroskopii konfokalnej oraz metod ilościowych. Następnie strukturę tę traktowano badanymi substancjami analogicznie, jak w przypadku metod *in vitro* – najbardziej obiecującymi (na podstawie wcześniejszych eksperymentów) USCLs L1 i C1 w stężeniach równych MBEC oraz najkorzystniejszą kombinacją FIC dla par L1-flukonazol i C1-flukonazol, a dodatkowo także stężeniami subinhibicyjnymi MBEC (1/2 MBEC). Ponieważ w metodzie wyznaczania MBEC z użyciem soli tetrazoliowej MTT uzyskano całkowitą eradykację żywych komórek biofilmu z powierzchni płytki polistyrenowej, spodziewanym efektem działania analizowanych związków w tych samych stężeniach była przynajmniej znaczna eradykacja biomasy biofilmu z tkanki. W żadnym przypadku nie udało się jednak uzyskać takiego rezultatu. Sugerowałoby to pewną istotną odmienność zachowania się *Candida* na powierzchni abiotycznej (płytce) oraz biotycznej (tkance), w tym sposobów pozyskiwania składników odżywczych (z dostarczanego podłoża vs z komórek epitelium) oraz możliwości inwazji i ekspansji (w przypadku błony śluzowej). Podobnie, nie udało się także eradykować biofilmu z powierzchni epitelium za pomocą kombinacji lipopeptyd-flukonazol. Należy jednak zauważyć, że otrzymywane wartości CFU/g tkanki w tym przypadku niejednokrotnie okazywały się najniższe w toku prowadzenia eksperymentów, a najczęściej zbliżone do pozostałych wartości CFU/g. Jest to warte podkreślenia z uwagi na fakt, że stężenia odpowiadające wartościom FIC wyznaczane były, zgodnie z metodologią, dla komórek w formie planktonowej, a nie biofilmu *Candida*. Oznacza to, że zastosowanie kombinacji stężeń USCL-flukonazol niższych, niż MIC dla każdego związku osobno, a także wielokrotnie niższych (przynajmniej 6 rzędów wartości) niż MBEC, skutkuje podobnym efektem wobec biofilmu *C. albicans* na tkance, co minimalne stężenia hamujące (zgodnie z metodami *in vitro*). Dołączenie do eksperymentów badania wpływu stężeń subinhibicyjnych na biofilm na powierzchni tkanki miało na celu wstępną weryfikację potencjalnie istniejących możliwości uwrażliwiania drobnoustrojów na działanie naturalnych komponentów immunologicznych za pomocą niskich stężeń USCLs. Dotychczas zaobserwowano możliwość wspomagania tzw. peptydów obronnych gospodarza (ang. *Host defence peptides*, HDPs), jak lizozym czy laktoferyna za pomocą dodatku subinhibicyjnych stężeń pewnych AMPs w miejscu zakażenia. Komórki epitelium pochwy niewątpliwie wydzielają wiele substancji o działaniu przeciwdrobnoustrojowym, w tym HDPs – istnieje więc szansa, że lipopeptydy mogłyby wykazywać działanie addytywne lub synergistyczne analogicznie jak w połączeniu z flukonazolem. Jednak również w przypadku zastosowania stężeń ½ MBEC, nie uzyskano znaczącej eradykacji biofilmu z powierzchni tkanki.

W modelu wykorzystującym BioFlux również udało się utworzyć dojrzałą strukturę biofilmu *C. albicans* wraz z obserwacją mikroskopową poszczególnych faz jego powstawania. Zastosowanie lipopeptydów L1 i C1 w stężeniach równych MBEC w warunkach mikroprzepływowych także w tym przypadku nie doprowadziło do eradykacji biofilmu. Szczegółowa analiza dynamiki wzrostu badanej struktury wykazała nieznaczny, choć zauważalny, wpływ jedynie ograniczający dalszy rozwój i przyrost biofilmu w mikrokanałach BioFlux w czasie 24 godzin inkubacji w obecności USCLs – zwłaszcza L1. Powód, dla którego obserwuje się pozytywny efekt hamujący takich związków jak lipopeptydy wobec biofilmu *Candida* na płytkach polistyrenowych *in vitro*, a którego próżno doszukiwać się przy zastosowaniu innych metod badania biofilmu *in vitro* (BioFlux) lub *ex vivo* (model tkankowy) pozostaje niewyjaśniony.

Dalsza weryfikacja tego typu doniesień jest oczywiście konieczna i wymaga bardziej szczegółowych badań przede wszystkim nad interakcjami grzybów drożdżopodobnych z komórkami gospodarza w mikrośrodowisku pochwy. Tym niemniej opisywane obserwacje są kolejnym argumentem przemawiającym za istnieniem niedoskonałości i braku dostatecznego przełożenia badania lekowrażliwości izolatów z VVC na rzeczywiste warunki panujące w trakcie kandydozy pochwy, co może wiązać się z opisywanymi niepowodzeniami terapeutycznymi i występowaniem RVVC.

6. WNIOSKI

1) Wybrane ultrakrótkie kationowe lipopeptydy w postaci związków oznaczanych skrótami L1, C1, L2 i C2 są skuteczne w hamowaniu wzrostu oraz eradykacji biofilmu utworzonego przez różne gatunki *Candida* izolowane z kandydozy pochwy i sromu.
2) Lipopeptydy wykazują znaczącą przewagę w zwalczaniu biofilmu *Candida* nad konwencjonalnie stosowanym klinicznie flukonazolem niezdolnym do eradykacji tej struktury.
3) Terapia skojarzona w postaci połączeń flukonazolu z poszczególnymi USCLs mogłaby być skuteczna wobec *Candida* z uwagi na addytywny lub synergistyczny efekt kombinacji tych związków.
4) Badane lipopeptydy o strukturze cyklicznej wykazują korzystniejszy efekt hamujący i eradykujący wobec *Candida* niż ich liniowe odpowiedniki, jednak cechują się one znacznie wyższą toksycznością i niską selektywnością. Poprawę działania analogów liniowych można stosunkowo łatwo uzyskać dzięki jednoczesnemu ich zastosowaniu z flukonazolem w stężeniach znacznie niższych i mniej toksycznych, niż przy stosowaniu tych związków osobno.
5) Nowo opracowany zwierzęcy model tkankowy *ex vivo* kandydozy pochwy jest funkcjonalny i użyteczny w badaniu tworzenia biofilmu *C. albicans* na powierzchni błony śluzowej oraz weryfikacji skuteczności działania lipopeptydów i ich połączeń z flukonazolem wobec tej struktury.
6) Występują kluczowe różnice pomiędzy wynikami uzyskanymi klasycznymi metodami badania biofilmu *in vitro* oraz metodą jego badania w warunkach przepływowych z użyciem BioFlux wraz z obserwacją zachowania się komórek *Candida* w czasie rzeczywistym.
7) Rezultaty badań nad wpływem USCLs prowadzonych standardowymi metodami laboratoryjnymi *in vitro* nie znalazły potwierdzenia w modelu tkankowym *ex vivo,* ani modelu biofilmu w warunkach przepływowych *in vitro*, co sugeruje potrzebę ujednolicenia metod badawczych oraz szerszej weryfikacji ich skuteczności i przełożenia na rzeczywiste warunki panujące w trakcie kandydozy pochwy.

7. BIBLIOGRAFIA

1. Mayer, F.L.; Wilson, D.; Hube, B. Candida albicans pathogenicity mechanisms. *Virulence* **2013**, *4*, 119–128, doi:10.1093/benz/9780199773787.article.b00119352.
2. Aniebue, U.U.; Nwankwo, T.O.; Nwafor, M.I. Vulvovaginal candidiasis in reproductive age women in Enugu Nigeria, clinical versus laboratory-assisted diagnosis. *Niger. J. Clin. Pract.* **2018**, *21*, 1017–1022, doi:10.4103/njcp.njcp_25_16.
3. Żyrek, D.; Wajda, A.; Czechowicz, P.; Nowicka, J.; Jaśkiewicz, M.; Neubauer, D.; Kamysz, W. The antimicrobial activity of omiganan alone and in combination against candida isolated from vulvovaginal candidiasis and bloodstream infections. *Antibiotics* **2021**, *10*, doi:10.3390/antibiotics10081001.
4. Sobel, J.D. Recurrent vulvovaginal candidiasis. *Am. J. Obstet. Gynecol.* **2016**, *214*, 15–21, doi:10.1016/j.ajog.2015.06.067.
5. Zeng, X.; Zhang, Y.; Zhang, T.; Xue, Y.; Xu, H.; An, R. Risk Factors of Vulvovaginal Candidiasis among Women of Reproductive Age in Xi'an: A Cross-Sectional Study. *Biomed Res. Int.* **2018**, *2018*, doi:10.1155/2018/9703754.
6. Sobel, J.D. Vulvovaginal candidosis. *Lancet* **2007**, *369*, 1961–1971, doi:10.1016/S0140-6736(07)60917-9.
7. Gonçalves, B.; Ferreira, C.; Alves, C.T.; Henriques, M.; Azeredo, J.; Silva, S. Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. *Crit. Rev. Microbiol.* **2016**, *42*, 905–927, doi:10.3109/1040841X.2015.1091805.
8. Das, K.H.; Mangayarkarasi, V.; Sen, M. Antifungal resistant in non-albicans candida species are emerging as a threat to antenatal women with vulvovaginal candidiasis. *Biomed. Pharmacol. J.* **2019**, *12*, 1369–1378, doi:10.13005/bpj/1765.
9. Rodríguez-Cerdeira, C.; Gregorio, M.C.; Molares-Vila, A.; López-Barcenas, A.; Fabbrocini, G.; Bardhi, B.; Sinani, A.; Sánchez-Blanco, E.; Arenas-Guzmán, R.; Hernandez-Castro, R. Biofilms and vulvovaginal candidiasis. *Colloids Surfaces B Biointerfaces* **2019**, *174*, 110–125, doi:10.1016/j.colsurfb.2018.11.011.
10. Hacioglu, M.; Guzel, C.B.; Savage, P.B.; Seher Birteksoz Tan, A. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against Candida spp. Isolated from vulvovaginal candidiasis. *Med. Mycol.* **2019**, *57*, 291–299, doi:10.1093/mmy/myy023.
11. Brandolt, T.M.; Klafke, G.B.; Gonçalves, C.V.; Bitencourt, L.R.; Martinez, A.M.B. de; Mendes, J.F.; Meireles, M.C.A.; Xavier, M.O. Prevalence of Candida spp. in cervical-

vaginal samples and the in vitro susceptibility of isolates. *Brazilian J. Microbiol.* **2017**, *48*, 145–150, doi:10.1016/j.bjm.2016.09.006.

12. Peters, B.M.; Yano, J.; Noverr, M.C.; Fidel, P.L. Candida Vaginitis: When Opportunism Knocks, the Host Responds. *PLoS Pathog.* **2014**, *10*, e1003965, doi:10.1371/journal.ppat.1003965.
13. Swidsinski, A.; Guschin, A.; Tang, Q.; Dörffel, Y.; Verstraelen, H.; Tertychnyy, A.; Khayrullina, G.; Luo, X.; Sobel, J.D.; Jiang, X. Vulvovaginal candidiasis: histologic lesions are primarily polymicrobial and invasive and do not contain biofilms. *Am. J. Obstet. Gynecol.* **2019**, *220*, 91.e1-91.e8, doi:10.1016/j.ajog.2018.10.023.
14. Yano, J.; Peters, B.M.; Noverr, M.C.; Fidel, P.L. Novel Mechanism behind the Immunopathogenesis of Vulvovaginal Candidiasis: "Neutrophil Anergy." *Infect. Immun.* **2018**, *86*, 1–12, doi:e00684-17.
15. Willems, H.M.E.; Ahmed, S.S.; Liu, J.; Xu, Z.; Peters, B.M. Vulvovaginal candidiasis: A current understanding and burning questions. *J. Fungi* **2020**, *6*, doi:10.3390/jof6010027.
16. Roselletti, E.; Perito, S.; Sabbatini, S.; Monari, C.; Vecchiarelli, A. Vaginal Epithelial Cells discriminate between yeast and hyphae of C. albicans in women who are colonized with or have vaginal candidiasis. *J. Infect. Dis.* **2018**, *10*, 1645–1654, doi:10.1093/asj/sjy179/5059000.
17. Tulasidas, S.; Rao, P.; Bhat, S.; Manipura, R. A study on biofilm production and antifungal drug resistance among Candida species from vulvovaginal and bloodstream infections. *Infect. Drug Resist.* **2018**, *11*, 2443–2448, doi:10.2147/IDR.S179462.
18. Harriott, M.M.; Lilly, E.A.; Rodriguez, T.E.; Fidel, P.L.; Noverr, M.C. Candida albicans forms biofilms on the vaginal mucosa. *Microbiology* **2010**, *156*, 3635–3644, doi:10.1099/mic.0.039354-0.
19. Lohse, M.B.; Gulati, M.; Johnson, A.D.; Nobile, C.J. Development and regulation of single-and multi-species Candida albicans biofilms. *Nat. Rev. Microbiol.* **2018**, *16*, 19–31, doi:10.1038/nrmicro.2017.107.
20. Czechowicz, P.; Nowicka, J. Antimicrobial activity of lipopeptides. *Postep. Mikrobiol.* **2018**, *57*, 213–227.
21. Neubauer, D.; Jaśkiewicz, M.; Sikorska, E.; Bartoszewska, S.; Bauer, M.; Kapusta, M.; Narajczyk, M.; Kamysz, W. Effect of disulfide cyclization of ultrashort cationic lipopeptides on antimicrobial activity and cytotoxicity. *Int. J. Mol. Sci.* **2020**, *21*, 1–30, doi:10.3390/ijms21197208.

22. Simonetti, O.; Arzeni, D.; Ganzetti, G.; Silvestri, C.; Cirioni, O.; Gabrielli, E.; Castelletti, S.; Kamysz, W.; Kamysz, E.; Scalise, G.; et al. In vitro activity of the lipopeptide derivative (Pal-lys-lys-NH 2), alone and in combination with antifungal agents, against clinical isolates of dermatophytes. *Br. J. Dermatol.* **2009**, *161*, 249–252, doi:10.1111/j.1365-2133.2009.09166.x.
23. Yuan, Y.; Zhou, F.; Su, H.; Zhang, Y. Structural design of microbicidal cationic oligomers and their synergistic interaction with azoles against Candida albicans. *Sci. Rep.* **2019**, *9*, 1–11, doi:10.1038/s41598-019-48322-x.
24. Desmyttere, H.; Deweer, C.; Muchembled, J.; Sahmer, K.; Jacquin, J.; Coutte, F.; Jacques, P. Antifungal activities of bacillus subtilis lipopeptides to two venturia inaequalis strains possessing different tebuconazole sensitivity. *Front. Microbiol.* **2019**, *10*, 1–10, doi:10.3389/fmicb.2019.02327.
25. Suchodolski, J.; Derkacz, D.; Muraszko, J.; Panek, J.J.; Jezierska, A.; Łukaszewicz, M.; Krasowska, A. Fluconazole and lipopeptide surfactin interplay during Candida albicans plasma membrane and cell wall remodeling increases fungal immune system exposure. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12040314.
26. Venisse, N.; Grégoire, N.; Marliat, M.; Couet, W. Mechanism-based pharmacokinetic-pharmacodynamic models of in vitro fungistatic and fungicidal effects against Candida albicans. *Antimicrob. Agents Chemother.* **2008**, *52*, 937–943, doi:10.1128/AAC.01030-07.
27. Arendrup, M.C.; Meletiadis, J.; Mouton, J.W.; Lagrou, K.; Hamal, P. Susceptibility testing of yeasts. *EClinical Microbiol. Infect.* **2017**, *3*, 14–16, doi:10.1111/j.1469-0691.1997.tb00893.x.
28. Pfaller, M.A.; Chaturvedi, V.; Espinel-Ingroff, A.; Ghannoum, M.A.; Gosey, L.L.; Odds, F.C.; Rex, J.H.; Rinaldi, M.G.; Sheehan, D.J.; Walsh, T.J.; et al. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts ; Approved Standard — Second Edition Serving the World ' s Medical Science Community Through Voluntary Consensus*; 2002; Vol. 22; ISBN 1562384694.
29. Brzozowska, P.; Czechowicz, P.; Nowicka, J. Porównanie metod oznaczania wrażliwości na flukonazol klinicznych szczepów Candida. *Forum Zakażeń* **2021**, *12*, 115–122, doi:10.15374/fz2021021.
30. Arendrup, M.C.; Prakash, A.; Metetiadis, J.; Sharma, C.; Chowdhary, A. Comparison of EUCAST and CLSI Reference Microdilution MICs of Eight and Associated Tentative. *Antimicrob. Agents Chemother.* **2017**, *61*, 1–10.

31. Naglik, J.R.; Fidel, P.L.; Odds, F.C. Animal models of mucosal Candida infection. *FEMS Microbiol. Lett.* **2008**, *283*, 129–139, doi:10.1111/j.1574-6968.2008.01160.x.
32. Krom, B.P.; Willems, H.M.E. In Vitro Models for Candida Biofilm Development. In *Candida Species: Methods and Protocols*; 2016; Vol. 1356, pp. VIII; 95–106 ISBN 978-1-4939-3051-7.
33. Pfaller, M.A.; Haturvedi, V.; Espinel-Ingroff, A.; Ghannoum, M.A.; Gosey, L.L.; Odds, F.C.; Rex, J.H.; Rinaldi, M.G.; Sheehan, D.J.; Walsh, T.J.; et al. *Reference method for broth dilution antifungal susceptibility testing of yeasts ; approved standard — second edition serving the world ' s medical science community through voluntary consensus*; 2002; Vol. 22; ISBN 1562384694.
34. Krom, B.P.; Cohen, J.B.; McElhaney-Feser, G.; Busscher, H.J.; van der Mei, H.C.; Cihlar, R.L. *Conditions for Optimal Candida Biofilm Development in Microtiter Plates [in:] Candida albicans methods and protocols*; Humana Press, a part of Springer ScienceþBusiness Media, LLC, 2009; ISBN 9781626239777.
35. Nilius, A.M. *Antimicrobial pharmacodynamics in theory and clinical practice*; 2002; Vol. 17; ISBN 9780824729257.
36. EUCAST Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. *Clin. Microbiol. Infect.* **2000**, *6*, 503–508, doi:10.1046/j.1469-0691.2000.00149.x.

8. STRESZCZENIE PRACY W JĘZYKU POLSKIM

Kandyzoza pochwy i sromu pozostaje drugim co do częstości zakażeniem ginekologicznym i dotyczy ok. 75% kobiet w wieku rozrodczym na całym świecie. Dotychczas diagnostyki tej infekcji dokonywano na podstawie objawów klinicznych, a leczenie dobierano w sposób empiryczny. Postępowanie takie obarczone jest stosunkowo dużą liczbą niepowodzeń terapeutycznych oraz ryzykiem nawrotów. Dodatkowe utrudnienie kliniczne stanowi fakt nadal nieznanego szczegółowego patomechanizmu rozwoju objawowej infekcji grzybiczej pochwy oraz ograniczonego zasobu konwencjonalnych leków przeciwgrzybiczych skutecznych wobec *C. albicans* oraz grzybów z grupy NCAC. W poszukiwaniu kluczowych czynników patogenności *Candida* na błonie śluzowej pochwy, duże znaczenie przypisuje się roli formowania wysoce opornej struktury biofilmu na powierzchni epitelium. Z tego powodu testowanie nowych opcji terapeutycznych opiera się m. in. na analizowaniu nowych związków pod kątem aktywności nie tylko przeciwgrzybiczej, ale i przeciwbiofilmowej. Obiecującymi substancjami spełniającymi te kryteria są ultrakrótkie kationowe lipopeptydy. Za te, posiadające największy potencjał wobec grzybów izolowanych z kandydozy pochwy uznano ostatnio dwie pary analogów o strukturze liniowej i cyklicznej w postaci: C_{16}-KKKK-NH_2 i C_{16}-CKKKKC-NH_2 oraz C_{16}-KRKK-NH_2 i C_{16}-CKRKKC-NH_2.

Celem pracy doktorskiej było określenie wpływu wymienionych związków oraz flukonazolu wobec 64 szczepów klinicznych i referencyjnych różnych gatunków *Candida* izolowanych z kandydozy pochwy oraz biofilmu tworzonego przez te grzyby. Dodatkowo zbadano możliwość terapii skojarzonej lipopeptydów z flukonazolem. Weryfikację rezultatów uzyskanych klasycznymi metodami laboratoryjnymi *in vitro* przeprowadzono z wykorzystaniem dwóch modeli badania biofilmów: nowo opracowanego modelu *ex vivo* z zastosowaniem tkanek zwierzęcych oraz modelu mikroprzepływowego uzyskanego dzięki technologii BioFlux.

Wszystkie cztery badane lipopeptydy okazały się skuteczne wobec analizowanej puli szczepów zarówno w formie planktonowej, jak i wobec biofilmu. Poza jednym wyjątkiem w postaci jednego szczepu *C. lusitaniae*, flukonazol również okazał się aktywny wobec *Candida*, jednak nie był zdolny do eradykowania struktury biofilmu. Badania nad wzajemnymi interakcjami azolu z lipopeptydami wykazały możliwość działania addytywnego obu rodzajów substancji – najprawdopodobniej dzięki wzajemnemu uzupełnianiu się ich różnych mechanizmów działania. Dzięki terapii skojarzonej możliwe byłoby użycie tych substancji w stężeniach znacznie niższych i mniej toksycznych, niż przy ich stosowaniu osobno.

Zastosowanie modelu *ex vivo* wykorzystującego fragmenty tkanki pochwy mysiej oraz modelu badania biofilmu *Candida* w warunkach mikroprzepływowych w BioFlux wykazało istnienie zasadniczych różnic w rezultatach uzyskanych poszczególnymi metodami. W modelach tych nie udało się uzyskać potwierdzenia wyników uzyskanych za pomocą standardowych metod laboratoryjnych *in vitro*. Dojrzały biofilm grzybiczy utworzony na powierzchni epitelium pochwy (zwierzęcej) oraz w mikrokanałach BioFlux nie był skutecznie eradykowany przez badane lipopeptydy w stężeniach równych MBEC ani przez ich połączenia z flukonazolem w stężeniach odpowiadających FIC. Przyczyny obserwowanych różnic w zachowaniu się i lekowrażliwości biofilmu *Candida* na płytkach polistyrenowych vs na błonie śluzowej pochwy i na powierzchni mikrokanałów pozostają nieznane.

Wyniki uzyskane w toku pracy badawczej wskazują na istnienie pewnych kluczowych, nieznanych mechanizmów zależnych od grzybów z rodzaju *Candida*, ale także zależnych od komórek gospodarza, istotnych w patomechanizmie kandydozy pochwy. Mechanizmy te są najprawdopodobniej odpowiedzialne za istnienie niezgodności rezultatów badania lekowrażliwości grzybów drożdżopodobnych różnymi metodami. Mogą być one także odpowiedzialne za obserwowane niepowodzenia terapeutyczne oraz rozwój nawrotowej postaci kandydozy pochwy. Sugeruje to potrzebę weryfikacji skuteczności obecnie stosowanych metod badawczych, ich ujednolicenie oraz ewentualną modyfikację tak, aby jak najlepiej odzwierciedlały one procesy realnie zachodzące podczas objawowego zakażenia grzybiczego w pochwie.

9. STRESZCZENIE PRACY W JĘZYKU ANGIELSKIM

Affecting approximately 75% of women of reproductive age worldwide, vulvovaginal candidiasis remains the second most common gynecological infection. Until now, this infection has been diagnosed on the basis of clinical symptoms, and treatment has been selected empirically. Such approach can lead to a relatively large number of therapeutic failures and carries a risk of recurrence. The still unknown detailed pathomechanism of symptomatic mycotic vulvovaginitis and the limited supply of conventional antifungal drugs effective against *C. albicans* and NCAC fungi constitute an additional clinical difficulty. The formation of a highly resistant biofilm structure on the surface of the epithelium is of great importance for research on the key factors of *Candida* pathogenicity on the vaginal mucosa. Therefore, testing of new therapeutic options is based, among others, on analyzing new compounds in terms of not only antifungal, but also antibiofilm activity. Ultrashort cationic lipopeptides are promising substances that meet the above two criteria. Two pairs of analogs with a linear and cyclic structure in the form of C_{16}-KKKK-NH_2 and C_{16}-CKKKKC-NH_2 and C_{16}-KRKK-NH_2 and C_{16}-CKRKKC-NH_2 have recently been recognized as having the greatest potential against fungi isolated from vulvovaginal candidiasis.

The aim of the doctoral dissertation was to determine the effect of the above compounds and of fluconazole on 64 clinical and reference strains of various *Candida* species isolated from vulvovaginal candidiasis and on the biofilm created by these fungi. In addition, the possibility of combination therapy including lipopeptides with fluconazole was investigated. The results obtained by classical *in vitro* laboratory methods were verified using two biofilm research models: a newly developed *ex vivo* model using animal tissues and the BioFlux microfluidic open model.

All four tested lipopeptides turned out to be effective against the analyzed pool of strains, both in planktonic and biofilm form. With a single exception (one strain of *C. lusitaniae*), fluconazole was also found to be active against *Candida* spp. but it was not able to eradicate the biofilm structure. Studies on the interactions of azoles with lipopeptides have shown the possibility of an additive effect of both substances – most likely owing to the mutual complementarity of their various mechanisms of action. In combination therapy, these substances could be used in concentrations much lower and less toxic than if administered separately.

The two models of investigating *Candida* biofilm, i.e. the *ex vivo* model using fragments of mouse vaginal tissue and the BioFlux microfluidic open model, showed significant

differences in the results obtained by individual methods. These models failed to confirm the results obtained by standard *in vitro* laboratory methods. The mature fungal biofilm formed on the epithelial surface of the (animal) vagina and in BioFlux microchannels was not effectively eradicated by the tested lipopeptides at concentrations equal to MBEC or by their combination with fluconazole at FIC concentrations. The reasons for the observed differences in the behavior and drug susceptibility of *Candida* biofilm on polystyrene plates vs. on the vaginal mucosa and on the surface of microchannels remain unknown.

The results obtained in the course of our research indicate the existence of some key, unknown mechanisms dependent both on *Candida* and on the host cells which play an important role in the pathomechanism of vaginal candidiasis. These mechanisms are most likely responsible for the discrepancies in the results of yeast-like fungi drug susceptibility testing obtained using different methods. They may also be responsible for the observed therapeutic failures and for the development of recurrent vulvovaginal candidiasis. This suggests the need for verification of the effectiveness of currently used research methods, their unification and possible modification so that they would best reflect the real processes occurring during symptomatic fungal infection in the vagina.

10. PUBLIKACJE

10.1. Artykuł nr 1

International Journal of
Molecular Sciences

MDPI

Review

Virulence Factors of *Candida* spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis

Paulina Czechowicz *, Joanna Nowicka and Grażyna Gościniak

Department of Microbiology, Faculty of Medicine, Wrocław Medical University, 50-368 Wrocław, Poland; joanna.nowicka@umw.edu.pl (J.N.); grazyna.gosciniak@umw.edu.pl (G.G.)

* Correspondence: paulina.czechowicz.umedwroc@gmail.com; Tel.: +71-784-13-01

Abstract: Vulvovaginal candidiasis (VVC) is one of the most common types of vaginal infections in women around the world and is often underestimated by both patients and doctors. Research on the pathogenesis of fungal vaginal infections over the last 20 years has resulted in a closer understanding of the virulence factors involved in *Candida* epithelial invasion and their mechanisms of action. Recently, attention was drawn to the enormous complexity of the interaction between yeast-like fungi and host cells, as well as the level of complexity of the host's response to infection and their impact on the course and treatment of VVC. Our work provides a broad description of already known and some new reports on *Candida* virulence factors (such as phenotypic switching or biofilm formation capacity) and their importance for tissue invasion in VVC. At the same time, we also focus on interactions with host cells and local innate immune mechanisms involved in the response to vaginal fungal invasion that are now considered equally important in this case. The presented review describes the most important aspects of the still unknown pathogenicity of *Candida* associated with vaginal infections.

Keywords: *Candida*; vaginal infections; VVC; biofilm; microorganism-host interaction

Citation: Czechowicz, P.; Nowicka, J.; Gosciniak, G. Virulence Factors of *Candida* spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis. *Int. J. Mol. Sci.* **2022**, *23*, 5895. https://doi.org/10.3390/ ijms23115895

Academic Editors: Jerome F. Strauss, III, Kimberly K. Jefferson and Andreas Burkovski

Received: 27 April 2022

Accepted: 23 May 2022

Published: 24 May 2022

1. Introduction

The first account of yeast fungus infections probably dates to the 5th century BC. The oldest documented reports indicating a clinical link between candidiasis and fungi were made in 1839. Since then, *Candida albicans* has been recognized as the main etiological factor of candidiasis and other diseases of the mucous membrane [1].

In the period between the discovery of fungi by the ancient Greeks to modern times, the view on the pathogenicity of these microorganisms has changed. It is now known that they are generally not pathogenic for people with a well-functioning immune system. However, in specific clinical situations they may become an etiological factor in severe, life-threatening infections [2]. It is worth noting that the incidence of infections caused by these microorganisms has been systematically increasing around the world, and mortality can reach 60–70% for some groups of patients [2–4]. All this makes fungal infections not only an epidemiological problem but a social one as well.

2. Overview of *Candida* spp.

2.1 Risk Factors Associated with Superficial and Invasive Candida Infections

Colonizing mucous membranes, mainly the oral cavity, intestines, vagina, and skin, fungi of the genus *Candida* are part of the human microbiota. As commensals, they do not cause infections in healthy people. They are considered opportunistic microorganisms which cause infections only in specific clinical situations and in the presence of favorable conditions. They mainly pose a threat to immunocompromised persons or patients hospitalized for a long time. People in these risk groups suffer from mucosal infections caused by switching commensal yeast-like fungi to pathogenic ones. Importantly, the risk factors described in detail below also create a predisposition to candidemia and other invasive candidiasis [5].

Factors predisposing to infection with fungi of the genus *Candida* can be classified into immunological and non-immunological [4]. The first group includes, among others, HIV infection. As the disease progresses, more than 90% of patients suffer from superficial fungal infections affecting mainly the oropharynx, primarily related to low levels of CD4+ lymphocytes, reduced activity of NK cells, and loss of T helper cells. The dominating species in this group of patients is *C. albicans*, but infections may also be caused by *Candida tropicalis, Candida krusei,* or *Candida parapsilosis* [4].

Immunosuppressive therapy, used, for example, in transplant patients, reduces the natural immune response, which makes the body more susceptible to all kinds of fungal infections, especially with *C. albicans*. The same is true for neoplastic diseases. The disease itself and the therapy used often lead to immunosuppression and disruption of the mechanisms of both humoral and cellular immune response [4]. The incidence of candidiasis among cancer patients treated in hospitals is higher compared to patients hospitalized for other causes [6].

A lowered level of leukocytes in the blood is an important risk factor, especially for patients with haematological disorders. It may result from the ongoing disease process, as well as from the therapy used (chemotherapy or antimicrobial treatment). It is worth adding that patients with chemotherapy-induced neutropenia are often in a serious condition that is additionally accompanied by the presence of other factors conducive to fungal infections (e.g., the use of broad-spectrum antibiotics and vascular catheters) [6].

Regarding non-immunological factors, it is worth mentioning disorders in the quantitative and qualitative composition of the microbiota, which promote the excessive multiplication of fungi. Diabetes and the associated high glycemia promote the colonization of fungi. In people with diabetes, much higher colonization of the oral mucosa with *Candida* fungi has been observed compared to the healthy population [3,4]. Reduced saliva flow or impaired neutrophil activity seem to be major factors here [3]. Hormonal disorders, especially in pregnant women, are also worth mentioning. Drugs that reduce the secretion of gastric juice or insufficient secretion of digestive enzymes promote the multiplication of fungi in the digestive tract. Inadequate diet, alcohol, lack of vitamins (especially vitamin A, vitamins B6, B12, and C) and microelements (selenium, zinc, iron), smoking, aging of the body, or chronic stress favor these types of infections [4,7]. Not without significance are also past surgeries, especially in the abdominal cavity or parenteral nutrition [8]. Patients with recurrent intestinal perforation or acute necrotizing pancreatitis have a higher risk of candidaemia [6]. The same applies to total parenteral nutrition—it increases the risk of candidiasis almost 4-fold [8]. The presence of a vascular catheter and its colonization may constitute the port of entry for the fungi to the bloodstream [6]. Past surgery, parenteral nutrition, or the use of endovascular catheters are all factors increasing the risk of (mainly systemic) infections [8].

Fungal infections often occur in extreme age groups: newborns, especially premature babies with low birth weight, and the elderly. Undoubtedly, the above is related to the functioning of the immune system—newborns do not have well-developed specific and nonspecific immune responses. In the case of older people, due to age, the immune system is no longer as efficient [9,10]. There may be additional factors in both groups. In the case of newborns, an important factor is the absence of microbiota in the gastrointestinal tract. A disruption in the process of acquiring a microbiota can lead to colonization by pathogenic microorganisms, including fungi. Hospitalization at an ICU, the use of vascular catheters, parenteral nutrition, and antibiotic therapy make them a group with a higher risk of infection [6,9]. It is worth adding that the risk of infection is higher for newborns with bodyweight of less than 1000 g than for newborns weighing more than 2500 g [6]. Similarly, in the case of the elderly, the use of immunosuppressive therapy, broad-spectrum antimicrobial drugs, or comorbidities such as diabetes often increase the risk of infections, especially in patients over 65 years of age [11].

The ability of fungi of the genus *Candida*, especially *C. albicans*, to colonize or infect various sites (tissues) in the human body depends on many pathogenic factors of these

microorganisms. Some of them, especially those important in the context of inducing vulvovaginitis, will be discussed in more detail later in the paper. It may be worth emphasizing. However, morphological changes and phenotypic switching, expression of a number of adhesins and invasins on the cell surface, the ability to form a biofilm, or the secretion of hydrolytic enzymes are considered the most important pathogenicity factors of these microorganisms. They allow colonization, adhesion, invasion, and damage to the host tissues. Their ability to adapt to the changing pH of an environment, as well as efficient systems for obtaining nutrients [12,13], are also of great significance.

2.2 Most Commonly Isolated Species

C. albicans is the species most often responsible for infections. However, it is worth noting that in recent years there has been an increased incidence of isolation of species other than *C. albicans*: *Candida glabrata*, *C. tropicalis*, *C. parapsilosis*, *Candida dubliniensis*, *Candida guilliermondii*, *C. krusei*, or *Candida kefyr* [2,14]. Epidemiological data confirm this trend worldwide [14]. Recent years witnessed a particular increase in the incidence of candidiasis caused by rarely isolated *C. krusei* or *C. guilliermondii* [15].

The suspected causes of the change in the epidemiology of fungal infections are the frequent use of compounds with antifungal activity, both in the prevention and empirical treatment of infections. Abuse or improper use of antifungal drugs are most likely causes of reduced susceptibility or even resistance of fungi to antifungal drugs. Increasingly, species other than *C. albicans* are responsible for infections, and, importantly, they demonstrate a reduced susceptibility to antifungal drugs [15,16]. When analyzing the frequency of isolation of yeast-like fungi from various clinical materials, Taei et al. showed that among NCAC (Non-*Candida albicans Candida*) species, *C. glabrata* was most often isolated (it accounted for 76% of all NCACs). The next most isolated were *C. krusei* (6.6%), *C. kefyr* (5.7%), *C. parapsilosis* (4.9%), and *C. tropicalis* (2.4%). The least common (0.8%) were *C. dubliniensis*, *C. guilliermondii,* and *Candida famata*. *C. albicans* accounted for 38.5% of all isolated fungi of the genus *Candida* [14]. According to the authors of the aforementioned paper, the increase in the frequency of NCAC isolation is a result of better laboratory diagnostics as well as previous exposure to polyenes and azoles or of reduced immunity, which is often related to the therapy used, e.g., cytostatics, in transplant or cancer patients. Diabetes was a common factor conducive to NCAC infections [14]. Research by Das et al. showed a similar rela- tionship. Of the 112 *Candida* fungi isolated from vaginal swabs, 58% were non-*C. albicans* species. Among them, *C. glabrata* (20%), *C. tropicalis* (19%), and *C. parapsilosis* (9%) were isolated most often. *C. albicans* was isolated in 42% of cases [17]. Despite *C. albicans* being the most commonly isolated, an increasing share of NCAC species and their dominance in complicated vulvovaginitis was observed. The authors explain the above primarily by prolonged antifungal treatment, diabetes, older age, and poor hygienic conditions. NCAC species were also shown to have a higher resistance to antifungal drugs compared to *C. albicans* [17]. Liu et al., who focused on the assessment of risk factors for NCAC candidiasis, showed that *C. albicans*, *C. tropicalis*, *C. glabrata*, *and C. parapsilosis* were most often isolated. The above species accounted for more than 96% of all isolated fungi of the genus *Candida*. NCAC and *C. albicans* were isolated in 53.5% and 46.5% of candidiasis cases, respectively. Among NCACs, *C. tropicalis* was most isolated, primarily from patients with haematological tumors. The authors have demonstrated a higher resistance among NCAC species, particularly of *C. tropicalis* isolates [18].

When discussing species other than *C. albicans*, *Candia auris* is worth mentioning. First isolated in Japan in 2009 from a patient with otitis, it has become a causative agent of invasive infections around the world. It is characterized by ease of spreading, resistance to antifungal agents and disinfectants, a wide range of pathogenic factors, and the fact that it causes severe infections with a high, estimated at even 72%, mortality rate [19–21]. Most often, it causes infections in patients who are artificially ventilated, hospitalized in the intensive care unit, catheterized, HIV-infected, diabetic and immunosuppressed. Other factors predisposing to *C. auris* infections include parenteral nutrition, previous

surgery, or long-term use of antimicrobials [19,20,22]. *C. auris* is currently a global threat, causing severe epidemic outbreaks of invasive infections most often associated with medical care [21].

2.3 Infections Caused by Fungi of the Genus Candida

Among the infections caused by *Candida* spp. fungi, one can distinguish surface infections and systemic infections [12,19,20,23]. Surface infections are not generally life-threatening [12]. Among them, one can mention infections of the skin and nails, mucous membrane of the mouth, throat, esophagus, intestines, and vagina [12,24]. Systemic infections, on the other hand, are characterized by a severe course and pose a direct threat to human life. Systemic candidiasis is associated with high mortality, and neutropenia, as well as damage to the gastrointestinal mucosa, are quoted as the most common factors predisposing to this type of infection. Other factors include the use of central venous catheters or antibacterial therapy, most often with broad-spectrum agents [12].

Skin infections are most often surface infections, while infections involving the dermis and subcutaneous layer occur very rarely. The changes most often affect skin folds, e.g., inguinal folds, and often occur in overweight people. *Candida* spp. may also be the causative agent of paronychia or onychomycosis [23]. Oral candidiasis is mainly caused by *C. albicans* and most often occurs in people with impaired immune function. The most common risk factors are HIV infection, followed by wearing prostheses and braces, old age, xerostomia, or poor oral hygiene [12,23]. Esophageal candidiasis is most often caused by *C. albicans* and occurs primarily in people with an impaired immune system or with concomitant diseases, e.g., diabetes. Often, it is manifested by odynophagia and dysphagia, but it can also be completely asymptomatic [23]. Intestinal mycosis is most often superficial, but it can also occur with intestinal perforation. Fungal infections of the intestines are most often associated with inflammatory bowel disease [23].

According to statistical data, about 75% of women have experienced vulvovaginal candidiasis (VVC) at least once in their lifetime, and in about 5–8% of them, it has a recurrent character (occurs at least four times per year) [12]. The pathomechanism of VVC and the pathogenicity of *Candida* spp. fungi associated with vaginal infections will be discussed further in the paper.

Invasive infections are most commonly caused by *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. kefyr* [23]. These fungi are some of the most common etiological factors of invasive infections, primarily in patients in intensive care units [25]. The entry of fungi into the bloodstream may result in the spread of fungi into tissues and organs [24]. It can cause, among others, meningitis, peritonitis, and abdominal infections, endocarditis, or infections affecting the patient's bones and joints [23]. The above-mentioned risk factors for invasive infections also include old age, parenteral nutrition, cancer, and immunosuppressive treatment [25].

Candida spp. fungi are also causative agents of infections associated with a foreign element (biomaterial) introduced into the human body. In this context, the adhesive properties of fungi and their ability to form biofilm are important. The possibility of fungal colonization in hip replacements, vascular catheters, urinary catheters, endotracheal tubes, dental implants, artificial valves, pacemakers, or even contact lenses has been demonstrated [26,27]. Infections that occur with the formation of biofilm often have a chronic, recurrent character and are characterized by a high mortality rate of more than 40% [27]. Biofilm plays an important role, protecting the fungal cells against the immune mechanisms of the human body as well as against antifungal agents. The therapeutic options for infections with biofilm-forming organisms are extremely limited [27]. The formation of biofilm structures on the biomaterial may result in blood infection or systemic infection of tissues and organs [28].

3. Vulvovaginal Candidiasis

As noted earlier, vulvovaginal candidiasis affects many women around the world. After bacterial infections, it is the second most common type of vaginal infection [12,29–31]. The clinical picture may vary, but most often, its symptoms include itching of the vagina and vulva, soreness, burning, and abnormal vaginal discharge. Other symptoms may include difficulty urinating and pain during sexual intercourse [29]. Data in the literature show that the incidence of this type of infection partially depends on the age of the woman, which also determines sexual maturity, hormonal activity, and the state of the vaginal microbiota [29,30,32]. The most common factors predisposing to VVC are pregnancy, the luteal phase of the menstrual cycle, the use of broad-spectrum antibiotics, the use of intrauterine devices, estrogen replacement therapy, immunosuppression, diabetes, or mechanical factors, e.g., the use of tight, synthetic underwear [31,33,34].

Research by Zeng et al. showed that women under 40 years of age had a higher risk of vaginal mycosis than older women [29]. It is also rarely diagnosed in patients before puberty [33]. Vulvovaginal candidiasis occurs primarily in young women of childbearing age and rarely in premenopausal women [30]. Almost 55% of women before the age of 25 will experience a VVC episode [33]. Sexual activity and physiological and tissue changes that are caused by reproductive hormones that occur in women during this period of life, seem to be important here. They increase susceptibility to *Candida* spp. infection [29].

To a large extent, the above has to do with the activity of sex hormones during the reproductive period and with low levels of estrogen and progesterone during menopause. During reproductive age, high levels of estrogen induce the hypertrophy of the vaginal mucosal epithelium as well as the secretion of glycogen by vaginal epithelial cells. Its fermentation to lactic acid by *Lactobacillus*, the main component of the vaginal microbiota, leads to a significant decrease in pH, which prevents the development of other microorganisms. In addition to ensuring proper vaginal pH, *Lactobacillus* bacteria, primarily *Lactobacilluis crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, and *Lactobacillus iners*, produce a number of substances, such as hydrogen peroxide or bacteriocins, which inhibit the multiplication of other microorganisms. They also compete for nutrients and receptors on vaginal epithelial cells with other microorganisms [30,32]. In menopausal women, a drop in estrogen levels and vaginal atrophy occurs, leading to a decrease in glycogen levels in vaginal epithelial cells and a neutral pH [32,34]. As Hoffmann points out, vulvovaginal candidiasis often occurs at lower pH values of vaginal secretions, which is the case for healthy women of childbearing age. Low hormone levels and vaginal atrophy are associated with a much lower risk of VVC in postmenopausal women [34]. It is generally accepted that a decrease in the level of lactobacilli or their complete absence predisposes women to VVC [30]. However, in his 2010 paper, McCelland showed that colonization of the vagina with *Lactobacillus* increases the risk of VVC by a factor of four [30,35]. Additionally, Ceccarani et al. showed that the vaginal microbiota of healthy women was dominated by *L. crispatus*. In a state of disease, the abundance of this species decreased while the number of *L. iners* increased significantly. The same relationship was also demonstrated by the mentioned authors for VVC. Additionally, high levels of glucose in the vagina of women with VVC were shown. On the one hand, glucose is a nutrient for *Candida* spp. However, on the other hand it promotes the expression of binding molecules in vaginal epithelial cells, thus increasing the adhesion of *Candida*. As the authors point out, high levels of glucose in the vagina may be associated with a reduction in the abundance of *L. crispatus* [36].

Data in the literature show that vaginal colonization with *Candida* spp. is greater in pregnant women (especially in the second and third trimester of pregnancy) than in non-pregnant women. The above state of affairs is most often explained by reduced cell-mediated immunity, high estrogen levels, and high glycogen levels, which promotes colonization with *Candida* spp. [37,38].

The diagnosis of vulvovaginal candidiasis is based on symptomatic vulvovaginitis with simultaneous isolation of *Candida* spp. from the clinical material (or from a vaginal

swab) in the absence of any other etiology [30,39]. As mentioned earlier, about 75% of women will experience vulvovaginal candidiasis (VVC) at least once in their lives, and in about 5–8% of them, it will be recurrent (at least four VVC per year) [12]. Recurrent vulvovaginal candidiasis affects nearly 138 million women annually [39].

In the vast majority of cases, the etiological factor of VVC is *C. albicans*. However, more and more frequently, the causative agents are species other than *C. albicans*, e.g., *C. glabrata*, *C. parapsilosis*, *C. tropicalis* or *C. krusei* [39]. An increase in the isolation of NCAC species in recurrent and complicated VVC was observed [17,39]. It is commonly known that species other than *C. albicans* are characterized by greater virulence and resistance to antifungal agents, which often leads to therapeutic failures [39].

4. Pathogenicity of Fungi of the Genus *Candida* spp. Related to Vaginal Infections—Virulence Factors

The detailed pathomechanism of vulvovaginal candidiasis remains unexplained. On the one hand, it is linked to imbalances in the vaginal microbiota and, on the other hand, to an abnormal immune response of the mucous membrane to the presence of fungi of the genus *Candida* [40–44]. Among the factors potentially responsible for the evolution of asymptomatically colonizing yeast-like fungi into invasive pathogens and, as a result, for the development of symptomatic vaginal infection are both dependent on the host cells and many factors of fungal virulence [43,45]. The pathogenicity of *Candida* spp. in VVC is related to the following: adhesive properties, both in relation to abiotic surfaces and to mucous membranes; the synthesis and secretion of many hydrolytic enzymes, among others, hydrolases or phospholipases; the formation of biofilm structure; the ability to grow in extreme environmental conditions; reversible filamentation; phenotypic switching [42,43,45–47]. Disputes are ongoing to determine which of these factors is the most important in the development of VVC, with many authors pointing to its multifactorial pathogenesis. Thus, the key task seems to be to gain knowledge about the interactions between vaginal epithelium and the fungi of the genus *Candida* and about the mechanisms of cell invasion by these microorganisms.

4.1 Adhesion

The adhesion ability is unquestionably the key factor in the virulence of *Candida* spp., necessary for both the initiation of colonization and the development of infection [42,47,48]. *Candida* spp. cells are capable of adhering to biotic surfaces, as well as to various types of biomaterials [42,44,47,49]. Adhesion is also the first stage of forming a biofilm structure, allowing mature biofilm to be formed on both types of surfaces. In the context of the pathogenesis of VVC, the ability of *Candida* spp. to adhere to the surface of the mucous membrane of the vaginal epithelium is important. This process most likely determines the development of the infection. However, according to epidemiological data, a symptomatic vaginal infection may also be associated with the adhesion of fungal cells to the surface of intrauterine devices, with the formation of a biofilm structure on them and with the invasion of the surrounding tissues [47].

Adhesion is a complex and multifactorial process. The earliest stages of adhesion consist of the interaction of yeast cells with the colonized surface by means of non-specific interactions associated primarily with cell hydrophobicity and the occurrence of electrostatic forces [8]. The degree of adhesion depends on the type and species of the microorganism, the response of host cells to the presence of a given strain, and the properties of the surface structure and its composition [42]. The surfaces of *C. albicans* and *C. glabrata* cells show a similar degree of hydrophobicity. In the case of *C. glabrata*, the degree of hydrophobicity appears to be more stable under changing environmental conditions, which is indicated as the reason for the twice higher tendency of these strains to adhere to acrylic surfaces of prostheses in comparison to *C. albicans* [42,50].

Further phases of adhesion are associated with the presence of adhesins—specific adhesive proteins [8,42,44,47,48]. The expression of adhesive proteins occurs in the fungal

cell wall. They mediate adhesion by recognizing and binding their specific ligands (laminin, fibronectin, collagen, fibrinogen, vitronectin or complement proteins) on the surface of the host cells [8,47]. Different adhesive proteins often demonstrate various expression on blastospores and hyphae in different species and have different mechanisms of action [48].

One of the adhesin groups characterized in most detail are Als proteins, encoded by a large family of *ALS* (agglutinin-like sequence) genes [8,42,47,48]. It is a group of eight proteins (Als1–7 and Als9) bound to glycosylphosphatidylinositol (GPI). Each consists of three domains: N-terminal, having a region binding to a specific substrate, centrally located tandem repeat sequences, and C-terminal, containing the GPI anchor sequence. *ALS* genes are present in *C. albicans* strains, and they were thought to be specific to this species. However, a family of five *ALS* genes in *C. parapsilosis* and three *ALS* genes in *C. tropicalis* was discovered. Additionally, Als3 homologues were found in *C. dubliniensis*, *C. guilliermondii* and in *C. lusitaniae* [8,42,48].

Two approaches are currently developed and used to assess the role of Als in adhesion. These include studying the expression of individual Als characteristics for *C. albicans* in *Saccharomyces cerevisiae* which do not demonstrate adhesive properties, and determining the degree of adhesion in mutants in which both copies of a given *ALS* gene have been removed. What is surprising is a quite frequent lack of compatibility of the results obtained using individual methods. Increased expression of Als5 and Als6 in *C. albicans* correlated with an increase in the degree of adhesion, while gene deletion for these proteins also resulted in increased adhesion. The authors of the tests indicate that perhaps deletion results in a compensatory increase in gene expression for other adhesins and hence the cumulative increase in the adhesion of such strains [48]. Confirmation of this hypothesis requires more detailed research, as does the determination of the exact role of individual Als proteins in adhesion [42,44,47,48].

Another group of adhesive proteins are epithelial adhesins (Epa), whose *EPA* gene expression is induced by the presence of nicotinic acid. Epa1, with the properties of lectin dependent on calcium ions, is considered the most important representative of this group of proteins [42,51]. Interestingly, it has been shown that gene deletion only for Epa1 results in an almost complete loss of adhesive abilities in a given strain [42]. Epa are associated with the adhesion of *C. glabrata* strains to epithelial cells and biomaterials. Unfortunately, the mechanism of their action and their role in adhesion are not very well characterized and require more in-depth analysis [8,42,51].

Another crucial factor in the adhesion of fungal cells to the epithelium is Hwp1 (hyphal wall protein 1)—the protein of the cell wall characteristic of the hyphae form [8,41,44,48]. Hwp1 is one of the key virulence factors of yeast-like fungi occurring as hyphae, displaying a unique mechanism of action. Hwp1 has a GPI anchor domain like adhesive Als and Epa, while its N-terminal sequence is a substrate for transglutaminases binding to epithelial cells. Thanks to these enzymes, Hwp1 (and the entire fungal cell) binds to various proteins on the surface of the host epithelium. It has also been proven that this adhesin can bind other adhesion proteins, e.g., Als1 and Als3 *C. albicans*, conditioning the auto-adhesion of blastospores-hyphae and hyphae-hyphae, a key interaction within the biofilm structure. Strains with both copies of the gene deleted for Hwp1 showed a drastically reduced virulence in a mouse model of oropharyngeal candidiasis, which indicated that this adhesin is a key virulence factor in *Candida* spp. [48].

Fungi of the genus *Candida* express many other adhesion proteins and non-protein factors with similar properties, such as Eap1, Iff4, Mp65, Ecm33, Utr2, Int1, or Mnt1 [8,48]. However, because they have not yet been fully characterized and their mechanism of action is unknown, they will not be discussed in more detail. Therefore, it is currently impossible to determine which of them and how exactly they interact with the mucous membrane of the vagina and what role they play in the pathogenesis of VVC.

4.2 Hydrolytic Enzymes

The production of hydrolytic enzymes serves *Candida* spp. strains both to obtain nutrients by digesting various molecules, and it is also a virulence factor. Enzymes facilitate tissue invasion and inactivation of the components of the host immune system [52]. Although most authors are predominantly interested in various hydrolases secreted extracellularly by yeast-like fungi, *Candida* strains also have the ability to produce endopeptidases [53]. The most important groups of hydrolytic enzymes include proteases, hemolysins, as well as lipases, and phospholipases [8,42,47,53–56].

Secreted aspartyl proteases (Sap) are the *Candida* enzyme group that has been characterized most extensively and in most detail. They are encoded by a large family of *SAP* genes. To date, ten genes have been identified in *C. albicans* (*SAP1–10*), three in *C. parapsilosis* (*SAPP1–3*), at least four in *C. tropicalis* (*SAPT1–4*), and more recently, seven genes in *C. dubliniensis* [24,42,47,50,54]. *C. glabrata* is the only one without these genes, and it is capable of producing 11 yapsins, proteolytic enzymes encoded by a cluster of 11 *YPS* genes. They are highly similar to Sap and *S. cerevisiae* yapsins, anchored on the cell surface, of fundamental importance in maintaining cell wall integrity, adhesion, and survival in macrophages [8,53,54]. Sap is associated with the formation of hyphae, increased adhesion, and phenotypic variability, so they facilitate colonization and invasion of host tissues. In addition, they can degrade defense proteins produced in response to the presence of fungi: immunoglobulins, α-macroglobulin, lactoperoxidase, collagen, creatine, mucin, fibronectin, or complement proteins (C3b, C4b, C5) [24,45,52,53,57]. Conventionally, they were classified into three groups based on substrate specificity and amino acid sequence homology: Sap1–3 with sequence homology of up to 67% and much greater importance in mucous membrane infections, Sap4–6 with sequence homology of nearly 90%, associated with systemic candidiasis, and Sap7 (and, according to some authors, also Sap10), which are the most distinguishable in this family of enzymes, whose functions have not been fully identified [53,57,58]. At the same time, Sap1–3 are produced by blastospores, while the expression of *SAP4–6* genes occurs mainly at the ends of hyphae [8,24,53]. Interestingly, contrary to their name, Sap9 and Sap10 are classified as endopeptidases and are anchored with GPI to the cell surface. Their functions are related, like *C. glabrata* and *S. cerevisiae* yapsins, to the maintenance of cell wall integrity and adhesion [53,54,58]. Knowledge about secretory proteases of fungi of the genus *Candida* is constantly expanding, and given the multitude of available in-depth studies on their structure and function as well as detection methods, the authors of this study will focus only on the potential role of Sap in the pathomechanism of vaginal mucosa infection [8,24,42,47,52–54,58,59].

Unlike other proteases, Sap have proteolytic activity only in an acidic environment, with a pH of four at most [47]. The fact that the normal pH value in the vaginal lumen is about four is the basis for the assumption that these enzymes may be one of the key virulence factors leading to the development of full-blown VVC. As already mentioned above, secretory aspartyl proteases possess wide substrate specificity. They can degrade many free and host cell-bound proteins that disrupt colonization and invasion by yeast-like fungi [56]. What seems to be the most important is the destruction by Sap of various compounds on the mucous membrane and the disturbance of homeostasis prevailing in this place, as well as the recognition and hydrolysis of inflammatory mediators produced in response [41,43]. The last two to three decades witnessed an abundance of studies aiming to determine whether *SAP* gene expression varies depending on the infection site. Such studies could explain the pathomechanism of various fungal infections by identifying specific enzymes that are crucial for developing candidiasis of a given type. This would make it possible to design experiments looking for ways to inhibit these Sap as part of a new antifungal therapy. Unfortunately, so far, the results of the above experiments have often been contradictory, and they failed to provide sufficiently reliable evidence documenting the role of individual Sap in various types of candidiasis.

When discussing mucosal infections caused by *Candida* spp., the most frequent comparison is between oral candidiasis and vaginal candidiasis. A group of authors has indicated

that aspartyl proteases can be found in the discharge of only sick women with VVC, as opposed to asymptomatic carriers [56,60]. However, most studies demonstrate that all described Sap can be isolated from *Candida*-containing samples taken both from patients and from only colonized individuals [58]. A study of *SAP* gene expression levels showed that Sap1–3 most likely play a key role in both oropharyngeal and VVC infections [24,53,58]. Mutants lacking all three genes caused significantly less damage to the mucosal epithelium and reduced the activity of pro-inflammatory cytokines in these sites. Confirmation was obtained in studies conducted on mouse models and on strains isolated from infected hu- mans. However, experiments using reconstituted human epithelium (RHE) cells contradict these reports [53]. Sap2 was the dominant protease of *C. albicans* isolated from oral and vaginal infections and colonizing these areas [24,58]. Sap1 (dominant) and Sap3—both closely related to the phenomenon of phenotypic switching—turned out to be much less common proteases in asymptomatic carriers. Comparing the results obtained during the study and literature data, the authors pointed to the colonization and infection of the vaginal epithelium as the likely main role of Sap3. In combination with Sap1, on the other hand, this role is to enable adaptation to changing environmental conditions and survival in the lumen of the vagina [58].

Next to Sap, an often-mentioned group of hydrolases are phospholipases. They hydrolyze the phospholipids of cell membranes, contributing to the induction of host cell lysis and further penetration into the tissue. They are directly related to the progression of fungal infection [8,42,45,47,50,52,53,55]. There are four classes of phospholipases, depending on the ester bond they cleave: A, B, C, and D. Almost all *Candida* spp. strains are capable of synthesizing various phospholipases belonging to all these classes. *C. albicans* produces much higher amounts of them than the representatives of the NCAC group [8,42,47,50,52,58]. The literature provides much less information about this group of enzymes, although they are an object of interest to an increasing number of researchers. Unfortunately, the few experiments looking for gene expression patterns for individual phospholipases, such as *PLB1*, *PLB2*, *PLC1*, or *PLD1*, did not allow us to draw valid conclusions about their role in the pathogenesis of mucous membrane infections [47,53,58].

Regarding yeast-like lipases produced by fungi, the literature also provides only a limited amount of information. They are involved in the hydrolysis and synthesis of triacylglycerols. Most likely, they increase adhesion and penetration of host tissues, as well as survival in macrophages [42,47,52,53,59]. There are currently ten known *LIP* genes in *C. albicans*, 2 in *C. parapsilosis,* and one lipase sequence in *C. tropicalis* [42,50,53,59]. However, preliminary reports indicate that the role of lipases in mucous membrane infections is limited and rather insignificant in VVC [53].

Among other enzymes acting as virulence factors of *Candida* spp. are hemolysins which are necessary for iron acquisition and survival in the host organism [41,42,45,47,50] and esterase with probable cytotoxic effects [55]. However, they are discussed sporadically, and there is a lack of detailed information about their structure and function or about their role in the development of individual types of candidiasis.

The experiments conducted using various research models and enzyme detection methods did not show any correlation between specific hydrolytic enzymes and infection, including VVC. It is highly likely that gene expression and their levels for different *Candida* enzymes change not only depending on the site of infectionbut also during disease progression, depending on its severity [58].

4.3 Adaptability to Changing Environmental Conditions, Dimorphism and Phenotypic Switching

For many years, it was thought that one of the factors distinguishing *C. albicans* from other *Candida* species is the ability of these strains to grow in various morphological forms. This ability is called dimorphism, where attention is drawn to the possibility of *C. albicans* being present in the form of blastospores or blastoconidia (terms used interchangeably) and filaments. Equally often, one can find the term polymorphic growth in connection with the occurrence of four forms: blastospores, germ tubes, pseudo-hyphae, and true

hyphae [8,24,42,44,50,59–64]. It has been confirmed that *C. dubliniensis* is also a truly polymorphic fungus [24,42,50,59,62,65]. At the same time, the possibility of forming pseudohyphae by *C. parapsilosis* and *C. tropicalis* is described [42,50,66]. An example of a *Candida* species lacking this ability is *C. glabrata*, which grows only in the form of extremely fine blastospores [42,50].

The phenomenon of dimorphism is closely related to the adaptability of *Candida* spp. to the surrounding microenvironment. The response to external signals, consisting in changing the morphology of fungal cells, is possible due to phenotypic switching. Typical examples include the ability of the described strains to adapt to changes in temperature, pH, oxygen availability, lack of nutrients, or the presence of serum by inducing filament growth. An important feature of phenotypic switching is its reversibility and the possibility of returning from the hyphae form to growing blastoconidia [8,60,61,63,64,66].

Studies show that the form of blastospores or yeast (Y form) is associated primarily with the colonization and spread of *Candida* in the host organism. Hyphae (H form) is considered invasive and capable of adhesion, secretion of proteolytic enzymes, and tissue destruction [8,24,43,56,60–64,66]. In addition, unlike blastospores, filaments have the ability to survive in macrophages and show reduced susceptibility or resistance to antifungal agents active against Y forms [50,60,64–66]. In this way, the importance of the phenomenon of phenotypic switching as a virulence factor with a significant role in infection is proved. The hypothesis that the presence of hyphae, and thus phenotypic switching, is required by *Candida* spp. for its virulence and for the development of infection is questioned [59–61,63,64]. Difficulties in confirming or denying this hypothesis concern the way in which the role of individual phenotypes in candidiasis is studied. In experiments on animal models, mainly mouse and rat models, comparisons are made between the virulence of wild strains and of mutants with the deletions of specific genes involved in changing the morphology from blastospores to filaments and vice versa. When conducting experiments in this way, two important issues should be kept in mind: the different pathogenesis of fungal infections in laboratory animals and the enormous complexity of molecular processes involved in phenotypic switching. The most common animal model of candidiasis is the mouse model of disseminated infection, in which the pathogen is applied directly into the caudal vein. Obviously, such an experiment cannot reflect the processes leading to the development of mucosal infection and endogenous infections. It does not provide sufficiently reliable information about the interactions of the Y and H forms with the epithelium [60,64]. Intensive genetic studies over the past two decades have provided a wealth of extremely valuable information on the regulation and causes of morphological and phenotypic changes in fungi of the genus *Candida*, although the authors point out that there is still more to be discovered than is known. The described phenomena involve both transcription factors promoting growth in the form of hyphae (such as Efg1, Cph1, Cph2, Tec1, Czf1, Ndt8, and Rim101) and negative hyphae-specific regulators (e.g., Tup1, Nrg1, Rfg1), as well as entire signal transduction pathways. At the same time, they are also involved in the regulation of other factors and processes related to other virulence factors of *Candida*. For this reason, it is not possible to distinguish reliably
enough whether the reduced virulence of the mutant *C. albicans* with gene deletion for Efg1 and Cph1 (which makes these strains unable to form filaments) is associated with the absence of a hyphae form. The reason may also be the reduced gene expression for the cell wall proteins—adhesins and Sap—which are also affected by the aforementioned Efg1 and Cph1 and their activation pathways [24,59,61,64]. Thus, the very definition of dimorphism and phenotypic switching as virulence factors with a specific role in developing candidiasis causes many controversies.

In the context of VVC of *Candida* etiology, the percentage of asymptomatic carriers of these microorganisms in the vagina should be taken into account. The above means that these fungi are tolerated by the immune system of the carrier women. *Candida* spp. is called commensal flora in such cases, although it is unclear whether the host organism benefits from its presence in this site [60,64]. Despite only scarce reports on the role of hyphae and

phenotypic switching in VVC, the authors indicate quite unequivocally that in the case of samples isolated from patients with an active infection, only filament forms are present, while in asymptomatic carriers, there are only blastopores. There are reports that also in in vitro cultures of VVC-isolated strains and systemic infections, the phenotypic switching level was higher than in healthy, only colonized subjects [43,56,59,60,63,64]. It is believed today that blastospores adhere to the vaginal epithelium and only then abruptly switch into hyphae—similarly to the development of infections in the oral cavity and gastrointestinal tract. Filaments are not tolerated by the immune system and induce a specific inflammatory response. Two ways of mucosal invasion are possible: by induction of endocytosis through hyphae (a mechanism not fully understood) and by active penetration into the tissue. In the oral cavity environment, both pathways have been observed, with endocytosis dominating initially. As regards GI candidiasis, only active penetration of the mucous membrane occurs [64]. Unfortunately, no similar observations have yet been made for VVC. The H-form shows the activity of more virulence factors than blastospores, such as enzymes from the Sap family, with an undetermined role in the pathogenesis of VVC. Recently, it has also been pointed out that higher filament virulence is associated with factors that allow disruption and avoidance of the host's immune response rather than with the expression of virulence factors [60]. Some authors explicitly suggest that the role of hyphae formation, while undoubtedly significant, is not crucial in the development of VVC [63].

Phenotypic switching enabling *Candida* fungi to grow in the form of blastoconidia and filaments is certainly not irrelevant for the symptomatic development of VVC. However, a detailed determination of the role of individual growth forms requires more thorough research and careful analysis, also taking into account other virulence factors associated with hyphae as well as molecular pathways involved in their regulation.

4.4 Biofilm

Biofilm is defined as a highly heterogeneous, multicellular, and multilayered three-dimensional structure in which cells are surrounded by an extracellular matrix (ECM), specialize in performing specific functions, and communicate with each other [8,42–44,46,47,49,50,67–70]. The great interest of researchers in biofilm structure is primarily related to its clinical significance—biofilm is highly resistant to antimicrobials and the host defense mechanisms [8,41,42,44,46,47,68,70,71]. At the same time, it is clearly indicated that it is the preferred and widespread form of growth of most microorganisms [44,47,50]. Hence, research on both the pathomechanism of various infections and their treatment must undoubtedly consider the biofilm-forming ability of the analyzed microorganisms.

Both bacteria and fungi, including *Candida* spp., can produce biofilm. Increasingly frequently, multi-species and mixed (bacterial-fungal) biofilms are studied [72–74]. They are widely described in the literature; hence their detailed characteristics will not be presented in this paper.

Among yeast-like fungi of the genus *Candida*, almost all clinically relevant species form biofilm. Classically, the process of forming this structure is divided into four phases: early (adhesion), intermediate (multiplication, filamentation, and ECM production), maturation, and spreading (dispersion) [8,44,69,71,75]. The development of individual phases, including their duration and the architecture of mature biofilm, depend on *Candida* species and on environmental conditions, e.g., the surface to which the cells adhere [8,41,42,69,71]. Nevertheless, the general basic structure of fungal biofilm and its characteristics always remain similar. Importantly, the results of observation of biofilm structure and its formation obtained in vitro on biomaterials have already been confirmed in in vivo and ex vivo animal models, including biotic surfaces, primarily the mucous membrane (vaginal, among others) [41,44,67,69,70,75].

Basically, all virulence factors of *Candida* spp. discussed in this chapter are to a higher or lower extent related to biofilm formation (Table 1). Adhesion is the first and decisive stage initiating the development of biofilm in a given microenvironment. Many

authors treat biofilm as a consequence of the existence of adhesion [8,41,44,47,50,67,75]. *Candida* spp. forms biofilm composed of blastospores and hyphae cells which serve as a scaffold for the entire structure and provide many proteins, such as the already mentioned adhesins [8,42,69,71,75]. Additionally, dispersion is possible owing to dimorphism and phenotypic switching, and blastospores detaching from the surface of mature biofilm show, among others, a higher tendency for adhesion [71,75]. Once again, attention should be paid to the multitude of complex dependencies and interactions of individual virulence factors and their components, as well as molecular mechanisms underlying their functioning. They are a significant obstacle for researchers in their efforts to define and isolate specific factors related to the pathomechanism of candidiasis.

Table 1. The most important virulence factors associated with *Candida* biofilm formation during VVC and their functions.

Factor	Genes	*Candida* Strains	Function in Biofilm Formation during VVC	References
Als proteins	family of *ALS*	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. dubliniensis* *C. guillermondii* *C. lusitaniae*	adhesion to VECs	[8,42,47,48]
Epa protein	*EPA*	*C. albicans* *C. glabrata*	adhesion to VECs	[8,42,51]
Hwp1 protein	*HWP1*	*Candida spp.*	adhesion to VECs	[8,41,44,48]
Sap enzymes	family of *SAP*	*C. albicans* *C. parapsilosis* *C. tropicalis* *C. dubliniensis*	adhesion to VECs, phenotypic switching, formation of hyphae and its adhesion and penetration into VECs	[24,42,47,50,54]
yapsins	*YPS*	*C. glabrata*	adhesion to VECs, phenotypic switching, formation of hyphae and its adhesion and penetration into VEC	[8,53,54]
lipases	*LIP*	*C. albicans* *C. parapsilosis* *C. tropicalis*	increasing adhesion to VECs	[42,50,53,59]

In the case of VVC, the hypothesis regarding the key role of biofilm in the development of full-blown infection and the transition of the commensal form of *Candida* spp. into pathogenic form is associated with two main observations. First, routine microbiological diagnostics, carried out using classical methods of identification and drug susceptibility testing (in vitro), most often indicates the susceptibility of yeast-like fungi isolated from the vagina to conventionally used antimycotics—mainly fluconazole. Meanwhile, clinicians more and more often observe therapy failures and recurrences of infections. The existence of biofilm in vaginal mucosa, a structure highly resistant and impermeable to most antimicrobials, could explain the ineffectiveness of antifungal drugs, as well as constitute a reservoir of persister cells and be the cause of VVC recurrence [40,41,45,47,49,70,72,76]. Secondly, in the case of the most common type of infection in the vagina, BV (bacterial vaginosis), it has already been proven that the phenomenon of biofilm formation is important for the development of infection and is its underlying cause. Hence the suspicion that a similar pathomechanism could underlie fungal vulvovaginitis [47,67,70,77]. A similar situation occurs in the presence of biomaterials in the vaginal environment, e.g., an intrauterine device. In this case, it is already clear that biofilm is the basic cause and source of fungal infection [47,78]. The above arguments are relatively often cited as evidence for the role of biofilm in the pathogenesis of VVC and RVVC [72]. The above produces further questions for researchers—is biofilm necessary for vaginal colonization and the development of candidiasis? Is it the cause of the transition of saprophytic fungi of the genus *Candida* into pathogens in the vaginal environment? Does the growth of fungi in the form of biofilm affect the host's immune response? [41,67,69,70] These questions remain unanswered. To our knowledge, the only report so far focusing on the observation of the *Candida* biofilm directly on the vaginal epithelium contradicts the hypothesis of the key role of this structure in the development of VVC, arousing skepticism in the scientific community about such a high interest in biofilm in the context of vaginal candidiasis [40,79].

Even if it is not the source of infection and does not determine the pathogenesis of infection, biofilm in VVC and RVVC remains an important factor in the virulence of fungi of the genus *Candida*. However, the significance of this phenomenon remains a hypothesis that requires many further in-depth studies on the interaction of fungi with the surface of the vaginal mucosa.

5. Pathogenicity of Fungi of the Genus *Candida* spp. Associated with Vaginal Infections—Host Immune Response

The role and significance of specific virulence factors of *Candida* are not the only factors that have remained unknown until today. After many years of intensive research on the immune mechanisms involved in the pathogenesis of VVC, there is still more to be discovered than is known. Nevertheless, especially in the last few years, several factors, and mechanisms potentially crucial for the development of fungal infection in the vagina have been identified, related to the host's response to the presence of *Candida*.

It turned out relatively quickly that the acquired cellular or humoral immune response plays no role in VVC. Similarly, searches for the potentially protective role of innate immune components, such as macrophage or dendritic cell activity, did not reveal their effect on the vagina [43,80–82]. What has now been proven, vaginal epithelial cells (VEC) should be considered the first line of the host's defense. On their surface, there are so-called PRRs (pattern recognition receptors) responsible for the recognition of *Candida*. Many authors emphasize that it is VEC that has the ability to distinguish and determine the fungi present on the vaginal mucosa as commensal or as pathogenic [43,80,81,83,84]. The most frequently cited hypothesis indicates the fungistatic action of epithelial cells, significantly limiting the development of infection and promoting the state of asymptomatic commensalism. The mechanisms underlying this phenomenon remain unknown. Annexin A1 can probably play a role here. The above way of VEC response predominates in women who are not susceptible to fungal infection, most often without a history of VVC/RVVC, in whom there is no further activation of the immune system [80,82]. This possibility is presented in Figure 1.

In women susceptible to infection (Figure 2), PRRs on the VEC surface recognize the

components of the *Candida* cell wall and, in response, begin the production of a number of pro-inflammatory cytokines and alarmins, which in turn have a chemotactic effect on the PMN (polymorphonuclear) neutrophils located in the tissue stroma. Strong infiltration of vaginal tissue by PMN has been repeatedly shown in women manifesting clinical symptoms of VVC. It has been possible to clearly demonstrate that it is the immune response associated with PMN that is the cause of the symptoms of infection. In this way, one of the strongest pieces of evidence for the immunopathological origin of vulvovaginal candidiasis was provided, with VVC being an infection resulting not so much from the presence of the pathogen but the massive inflammatory response of the host organism and the resulting damage [43,80,81,84,85].

PMN cells migrating to the site of infection in response to the signals sent are not capable of effectively reducing or eliminating fungal cells. It has also been confirmed that they are not responsible for the damage to vaginal tissue associated with the infection that is observed during VVC [80,81]. Therefore, it is clear that the mechanism described above cannot be the only one underlying the infection in question. The virulence factors of fungi of the genus *Candida* and the resulting inflammatory response are also crucial. The most important in the immune context now seems to be phenotypic switching and hyphae growth. One of the most important factors associated with hyphae and identified recently is candidalysin, a toxin with lytic activity encoded by the *ECE1* (extent of cell elongation 1) gene. It works in two ways—on the one hand, it has the ability to activate the NLRP3 inflammasome; on the other hand, it causes damage to vaginal tissue directly [80,81,85]. NLRP3 is an intracellular receptor complex that performs a protective function in most types of candidiasis. In the vagina, however, after its oligomerization, caspase-1 and a further signal path are activated, because of which IL-1β, the main effector of inflammation, as well as alarmin S100A8, are produced and released. As a result, large amounts of many different pro-inflammatory cytokines, chemokines, and alarmins are released, which are also responsible for the recruitment of PMN to the site of infection [43,60,80,81,84–86]. Since PMNs are not able to effectively kill fungal cells in this case, the next step is their death and granular release, which, in positive feedback, causes further cytokine release and chemotaxis of subsequent PMNs, intensifying the inflammation [80,81]. In addition to candidalysin, other hyphae-related factors are also indicated that could act similarly, such as some Sap (although their role remains controversial), Als, lipases, or SAA3, activating NLRP3 [60,80,81,85,86].

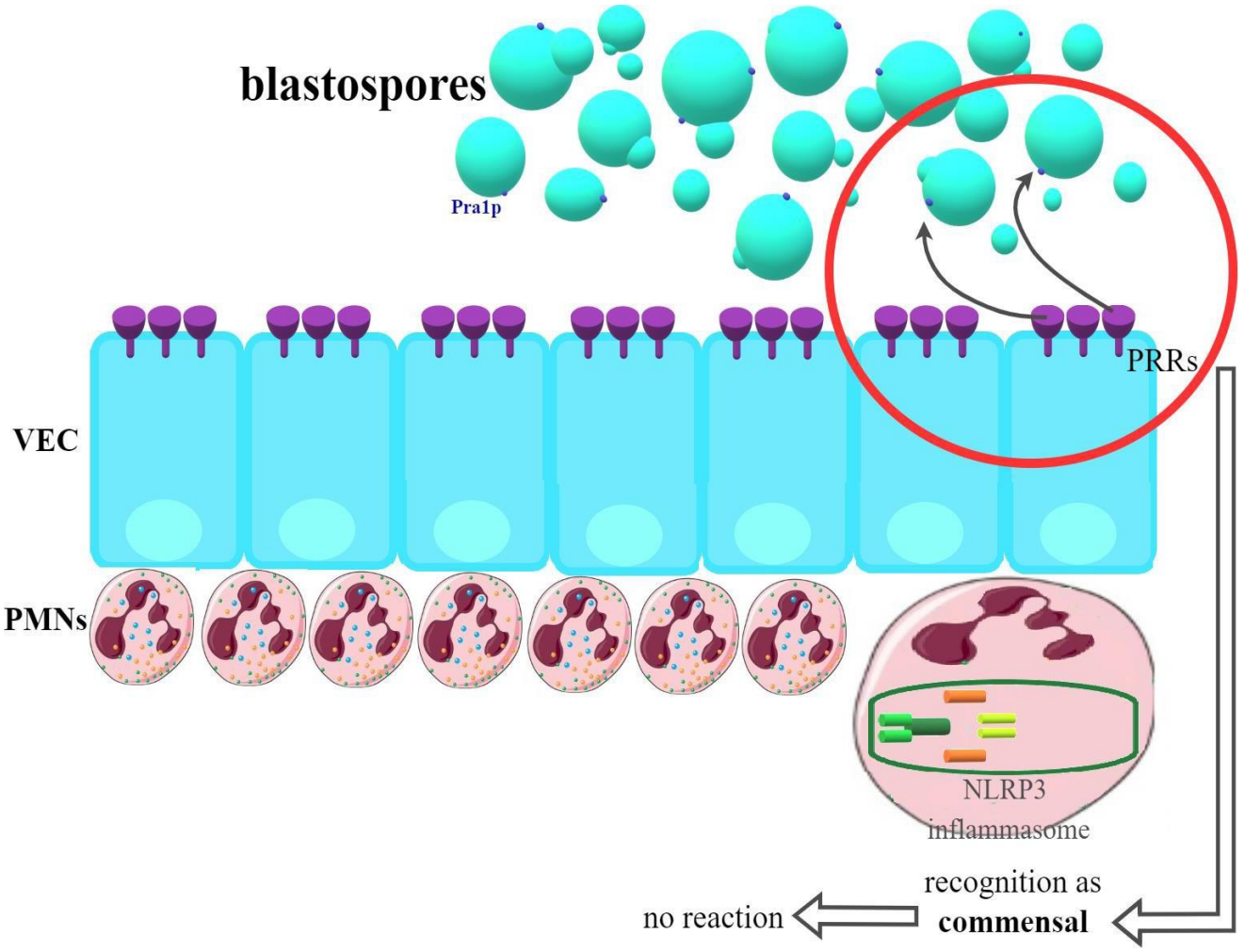


Figure 1. Recognition of *Candida* blastospores by VEC (vaginal epithelial cells) in women not susceptible to VVC (vulvovaginal candidiasis). PRRs (pattern recognition receptors) classify blastospores as commensals—no immunological reaction is provided.

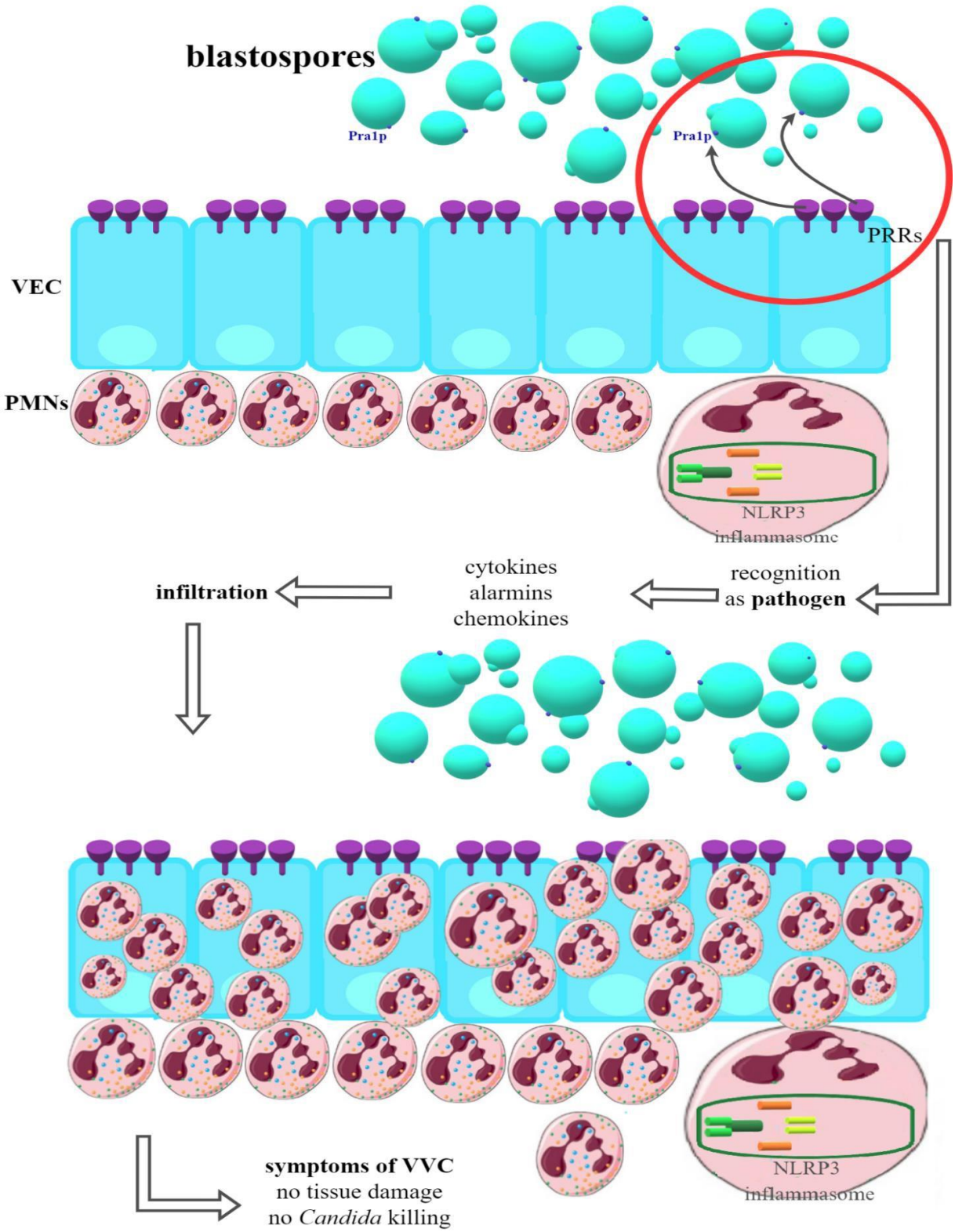


Figure 2. Recognition of *Candida* blastospores by VEC (vaginal epithelial cells) in women susceptible to VVC (vulvovaginal candidiasis). PRRs (pattern recognition receptors) classify blastospores as pathogens—production of proinflammatory cytokines, chemokines and alarmins is started. PMNs (polimorfonuclears) infiltrate VECs but are unable to kill *Candida* or directly damaging tissue. This state relates to clinical symptoms of VVC (detailed mechanism still unknown).

Candidalysin also causes direct damage to the VEC—it is now considered a key factor responsible for this effect. Experiments were carried out, which proved that the mere presence of hyphae (in the absence of toxin-producing ability) does not cause damage to the vaginal tissues during *Candida* infection. Therefore, it is highly probable that this peptide is the main factor behind the invasiveness of the hyphae form of yeast-like fungi and their ability to penetrate deep into the tissues [80,81,84]. In addition to its obvious clinical significance, VEC damage is also associated with yet another relatively recently discovered factor. It has been proven that VEC damage involves the release of, among others, heparan sulfate (HS). Observations confirmed that HS might be responsible for the so-called neutrophil anergy or inability to produce an immune response to an immunogen. The antifungal activity of PMN is conditioned by the specific recognition of fungal cells, which occurs through Mac1 present on neutrophils, binding to Pra1p (pH-regulated antigen 1 protein) in *Candida*. It is worth mentioning that this protein is present in a much higher density in hyphae cells than in blastospores. In other candidiasis types, Mac1 activation promotes the killing of fungal cells by creating so-called NETs (neutrophil extracellular traps). In VVC, this phenomenon does not occur, and it has been proven that HS acts as a competitive ligand for Mac1, blocking the target Pra1p binding point, which prevents the killing of *Candida* and is most likely responsible for the anergy phenomenon. Recent reports also focus on the role of estrogens in the development of infection. Their large amount also causes an increase in the release of HS and thus may contribute to additional impairment of cidal functions of PMNs. A possible role of estrogens in the direct activation of the NLRP3 inflammasome is also indicated [80,81,86]. The described hypothesis is presented in Figure 3.

The above interactions are only the first step in identifying key immune factors relevant to VVC immunopathogenesis. Researchers are currently interested in so many other immune components and mechanisms potentially involved in the host response to the presence of *Candida* on the vaginal mucosa that an attempt at listing them is far beyond the scope of this paper. However, it is worth emphasizing that there is no longer any doubt that for the development of symptomatic vaginal candidiasis, the action of both components associated with the innate immune response and the activity of a number of interrelated virulence factors of yeast-like fungi is necessary.

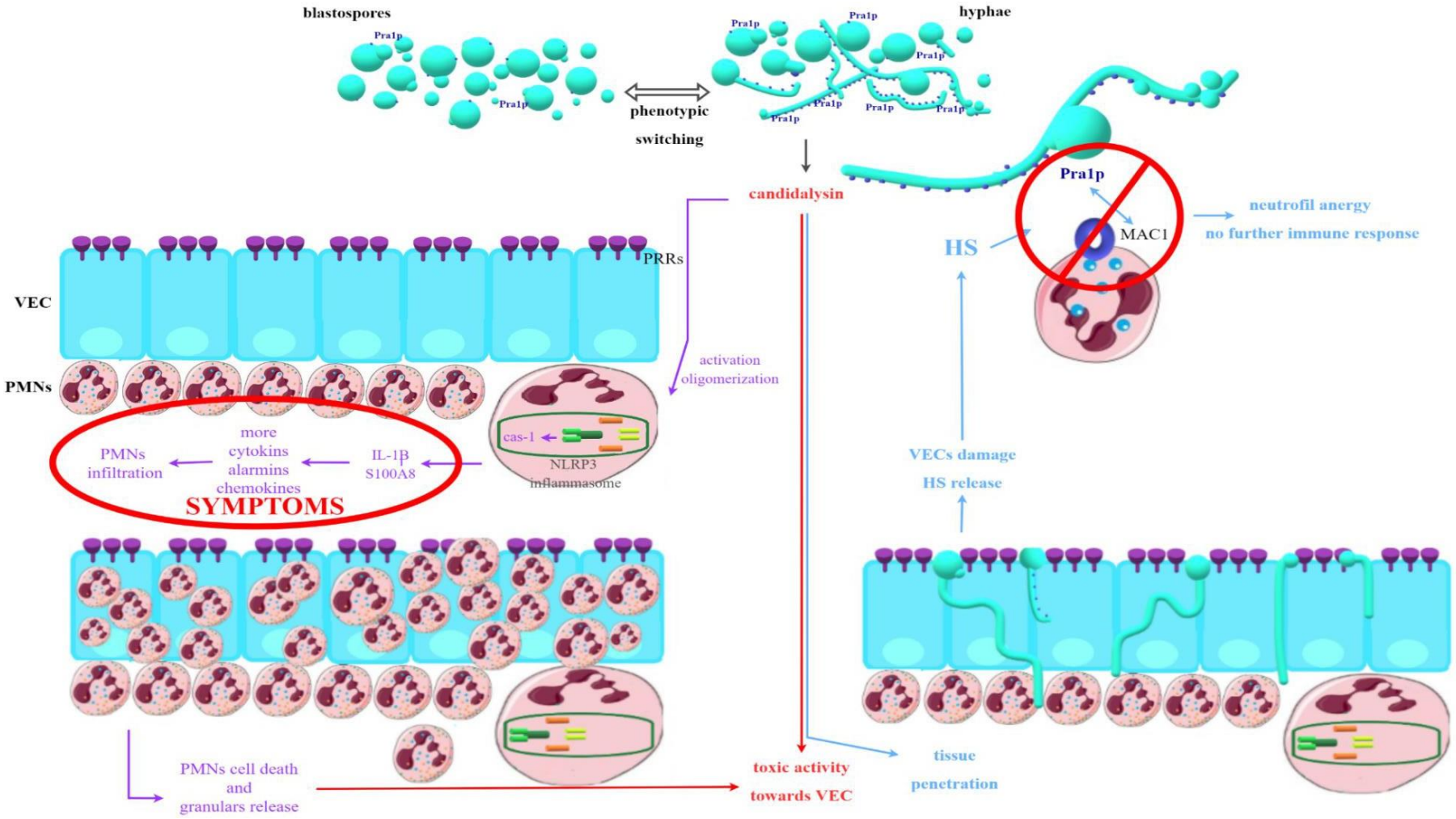


Figure 3. Hypothetical explanation of processes underlying tissue damage and clinical symptoms during VVC (vulvovaginal candidiasis) associated with production and release of candidalysin.

6. Summary

The above-listed factors and mechanisms are not the only ones currently being investigated in the context of VVC pathogenesis. In addition to the enormous complexity of molecular and immunological processes involved in the described phenomena and the difficulties associated with determining the specific functions of individual components, both related to *Candida* and the host's immune system, problems also arise with ensuring reliable experiments in the research laboratory. The tools most commonly used to assess fungus-host interactions are mouse models and, less frequently, rat models. Many of the discussed mechanisms were observed in an animal model and then confirmed in studies using clinical material taken from female volunteers. However, there are many more laboratory observations, mainly regarding mice, waiting to be verified in human studies. However, the proper and reliable design of such studies continues to be difficult. A similar situation applies to experiments using reconstituted vaginal epithelial cells. Despite the above, recent years have undoubtedly brought many milestones in understanding the pathomechanism of the development of VVC. Although there is still a long way to go to understand the development of VVC fully, the identified factors and mechanisms are already the goal of new antifungal therapies under development, aimed both at fighting an active infection and preventing its development.

Author Contributions: P.C. and J.N. wrote the manuscript; G.G. reviewed the manuscript and man- aged the supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subvention according to number of STM.A130.20.102 from the IT Simple system of Wroclaw Medical University as well as financed from the funds granted by the Ministry of Education and Science in the „Regional Initiative of Excellence" programme for the years 2019–2022, project number 016/RID/2018/19, the amount of funding 9 354 023,74 PLN.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Yang, B.; Rao, R. Emerging Pathogens of the Candida Species. *Intech Open Sci.* 2012, *1*, 3–18. [CrossRef]
2. Singh, D.K.; Tóth, R.; Gácser, A. Mechanisms of Pathogenic Candida Species to Evade the Host Complement Attack. *Front. Cell. Infect. Microbiol.* 2020, *10*, 94. [CrossRef] [PubMed]
3. Rodrigues, C.F.; Rodrigues, M.E.; Henriques, M. *Candida* sp. Infections in patients with diabetes mellitus. *J. Clin. Med.* 2019, *8*, 76. [CrossRef] [PubMed]
4. Singh, S.; Fatima, Z.; Hameed, S. Predisposing factors endorsing Candida infections. *Infez. Med.* 2015, *23*, 211–223. [PubMed]
5. Wróblewska, M.; Sulik-Tyszka, B. Candida auris—Epidemiology and laboratory diagnostics of infections. *Diagnostyka Lab.* 2017, *53*, 235–240. [CrossRef]
6. Alberto Cortés, J.; Fernanda Corrales, I. Invasive Candidiasis: Epidemiology and Risk Factors. In *Fungal*

Infection; IntechOpen: London, UK, 2018; pp. 33–46.

7. Vila, T.; Sultan, A.S.; Montelongo-Jauregui, D.; Jabra-Rizk, M.A. Oral candidiasis: A disease of opportunity. *J. Fungi* 2020, *6*, 15. [CrossRef]
8. Sikora, M. Czynniki wirulencji grzybów z rodzaju candida istotne w patogenezie zakażeń wyste˛puja˛cych u pacjentów z˙ywionych pozajelitowo. *Postep. Mikrobiol.* 2015, *3*, 224–234.
9. Arsenault, A.B.; Bliss, J.M. Neonatal Candidiasis: New Insights into an Old Problem at a Unique Host-Pathogen Interface. *Curr. Fungal Infect. Rep.* 2015, *9*, 246–252. [CrossRef]
10. Flevari, A.; Theodorakopoulou, M.; Velegraki, A.; Armaganidis, A.; Dimopoulos, G. Treatment of invasive candidiasis in the elderly: A review. *Clin. Interv. Aging* 2013, *8*, 1199–1208. [CrossRef]
11. Dekkers, B.G.J.; Veringa, A.; Marriott, D.J.E.; Boonstra, J.M.; van der Elst, K.C.M.; Doukas, F.F.; McLachlan, A.J.; Alffenaar, J.W.C. Invasive Candidiasis in the Elderly: Considerations for Drug Therapy. *Drugs Aging* 2018, *35*, 781–789. [CrossRef]
12. Mayer, F.L.; Wilson, D.; Hube, B. Candida albicans pathogenicity mechanisms. *Virulence* 2013, *4*, 119–128. [CrossRef] [PubMed]
13. Lopes, J.P.; Lionakis, M.S. Pathogenesis and virulence of Candida albicans. *Virulence* 2022, *13*, 89–121. [CrossRef] [PubMed]
14. Taei, M.; Chadeganipour, M.; Mohammadi, R. An alarming rise of non-albicans Candida species and uncommon yeasts in the clinical samples; a combination of various molecular techniques for identification of etiologic agents. *BMC Res. Notes* 2019, *12*, 779. [CrossRef] [PubMed]
15. Aydemir, Ö.; Demiray, T.; Körog˘lu, M.; Aydemir, Y.; Altındis¸, M. Emerge of non-albicans Candida species; evaluation of Candida species and antifungal susceptibilities according to years. *Biomed. Res.* 2017, *28*, 2820–2825.
16. Lindberg, E.; Hammarström, H.; Ataollahy, N.; Kondori, N. Species distribution and antifungal drug susceptibilities of yeasts isolated from the blood samples of patients with candidemia. *Sci. Rep.* 2019, *9*, 12–17. [CrossRef]
17. Das, K.H.; Mangayarkarasi, V.; Sen, M. Antifungal resistant in non-albicans candida species are emerging as a threat to antenatal women with vulvovaginal candidiasis. *Biomed. Pharmacol. J.* 2019, *12*, 1369–1378. [CrossRef]
18. Liu, F.; Zhong, L.; Zhou, F.; Zheng, C.; Zhang, K.; Cai, J.; Zhou, H.; Tang, K.; Dong, Z.; Cui, W.; et al. Clinical features, strain distribution, antifungal resistance and prognosis of patients with non-albicans candidemia: A retrospective observational study. *Infect. Drug Resist.* 2021, *14*, 3233–3246. [CrossRef]
19. Ahmad, S.; Alfouzan, W. Candida auris: Epidemiology, diagnosis, pathogenesis, antifungal susceptibility, and infection control measures to combat the spread of infections in healthcare facilities. *Microorganisms* 2021, *9*, 807. [CrossRef]
20. Grondalska, D.; Kmieciak, W. Candida auris—Nowy patogen grzybiczy. *Post. Mikrobiol* 2019, *191*, E865.
21. Garcia-Bustos, V.; Ruiz-Saurí, A.; Ruiz-Gaitán, A.; Sigona-Giangreco, I.A.; Cabañero-Navalon, M.D.; Sabalza-Baztán, O.; Salavert-Lletí, M.; Tormo, M.Á.; Pemán, J. Characterization of the Differential Pathogenicity of Candida auris in a Galleria mellonella Infection Model. *Microbiol. Spectr.* 2021, *9*, e0001321. [CrossRef]
22. Keighley, C.; Garnham, K.; Harch, S.A.J.; Robertson, M.; Chaw, K.; Teng, J.C.; Chen, S.C.A. Candida auris: Diagnostic Challenges and Emerging Opportunities for the Clinical Microbiology Laboratory. *Curr. Fungal Infect. Rep.* 2021, *15*, 116–126. [CrossRef] [PubMed]
23. Talapko, J.; Juzbašic´, M.; Matijevic´, T.; Pustijanac, E.; Bekic´, S.; Kotris, I.; Škrlec, I. Candida albicans-the virulence factors and clinical manifestations of infection. *J. Fungi* 2021, *7*, 79. [CrossRef] [PubMed]
24. Staniszewska, M.; Bondaryk, M.; Kowalska, M.; Magda, U.; Łuka, M.; Ochal, Z.; Kurza˛tkowski, W. Pathogenesis and treatment of fungal infections by Candida spp. [Patogeneza i leczenie zakażeń *Candida* spp.]. *Postep. Mikrobiol.* 2014, *53*, 229–240.
25. Khan, F.; Bamunuarachchi, N.I.; Pham, D.T.N.; Tabassum, N.; Khan, M.S.A.; Kim, Y.M. Mixed biofilms of pathogenic Candida- bacteria: Regulation mechanisms and treatment strategies. *Crit. Rev. Microbiol.* 2021, *45*, 699–727. [CrossRef] [PubMed]
26. Ramage, G.; Martínez, J.P.; López-Ribot, J.L. Candida biofilms on implanted biomaterials: A clinically

significant problem. *FEMS Yeast Res.* 2006, *6*, 979–986. [CrossRef] [PubMed]

27. Vera-González, N.; Shukla, A. Advances in Biomaterials for the Prevention and Disruption of Candida Biofilms. *Front. Microbiol.* 2020, *11*, 2251. [CrossRef]
28. Peng, Z.; Tang, J. Intestinal Infection of Candida albicans: Preventing the Formation of Biofilm by *C. albicans* and Protecting the Intestinal Epithelial Barrier. *Front. Microbiol.* 2022, *12*, 783010. [CrossRef]
29. Zeng, X.; Zhang, Y.; Zhang, T.; Xue, Y.; Xu, H.; An, R. Risk Factors of Vulvovaginal Candidiasis among Women of Reproductive Age in Xi'an: A Cross-Sectional Study. *Biomed Res. Int.* 2018, *2018*, 9703754. [CrossRef]
30. Achkar, J.M.; Fries, B.C. Candida infections of the genitourinary tract. *Clin. Microbiol. Rev.* 2010, *23*, 253–273. [CrossRef]
31. Aniebue, U.U.; Nwankwo, T.O.; Nwafor, M.I. Vulvovaginal candidiasis in reproductive age women in Enugu Nigeria, clinical versus laboratory-assisted diagnosis. *Niger. J. Clin. Pract.* 2018, *21*, 1017–1022. [CrossRef]
32. Auriemma, R.S.; Scairati, R.; del Vecchio, G.; Liccardi, A.; Verde, N.; Pirchio, R.; Pivonello, R.; Ercolini, D.; Colao, A. The Vaginal Microbiome: A Long Urogenital Colonization Throughout Woman Life. *Front. Cell. Infect. Microbiol.* 2021, *11*, 613. [CrossRef] [PubMed]
33. Lema, V.M. Recurrent Vulvo-Vaginal Candidiasis: Diagnostic and Management Challenges in a Developing Country Context. *Obstet. Gynecol. Int. J.* 2017, *7*, 00260. [CrossRef]
34. Hoffmann, J.N.; You, H.M.; Hedberg, E.C.; Jordan, J.A.; McClintock, M.K. Prevalence of bacterial vaginosis and Candida among postmenopausal women in the United States. *J. Gerontol. Ser. B Psychol. Sci. Soc. Sci.* 2014, *69*, S205–S214. [CrossRef] [PubMed]
35. McClelland, R.S.; Richardson, B.A.; Hassan, W.M.; Graham, S.M.; Kiarie, J.; Baeten, J.M.; Mandaliya, K.; Jaoko, W.; Ndinya-Achola, J.O.; Holmes, K.K. Prospective study of vaginal bacteria flora and other risk factors for vulvovaginal candidiasis. *J. Infect. Dis.* 2009, *199*, 1883–1890. [CrossRef]
36. Ceccarani, C.; Foschi, C.; Parolin, C.; D'Antuono, A.; Gaspari, V.; Consolandi, C.; Laghi, L.; Camboni, T.; Vitali, B.; Severgnini, M.; et al. Diversity of vaginal microbiome and metabolome during genital infections. *Sci. Rep.* 2019, *9*, 14095. [CrossRef]
37. Konadu, D.G.; Owusu-Ofori, A.; Yidana, Z.; Boadu, F.; Iddrisu, L.F.; Adu-Gyasi, D.; Dosoo, D.; Awuley, R.L.; Owusu-Agyei, S.; Asante, K.P. Prevalence of vulvovaginal candidiasis, bacterial vaginosis and trichomoniasis in pregnant women attending antenatal clinic in the middle belt of Ghana. *BMC Pregnancy Childbirth* 2019, *19*, 1–10. [CrossRef]
38. Ang, X.Y.; Mageswaran, U.M.; Chung, Y.L.F.; Lee, B.K.; Azhar, S.N.A.; Roslan, N.S.; Saufian, I.F.B.; Mustaffa, N.S.; Kalam, E.M.; Ibrahim, A.F.; et al. Probiotics Reduce Vaginal Candidiasis in Pregnant Women via Modulating Abundance of Candida and Lactobacillus in Vaginal and Cervicovaginal Regions. *Microorganisms* 2022, *10*, 285. [CrossRef]
39. Z˙ yrek, D.; Wajda, A.; Czechowicz, P.; Nowicka, J.; Jas´kiewicz, M.; Neubauer, D.; Kamysz, W. The antimicrobial activity of omiganan alone and in combination against candida isolated from vulvovaginal candidiasis and bloodstream infections. *Antibiotics* 2021, *10*, 1001. [CrossRef]
40. Swidsinski, A.; Guschin, A.; Tang, Q.; Dörffel, Y.; Verstraelen, H.; Tertychnyy, A.; Khayrullina, G.; Luo, X.; Sobel, J.D.; Jiang, X. Vulvovaginal candidiasis: Histologic lesions are primarily polymicrobial and invasive and do not contain biofilms. *Am. J. Obstet. Gynecol.* 2019, *220*, e1–e91. [CrossRef]
41. Rodríguez-Cerdeira, C.; Gregorio, M.C.; Molares-Vila, A.; López-Barcenas, A.; Fabbrocini, G.; Bardhi, B.; Sinani, A.; Sánchez-Blanco, E.; Arenas-Guzmán, R.; Hernandez-Castro, R. Biofilms and vulvovaginal candidiasis. *Colloids Surf. B Biointerfaces* 2019, *174*, 110–125. [CrossRef]
42. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D.W.; Azeredo, J. Adherence and biofilm formation of non-Candida albicans Candida species. *Trends Microbiol.* 2011, *19*, 241–247. [CrossRef] [PubMed]
43. Peters, B.M.; Yano, J.; Noverr, M.C.; Fidel, P.L. Candida Vaginitis: When Opportunism Knocks, the Host Responds. *PLoS Pathog.* 2014, *10*, e1003965. [CrossRef] [PubMed]
44. Nobile, C.J.; Johnson, A.D. *Candida albicans* Biofilms and Human Disease. *Annu. Rev. Microbiol.* 2015, *69*, 71–92. [CrossRef] [PubMed]
45. Hacioglu, M.; Guzel, C.B.; Savage, P.B.; Seher Birteksoz Tan, A. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against Candida spp. Isolated from vulvovaginal

candidiasis. *Med. Mycol.* 2019, *57*, 291–299. [CrossRef] [PubMed]

46. Tulasidas, S.; Rao, P.; Bhat, S.; Manipura, R. A study on biofilm production and antifungal drug resistance among Candida species from vulvovaginal and bloodstream infections. *Infect. Drug Resist.* 2018, *11*, 2443–2448. [CrossRef]

47. Gonçalves, B.; Ferreira, C.; Alves, C.T.; Henriques, M.; Azeredo, J.; Silva, S. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit. Rev. Microbiol.* 2016, *42*, 905–927. [CrossRef]

48. Zhu, W.; Filler, S.G. Interactions of Candida albicans with epithelial cells. *Cell. Microbiol.* 2010, *12*, 273–282. [CrossRef]

49. Paiva, L.C.F.; Vidigal, P.G.; Donatti, L.; Svidzinski, T.I.E.; Consolaro, M.E.L. Assessment of in vitro biofilm formation by Candida species isolates from vulvovaginal candidiasis and ultrastructural characteristics. *Micron* 2012, *43*, 497–502. [CrossRef]

50. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D.W.; Azeredo, J. Candida glabrata, Candida parapsilosis and Candida tropicalis: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* 2012, *36*, 288–305. [CrossRef]

51. Ielasi, F.S.; Decanniere, K.; Willaert, R.G. The epithelial adhesin 1 (Epa1p) from the human-pathogenic yeast Candida glabrata: Structural and functional study of the carbohydrate-binding domain. *Acta Crystallogr. Sect. D Biol. Crystallogr.* 2012, *68*, 210–217. [CrossRef]

52. Staniszewska, M.; Bondaryk, M.; Siennicka, K.; Piłat, J.; Schaller, M.; Kurzą̨tkowski, W. Role of aspartic proteinases in Candida albicans virulence. Part I. Substrate specificity of Aspartic proteinases and Candida albicans pathogenesis (Postepy Mikrobiologii (2012) (131)). *Postep. Mikrobiol.* 2012, *51*, 236.

53. Vylkova, S.; Lorenz, M.C. Encounters with Mammalian Cells: Survival Strategies of Candida Species. In *Candida and Candidiasis*, 2nd. ed.; Calderone, R.A., Clancy, C.J., Eds.; American Society of Microbiology: Washington, DC, USA, 2012; pp. 261–282.

54. Sikora, M.; Dabkowska, M.; Swoboda-Kopec, E.; Jarzynka, S.; Netsvyetayeva, I.; Jaworska-Zaremba, M.; Pertkiewicz, M.; Mlynarczyk, G. Differences in proteolytic activity and gene profiles of fungal strains isolated from the total parenteral nutrition patients. *Folia Microbiol.* 2011, *56*, 143–148. [CrossRef] [PubMed]

55. Tellapragada, C.; Eshwara, V.K.; Johar, R.; Shaw, T.; Malik, N.; Bhat, P.V.; Kamath, A.; Mukhopadhyay, C. Antifungal Susceptibility Patterns, In Vitro Production of Virulence Factors, and Evaluation of Diagnostic Modalities for the Speciation of Pathogenic Candida from Blood Stream Infections and Vulvovaginal Candidiasis. *J. Pathog.* 2014, *2014*, 142864. [CrossRef] [PubMed]

56. Sobel, J.D. Vulvovaginal candidosis. *Lancet* 2007, *369*, 1961–1971. [CrossRef]

57. Jafari, M.; Salari, S.; Pakshir, K.; Zomorodian, K. Exoenzyme activity and possibility identification of Candida dubliniensis among Candida albicans species isolated from vaginal candidiasis. *Microb. Pathog.* 2017, *110*, 73–77. [CrossRef]

58. Naglik, J.R.; Rodgers, C.A.; Shirlaw, P.J.; Dobbie, J.L.; Fernandes-Naglik, L.L.; Greenspan, D.; Agabian, N.; Challacombe, S.J. Differential Expression of Candida albicans Secreted Aspartyl Proteinase and Phospholipase B Genes in Humans Correlates with Active Oral and Vaginal Infections. *J. Infect. Dis.* 2003, *188*, 469–479. [CrossRef]

59. Calderone, R.A.; Fonzi, W. A Virulence factors in Candida albicans. *TRENDS Microbiol.* 2001, *9*, 327–335. [CrossRef]

60. Cassone, A. Vulvovaginal Candida albicans infections: Pathogenesis, immunity and vaccine prospects. *BJOG Int. J. Obstet. Gynaecol.* 2015, *122*, 785–794. [CrossRef]

61. Sudbery, P.; Gow, N.; Berman, J. The distinct morphogenic states of Candida albicans. *Trends Microbiol.* 2004, *12*, 317–324. [CrossRef]

62. Moran, G.; Coleman, D.; Sullivan, D. An Introduction to the Medically Important Candida Species. In *Candida and Candidiasis*, 2nd ed.; Calderone, R.A., Clancy, C.J., Eds.; American Society of Microbiology: Washington, DC, USA, 2012; pp. 11–25.

63. Consolaro, M.E.L.; Albertoni, T.A.; Svidzinski, A.E.; Peralta, R.M.; Svidzinski, T.I.E. Vulvovaginal candidiasis is associated with the production of germ tubes by Candida albicans. *Mycopathologia* 2005, *159*, 501–507. [CrossRef]

64. Sudbery, P.E. Growth of Candida albicans hyphae. *Nat. Rev. Microbiol.* 2011, *9*, 737–748. [CrossRef] [PubMed]

65. Tang, Y.; Yu, F.; Huang, L.; Hu, Z. The changes of antifungal susceptibilities caused by the phenotypic switching of Candida species in 229 patients with vulvovaginal candidiasis. *J. Clin. Lab. Anal.* 2019, *33*, e22644. [CrossRef] [PubMed]

66. Lastauskiene˙, E.; Cˇ eputyte˙, J.; Girkontaite˙, I.; Zinkevicˇiene˙, A. Phenotypic Switching of Candida guilliermondii is Associated with Pseudohyphae Formation and Antifungal Resistance. *Mycopathologia* 2015, *179*, 205–211. [CrossRef] [PubMed]

67. Harriott, M.M.; Noverr, M.C. Importance of Candida-bacterial polymicrobial biofilms in disease. *Trends Microbiol.* 2011, *19*, 557–563. [CrossRef] [PubMed]

68. Silva, S.; Henriques, M.; Martins, A.; Oliveira, R.; Williams, D.; Azeredo, J. Biofilms of non-Candida albicans Candida species: Quantification, structure and matrix composition. *Med. Mycol.* 2009, *47*, 681–689. [CrossRef] [PubMed]

69. Harriott, M.M.; Lilly, E.A.; Rodriguez, T.E.; Fidel, P.L.; Noverr, M.C. Candida albicans forms biofilms on the vaginal mucosa. *Microbiology* 2010, *156*, 3635–3644. [CrossRef]

70. Muzny, C.A.; Schwebke, J.R. Biofilms: An underappreciated mechanism of treatment failure and recurrence in vaginal infections. *Clin. Infect. Dis.* 2015, *61*, 601–606. [CrossRef]

71. Desai, J.V.; Mitchell, A.P. Candida albicans biofilm development and its genetic controls. *Microbiol. Spectr.* 2015, *3*, 139–148. [CrossRef]

72. McKloud, E.; Delaney, C.; Sherry, L.; Kean, R.; Williams, S.; Metcalfe, R.; Thomas, R.; Richardson, R.; Gerasimidis, K.; Nile, C.J.; et al. Recurrent Vulvovaginal Candidiasis: A Dynamic Interkingdom Biofilm Disease of Candida and Lactobacillus. *mSystems* 2021, *6*, e00622-21. [CrossRef]

73. Thein, Z.M.; Seneviratne, C.J.; Samaranayake, Y.H.; Samaranayake, L.P. Community lifestyle of Candida in mixed biofilms: A mini review. *Mycoses* 2009, *52*, 467–475. [CrossRef]

74. Rodrigues, M.E.; Gomes, F.; Rodrigues, C.F. Candida spp./bacteria mixed biofilms. *J. Fungi* 2020, *6*, 5. [CrossRef] [PubMed]

75. Lohse, M.B.; Gulati, M.; Johnson, A.D.; Nobile, C.J. Development and regulation of single-and multi-species Candida albicans biofilms. *Nat. Rev. Microbiol.* 2018, *16*, 19–31. [CrossRef] [PubMed]

76. Tomaszewski, J. The statement of polish gynecological society experts on the etiology and treatment of recurrent vulvovaginal candidiasis. *Ginekol. Pol.* 2011, *82*, 869–873.

77. Sherry, L.; Kean, R.; Mckloud, E.; Donnell, L.E.O.; Metcalfe, R.; Jones, B.L. Biofilms formed by isolates from patients are heterogeneous and insensitive to fluconazole. *Antimicrob Agents Chemother* 2017, *61*, e01065-17. [CrossRef] [PubMed]

78. Auler, M.E.; Morreira, D.; Rodrigues, F.F.O.; Abrão, M.S.; Margarido, P.F.R.; Matsumoto, F.E.; Silva, E.G.; Silva, B.C.M.; Schneider,R.P.; Paula, C.R. Biofilm formation on intrauterine devices in patients with recurrent vulvovaginal candidiasis Biofilm formation on IUDs in patients with vulvovaginal candidiasis. *Med. Mycol.* 2010, *48*, 211–216. [CrossRef]

79. Sobel, J.D. Editorial Commentary: Vaginal Biofilm: Much Ado about Nothing, or a New Therapeutic Challenge? *Clin. Infect. Dis.*2015, *61*, 607–608. [CrossRef]

80. Yano, J.; Peters, B.M.; Noverr, M.C.; Fidel, P.L. Novel Mechanism behind the Immunopathogenesis of Vulvovaginal Candidiasis: "Neutrophil Anergy. " *Infect. Immun.* 2018, *86*, e00684-17. [CrossRef]

81. Willems, H.M.E.; Ahmed, S.S.; Liu, J.; Xu, Z.; Peters, B.M. Vulvovaginal candidiasis: A current understanding and burning questions. *J. Fungi* 2020, *6*, 27. [CrossRef]

82. Fidel, P.L. History and update on host defense against vaginal candidiasis. *Am. J. Reprod. Immunol.* 2007, *57*, 2–12. [CrossRef]

83. Roselletti, E.; Perito, S.; Sabbatini, S.; Monari, C.; Vecchiarelli, A. Vaginal Epithelial Cells discriminate between yeast and hyphae of *C. albicans* in women who are colonized with or have vaginal candidiasis. *J. Infect. Dis.* 2018, *10*, 1645–1654. [CrossRef]

84. Richardson, J.P.; Willems, H.M.E.; Moyes, D.L.; Shoaie, S.; Barker, K.S.; Tan, L.; Palmer, G.E.; Hube, B.; Naglik, J.R.; Peters, M. Candidalysin Drives Epithelial Signaling, Neutrophil Recruitment, and Immunopathology at the Vaginal Mucosa. *Infect. Immun.* 2018, *86*, e00645-17. [CrossRef] [PubMed]

85. Roselletti, E.; Perito, S.; Gabrielli, E.; Mencacci, A.; Pericolini, E.; Sabbatini, S.; Cassone, A.; Vecchiarelli, A. NLRP3 inflammasome is a key player in human vulvovaginal disease caused by Candida albicans. *Sci. Rep.* 2017, *7*, 17877. [CrossRef] [PubMed]

86. Bruno, V.M.; Shetty, A.C.; Yano, J.; Fidel, P.L.; Noverr, M.C.; Peters, B.M. Transcriptomic Analysis of Vulvovaginal Candidiasis Identifies a Role for the NLRP3 Inflammasome. *mBio* 2015, *6*, e00182-15. [CrossRef] [PubMed]

10.2. Artykuł nr 2

pharmaceutics

MDPI

Article

Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal *Candida* spp.

Paulina Czechowicz [1,*], Damian Neubauer [2], Joanna Nowicka [1], Wojciech Kamysz [2] and Grażyna Gościniak [1]

[1] Department of Microbiology, Faculty of Medicine, Wrocław Medical University, 51-368 Wrocław, Poland; joanna.nowicka@umed.wroc.pl (J.N.); grazyna.gosciniak@umed.wroc.pl (G.G.)

[2] Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University of Gdan´sk, 80-210 Gdansk, Poland; damian.neubauer@gumed.edu.pl (D.N.); wojciech.kamysz@gumed.edu.pl (W.K.)

* Correspondence: paulina.czechowicz.umedwroc@gmail.com; Tel.: +48-71-784-13-01

Abstract: Vulvovaginal candidiasis (VVC) occurs in over 75% of women at least once during their lifetime and is an infection that significantly affects their health. *Candida* strains resistant to standard azole antifungal therapy and relapses of VVC are more and more common. Hypothetically, biofilm is one of the main reasons of relapses and failure of the therapy. Ultrashort cationic lipopeptides (USCLs) exhibit high antimicrobial activities. Our previous study on USCLs revealed that disulfide cyclization can result in selective antifungal compounds. Therefore, four USCL were selected and their antifungal activity were studied on 62 clinical strains isolated from VVC. The results confirmed previous premises that cyclic analogs have increased selectivity between fungal cells and keratinocytes and improved anticandidal activity compared to their linear analogs against both planktonic and biofilm cultures. On the other hand, linear lipopeptides in combination with fluconazole showed a synergistic effect. It was found that the minimum inhibitory concentrations of the tested compounds in combination with fluconazole were at least four times lower than when used separately. Our results indicate that combination therapy of VVC with USCLs and fluconazole at low non-toxic concentrations can be beneficial owing to the synergistic effect. However, further in vivo studies are needed to confirm this hypothesis.

Keywords: *Candida*; biofilm; Vulvovaginal candidiasis; synergy; lipopeptides; cationic lipopeptides; fluconazole

Citation: Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gosciniak, G. Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal *Candida* spp. *Pharmaceutics* **2021**, *13*, 1589. https://doi.org/10.3390/pharmaceutics13101589

Academic Editor:Sanna Maria Sillankorva and Jennifer Fiegel

Received: 18 August 2021

Accepted: 28 September 2021

Published: 30 September 2021

1. Introduction

Vulvovaginal candidiasis (VVC) is the second most common type of vaginal infection, significantly reducing the quality and comfort of women's lives. According to estimates, more than 75% of women in childbearing age worldwide will experience a symptomatic episode of VVC at least once during their lifetime. Conventional treatments often result in therapeutic failure and/or recurrence of the infection [1–6]. Typical recurrent VVC (RVVC), defined as four or more episodes per year, affects at least 10% of patients. At the same time, *Candida* spp. can colonize the vagina and about 1/5 of women are asymptomatic carriers [1,2,4,5]. Generally this fungi are widespread commensals that can be part of the microbiota of mucous membranes and skin where they can cause opportunistic infections, especially in immunocompromised individuals [7]. In the case of VVC, in addition to states of compromised immunity, the most common risk factors are pregnancy, hormone replacement therapy, diabetes, antibiotic therapy, and steroid therapy [1,2]. Meanwhile, the detailed pathomechanism of vaginal mucosa invasion by yeast-like fungi remains unclear [2,3,8]. *Candida albicans* remains the most common etiological factor of VVC, but for many years an increasing percentage of NCAC (Non-*Candida albicans Candida*) fungi has been observed among vaginal isolates, such as *Candida glabrata*, *Candida parapsilosis* or

Candida lusitaniae [5,9–11]. Many researchers are inclined to the hypothesis that the ability to form a highly resistant biofilm structure by these strains is one of the causes of therapy failure in VVC [2,3,5,8,9,11,12]. Among virulence factors, the ability to create this structure is one of the most important for their pathogenicity. It seems to be essential primarily in the case of many candidiasis as well as infections related to biomaterials (artificial valves, endoprostheses, intravascular or urinary catheters). What is worth emphasizing is that biofilms are characterized by a high resistance to antifungal drugs, even at very high concentrations [13]. It is noteworthy that the formation of polymicrobial biofilms by *Candida* and bacterial strains is a frequent issue. The most common bacterial strain being isolated from vaginal mixed biofilm is *Lactobacillus* sp. [14]. Hence, research into new antifungal therapies also focuses on anti-biofilm activity.

One of the promising classes of compounds is lipopeptides [15–18]. They consist of a peptide fragment and conjugated lipid residue (s) and can be divided into subclasses. Ultrashort cationic lipopeptides (USCLs) are among the most effective against fungal strains and consist of a peptide with at most seven amino acid residues with a net positive charge owing to the occurrence of basic amino acids such as arginine or lysine. The most common hydrophobic fragment of USCLS is a fatty acid chain. In effect, USCLs are amphiphilic and can easily interact with the negatively charged pathogen membrane. In the case of fungi, lipopeptides interact with negatively charged residues of sialic acid and phosphatidylinositol found in the cell membrane of these microorganisms [19,20]. Their mode of action is based on the permeabilization of the membrane bilayers, which leads to cell death [16–18,21]. They can exhibit plenty of biological properties, such as antibacterial, antifungal, antibiofilm, antiadhesive, anticancer, and surface activities [22]. On the other hand, USCLs can be noticeably lytic to erythrocytes and cytotoxic to normal human cells [16,18,21].

One of the well-studied USCL with a proven antimicrobial activity is C_{16}-KKKK-NH_2 (C_{16}-palmitic acid) that contains four L-lysine (K-L-lysine) residues [23–27]. Our previous study on this lipopeptide and its analogs revealed that the substitution of L-lysine by L-arginine (R-L-arginine) residue and disulfide-cyclization can result in compounds with improved antimicrobial activity and selectivity between *Candida* strains and human cells [16]. The lipopeptides with cysteine residues (C-L-cysteine) were cyclized through intramolecular disulfide bridge formation [16]. Based on our previous results, four lipopeptides with the most favorable antifungal properties were selected for further study—C_{16}-KKKK-NH_2, C_{16}-KRKK-NH_2, C_{16}-CKKKKC-NH_2, and C_{16}-CKRKKC-NH_2. Undoubtedly, compounds with different modes of action can potentiate each other's antifungal activity. It was found that a combination of some lipopeptides (e.g., surfactin) with common antifungal drugs (e.g., azoles) can result in a synergistic effect [28–36].

In the context of fungal infections, interactions between conventionally used fluconazole and various AMPs (Antimicrobial peptides) are being investigated more and more frequently [30,37–42]. The literature includes several studies on the impact of cationic ultrashort lipopeptides on fungi, including, for example, dermatophytes, but to the best of our knowledge none of these reports concerned isolates from VVC [43].

The aim of the study was to determine the antifungal activity of two linear lipopeptides: C_{16}-KKKK-NH_2 (**L1**), C_{16}-KRKK-NH_2 (**L2**) and their two cyclic analogs: C_{16}-CKKKKC-NH_2 (**C1**) and C_{16}-CKRKKC-NH_2 (**C2**) against 62 clinical strains of various species of *Candida* isolated from Vulvovaginal candidiasis, both in planktonic and biofilm form. Moreover, studies on the potential synergistic or additive effects of combinations of fluconazole with these compounds have been carried out.

2. Materials and Methods

2.1 Chemicals and Reagents

Chemicals and reagents used in lipopeptides synthesis were: polystyrene resin and amino acids purchased from Orpegen Peptide Chemicals GmbH, Heidelberg, Germany; piperidine, *N*,N^t-diisopropylcarbodiimide (DIC), OxymaPure, triisopropylsilane (TIS) from

Iris Biotech GmbH, Marktredwitz, Germany; *N*,*N*-dimethylformamide (DMF) and diethyl ether from POCH, Avantor, Gliwice, Poland; dichloromethane (DCM) and acetic acid from Chempur, Piekary Slaskie, Poland; hexadecanoic acid (C16) and 1,2-ethanedithiol (EDT) from Merck, Darmstadt, Germany; and trifluoroacetic acid (TFA) from Apollo Scientific, Denton, UK.

2.2 Lipopeptides Synthesis

The compounds were obtained by using the method reported previously by Neubauer et al. [16]. Lipopeptides were synthesized manually by solid-phase Fmoc/tBu methodology. Polystyrene resin modified by Rink Amide linker was used as the solid support (loading ca. 1.0 mmol/g). Deprotection of the Fmoc group was performed with a 20% (*v*/*v*) piperidine solution in DMF for 15 min. Acylation was conducted with a mixture of DIC:OxymaPure:Fmoc-AA-OH (mole ratio 1:1:1) dissolved in DMF:DCM (1:1, *v*/*v*) in fourfold excess based on the resin for 1.5 h. Fmoc-L-Arg (Pbf)-OH, Fmoc-L-Lys (Boc)-OH, Fmoc-L-Cys (Trt)-OH, and hexadecanoic acid were used in coupling reactions. After deprotection and coupling reactions, the resin was rinsed with DMF and DCM and subsequently the chloranil test was carried out. The peptides were cleaved from the resin using one of the mixtures: (A) TFA, EDT, TIS and deionized water (92.5:2.5:2.5:2.5, *v*/*v*/*v*/*v*); or (B) TFA, TIS, and deionized water (95:2.5:2.5, *v*/*v*/*v*). Mixture A was used with peptides containing a cysteine residue, whereas mixture B was used for the remaining peptides. Cleavage was accomplished within 1.5 h under stirring. Then the peptides were precipitated with cooled diethyl ether and lyophilized. The crude peptide with cysteine was dissolved in 20% (*v*/*v*) acetic acid solution (0.5 g/L) and oxidized with iodine to obtain the peptide with intramolecular disulfide bridge. The peptides were purified by RP-HPLC. Pure fractions (>95%, HPLC) were collected and lyophilized. The identity of all compounds was confirmed by mass spectrometry (ESI–MS). The sequences of the synthesized lipopeptides were as follows: linear C_{16}-KKKK-NH_2 (L1) and C_{16}-KRKK-NH_2 (L2), cyclic: C_{16}-CKKKKC-NH_2 (C1) and C_{16}-CKRKKC-NH_2 (C2).

2.3 Candida Strains

Microbiological assays were performed on 62 clinical isolates of various *Candida* species. All strains were originally isolated from the vaginas of women with Vulvovaginal candidiasis and were deposited in the Internal Collection of the Department of Microbiology, Wroclaw Medical University. Two reference strains of *C. albicans* ATCC 90028 and *C. glabrata* ATCC 15126 (PCM, Polish Academy of Sciences, Wroclaw) were included in all experiments. The vast majority of strains were identified as *C. albicans* (52), while the remaining strains belonged to the NCAC group: i.e., *C. glabrata* (5), *C. lusitaniae* (2), *C. kefyr* (2), and *C. parapsilosis* (1).

The study protocol was approved by the local Bioethics Committee of Wrocław Medi- cal University (No. 774/2018, approval date: 27 December 2018). All experiments were performed in accordance with relevant guidelines and regulations.

2.4 Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) of fluconazole and four lipopeptides against *Candida* strains were determined. The research was carried out in accordance with Clinical and Laboratory Standards Institute guidelines [44]. Suspensions of *Candida* strains (subcultured for 24 h on Sabouraud Dextrose Agar with chloramphenicol at a concentration of 100 mg/L) in sterile 0.9% NaCl were diluted in RPMI 1640 (Merck, KGaA, Darmstadt, Germany) to a final concentration of 1–5 × 10^3 CFU per mL. The test compounds dissolved in DMSO (Merck, KGaA, Darmstadt, Germany) for fluconazole and in sterile distilled water for lipopeptides were diluted in RPMI 1640 on 96-well polystyrene plates to a final range of concentrations 0.125–128 µg/mL (fluconazole) and 0.5–256 µg/mL (USCLs). After the addition of inoculums, all plates were incubated for 24 h at 37 °C. In the case of fungistatic fluconazole the MIC value is defined as the concentration that inhibits at least

50% of fungal growth. In order to determine the most accurate MIC end-point value, cell densities were determined spectrophotometrically at 530 nm (BiochromAsys UVM 340 Microplate Spectrophotometer, Biochrom Ltd., Holliston, MA, USA). To calculate the MIC value, the following equation was used: $(OD_{well}\text{-}OD_{background})/(OD_{K+}\text{-}OD_{K-}) \times 100\%$, where OD_{well} is the absorbance of the well being assessed, OD_{K-} is the value for the negative control (background), and OD_{K+} is the value obtained in the control positive (strain growth control). Minimum inhibitory concentrations of lipopeptides were the lowest concentrations at which inhibition of fungal growth was noticeable. All experiments were conducted in triplicate.

2.5 Minimum Biofilm Eradication Concentration

The determination of minimum biofilm eradication concentrations (MBECs) of all five compounds was performed on 96-well polystyrene flat-bottom plates. Twenty-four- hour cultures of *Candida* were diluted with RPMI 1640 to obtain a final concentration of 1–5 × 10^6 cells per mL and 100 µL of cell suspension was added into each well of the test plate. The plates were incubated for 24 h at 37 °C in order to form a mature biofilm. After incubation, the biofilms were rinsed three times with sterile 0.9% NaCl. Subsequently, fluconazole was added with a final range of concentrations of 1–512 µg/mL, while the lipopeptides concentration ranged between 0.5 and 256 µg/mL. The plates were again incubated overnight at 37 °C. Visualization of results was carried out with a MTT solution (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), Merck, KGaA, Darmstadt, Germany), which is reduced by metabolically active sessile cells of biofilm to purple/navy blue formazan compounds [45]. Yellow MTT solution was added to each well of the plates and incubated for 3 h in the dark at 37 °C. MBECs were the lowest concentrations of the compounds at which no color change was observed (no metabolically active yeast cells were present) as compared to the positive and negative controls. All experiments were conducted in triplicate.

2.6 Fractional Inhibitory Concentration Index

The checkerboard method was used to determine fractional inhibitory concentration index (FICi) [46]. Each lipopeptide in combination with fluconazole were prepared on a 96-well polystyrene plate with concentrations serially diluted from 2× MIC to 1/64 MIC for every strain. In each well of the plates prepared as above, different lipopeptide-fluconazole concentrations were obtained. Inoculums of *Candida* strains were prepared as described for the determination of MIC (final concentration of 1–5 × 10^3 CFU per·mL in RPMI 1640). After the yeast suspension was added, the plates were incubated for 24 h at 37 °C. Inhibition of *Candida* growth was assessed visually. To calculate the FIC index, the following formula was used:

$$\frac{A}{\text{MIC } of\ A} + \frac{B}{\text{MIC } of\ B} = \text{FIC}_A + \text{FIC}_B = \text{FIC}, \quad (1)$$

$$\text{FIC index} = \frac{\sum \text{FIC}}{n} \quad (2)$$

MIC values of compound *A* (lipopeptide) and *B* (fluconazole) were obtained in the first part of the research. *A* and *B* values were concentrations of the compounds determined using the checkerboard method. The sum of the ratios of these values (FIC_A, FIC_B) was FIC and after it was divided by *n* (number of FICs), the FIC index was obtained. Interpretation of the results was consistent with EUCAST guidelines [47] as follows: FICi ≤ 0.5 indicates synergy (SYN), >0.5 to ≤1.0-addition (ADD), >1.0 to ≤2.0 indifference (IND), and FICi > 2.0 means antagonism (ANT).

3. Results

3.1 Minimum Inhibitory Concentration

Fluconazole and four tested lipopeptides exhibited antimicrobial activity against planktonic cultures of all *Candida* strains (Figure 1).

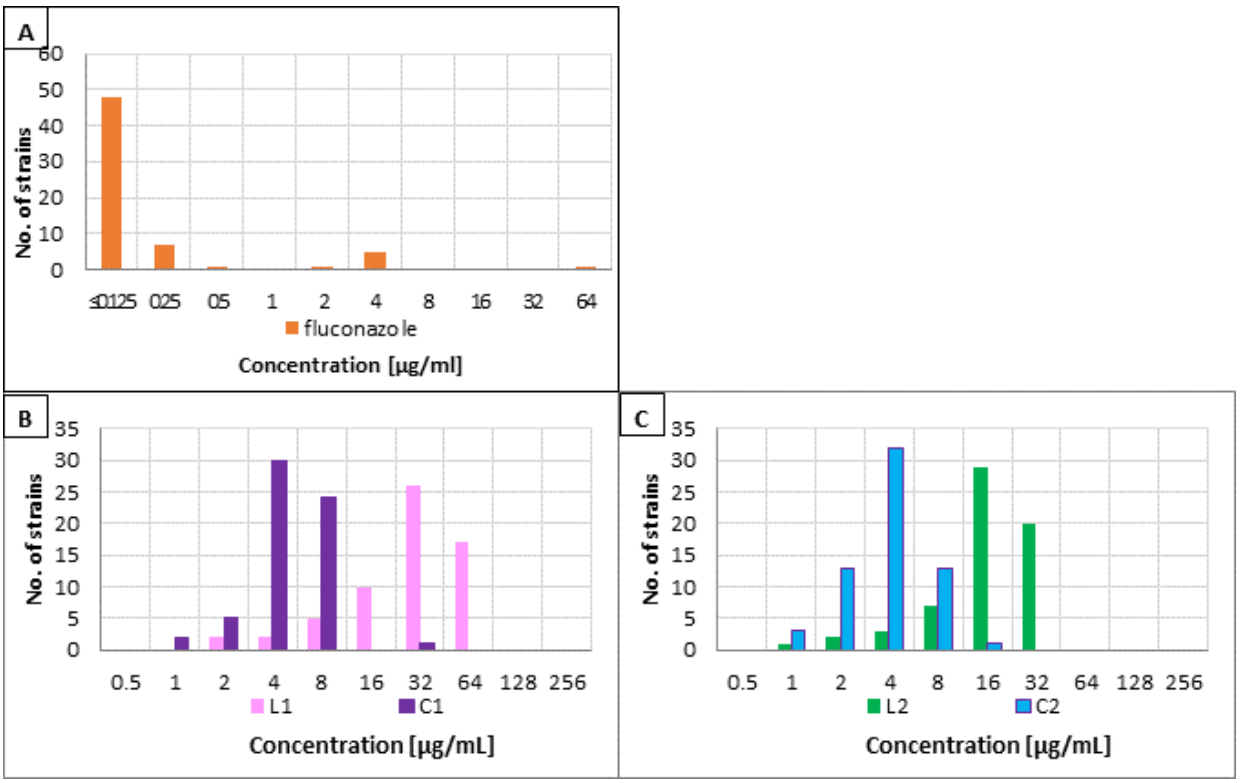


Figure 1. MIC value distribution of the tested compounds: (**A**) fluconazole, (**B**) lipopeptides L1 and C1, and (**C**) lipopeptides L2 and C2.

The most common MIC of fluconazole was ≤ 0.125 µg/mL and was determined for 77% of the strains (48/62). All of them were *C. albicans* and only single strains of this species demonstrated a slightly higher MIC, not exceeding 2 µg/mL. One isolate (*C. lusitaniae*) was resistant to fluconazole (MIC = 64 µg/mL). The remaining NCAC strains exhibited minimum inhibitory concentrations of tested azole of 4 µg/mL (all five *C. glabrata* isolates) or less. The distribution of MIC values of fluconazole is presented below in Figure 1A.

Minimum inhibitory concentrations of lipopeptide L1 were in the range 2–64 µg/mL, with 32 µg/mL as the most common value (26/62, ≈42%). No significant differences among various *Candida* species were observed. The cyclic analog of this lipopeptide (C1) exhibited MIC distribution in the lower concentration range between 1 and 32 µg/mL. For almost half (30/62, ≈48%) of the tested strains, MIC of C1 was 4 µg/mL and for 26 isolates (≈42%) it was twice as high 8 µg/mL. No deviations in MICs were observed between different *Candida* species. MIC distribution for L1 and C1 is presented below in Figure 1B.

The second pair of lipopeptides, the one with arginine residue, exhibited less pronounced differences in MIC distribution. MIC concentration range for L2 was 1–32 µg/mL, while for C2 it was 1–16 µg/mL. For nearly 50% (29/62, ≈47%) of strains, minimum inhibitory concentrations of linear lipopeptides were 16 µg/mL, followed by 32 µg/mL (20/62, ≈32%). The most frequent concentration of the cyclic compound was 4 µg/mL (32/62, ≈52%). As with the first pair of lipopeptides, no difference in MIC distribution was observed for individual *Candida* species. MIC distribution of L2 and C2 lipopeptides is displayed in Figure 1C.

To evaluate lipopeptides selectivity, selectivity indices (SI) were calculated as the ration of CM_{50} to GM. Previous results of cytotoxicity (IC50) against HaCaT cell line (immortalized human keratinocytes) were used for this calculation [16]. The results are presented in Table 1.

Table 1. Geometric mean of MICs (GM_MIC), IC_{50}, and selectivity indices (SI) of four tested lipopeptides.

Lipopeptide	GM_MIC [μg/mL]	IC_{50} [16]	SI
L1	26.46	23.5 ± 1.3	0.89
C1	4.89	26.9 ± 1.9	5.50
L2	15.47	4.3 ± 0.9	0.28
C2	3.83	33.8 ± 3.1	8.83

3.2 *Minimum Biofilm Eradication Concentration*

Fungistatic fluconazole failed to eradicate the biofilm of *Candida* strains. For almost all strains (58/62, ≈94%), MBEC values were extremely high (512 μg/mL) and for the remaining isolates, eradication concentrations were even higher. The distribution of MBECs is presented below in Figure 2A.

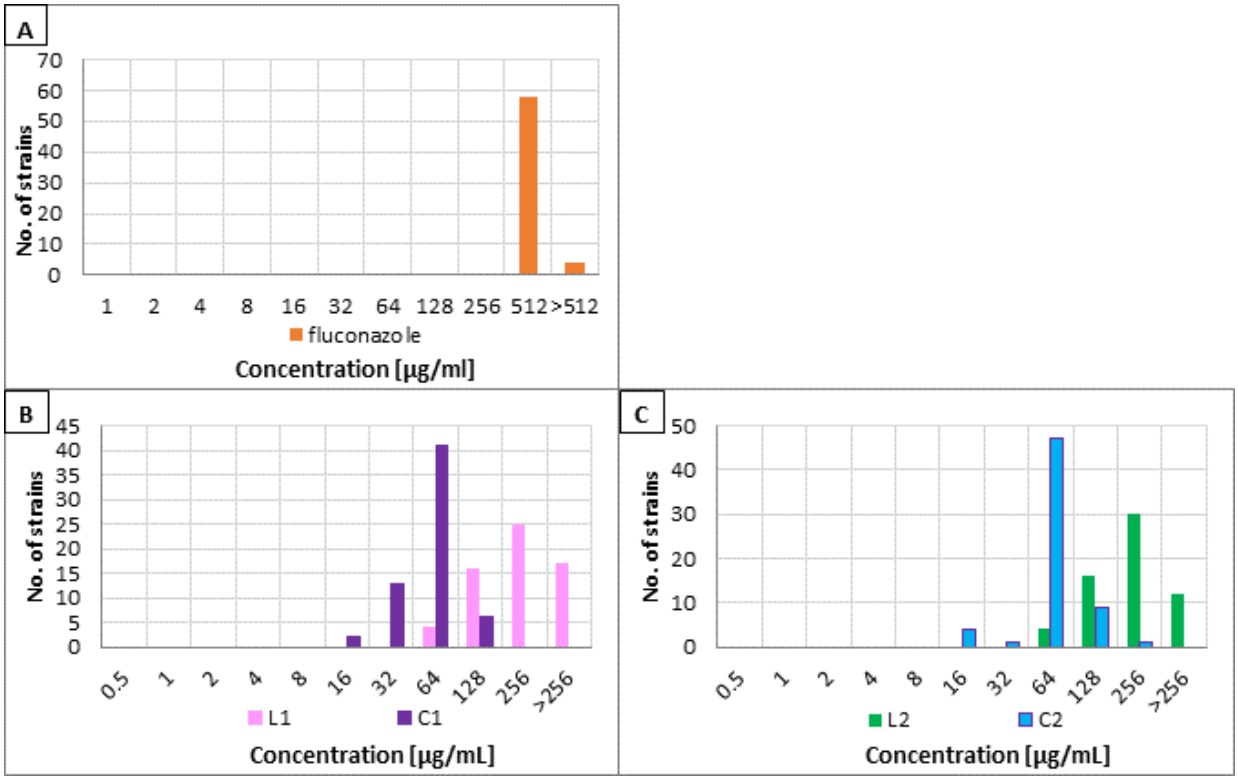


Figure 2. MBEC value distribution of the tested compounds: (**A**) fluconazole, (**B**) lipopeptides L1 and C1, and (**C**) lipopeptides L2 and C2.

In contrast to fluconazole, all four lipopeptides proved to be effective in biofilm eradication. In the case of the first pair of lipopeptides L1 and C1 (consisting of lysine residues only), similarly to MICs, the obtained MBECs were significantly lower for cyclic lipopeptide than for the linear analog. The most common MBEC value for L1 was 256 μg/mL (25/62, ≈40%), followed by 128 μg/mL (16/62, ≈26%) and concentration above 256 μg/mL (17/62, ≈27%). On the other hand, for the vast majority of strains (41/62, ≈66%), minimum biofilm eradication concentrations of the second lipopeptide (C2) were 64 μg/mL. Again, no differences in MBECs were observed in terms of species. The results of MBEC value determination are shown in Figure 2B.

The MBECs of lipopeptides L2 and C2 were very similar to those described above. In the case of cyclic lipopeptide, MBEC value of 64 μg/mL was definitely dominant (47/62, ≈76%). For the linear parent molecule, the values were more distributed. The most common MBEC was 256 μg/mL (30/62, ≈48%) followed by 128 μg/mL (16/62, ≈26%)

and >256 μg/mL (12/62, ≈19%). No differences for *C. albicans* versus NCAC fungi were observed. The results are presented below in Figure 2C.

Geometric means of MBECs were calculated. For MBEC values of USCLs >256 μg/mL, 512 μg/mL was taken into calculations. In the case of fluconazole, 1024 μg/mL value was used respectively for MBECs >512 μg/mL. Importantly, although this mathematical operation includes resistant strains in the calculations, their precise effective concentrations are not known. The calculated GM_MBECs of linear lipopeptides were similar to each other, as well as for the both cyclic analogues. In the case of L1 it was 236.73 μg/mL and 223.86 μg/mL for the L2 lipopeptide. GM_MBEC of lipopeptide with an arginine residue (C2) was 65.45 μg/mL and 56.59 μg/mL for C1.

3.3 Fractional Inhibitory Concentration Index (FICi)

To initially asses the interaction of each fluconazole–lipopeptide combination, 24 of the tested strains were randomly selected of which 15 isolates were *C. albicans* and the remaining 9 were from the NCAC group. The interpretation of FIC indices was as follows: FICi ≤ 0.5 indicates synergy (SYN), >0.5 to ≤1.0-addition (ADD), >1.0 to ≤2.0 means indifference (IND), and FICi > 2.0 means antagonism (ANT) [47]. The results are collected in Figure 3.

Although no synergy was observed among the tested fluconazole–lipopeptide pairs against *Candida* strains, an additive effect was determined, especially in the case of *C. albicans* isolates. The distribution of the results on the histograms (Figure 3) clearly indicates that the additive effect is more frequent for both linear lipopeptides combined with fluconazole: L1 (15/24, ≈63%) and L2 (11/24, ≈46%). For cyclic analogs combined with fluconazole, the dominant result was indifference: ≈71% (17/24) for C1 and ≈58% (14/24) for C2. A simultaneous use of cyclic USCLs with fluconazole has an antagonis- tic or neutral effect against strains of the NCAC group, e.g., *C. glabrata* and *C. kefyr*, in contrast to their linear counterparts. The FIC index determined for *C. albicans* isolates was in agreement with the overall results, with an additive effect obtained mostly for linear lipopeptides.

Based on the obtained results, two lipopeptides were selected for further experiments: linear L1 and its cyclic analog C1. Both USCLs showed more favorable effects (Figure 3, additive effect) against *Candida* in combination with fluconazole than lipopeptides with arginine residue.

The FIC indices of combinations of fluconazole–L1 and fluconazole–C1 were determined against the remaining 40 isolates. The results (64 isolates, reference strains included) are shown in Figures 4 and 5.

In the case of the linear lipopeptide, the results obtained for all 64 strains were consistent with those described for 24 isolates (Figure 3). Altogether, an additive effect with fluconazole was dominant (54/64, ≈84%). A similar result was observed for *C. albicans* strains (48/53, ≈91%). Among NCAC fungi, additive effect was the most frequent as well (6/11, ≈55%), but it is difficult to draw strict conclusions due to a relatively small pool of strains. At first, cyclic C2 seemed to have an indifferent effect in combination with the tested azole, but a study on 64 strains revealed that addition is the most common result (34/64, ≈53%) in reference to all *Candida* species and also in the case of *C. albicans* species. Unfortunately, this pair of USCL–fluconazole had still a predominantly neutral effect against NCAC group (7/11, ≈64%).

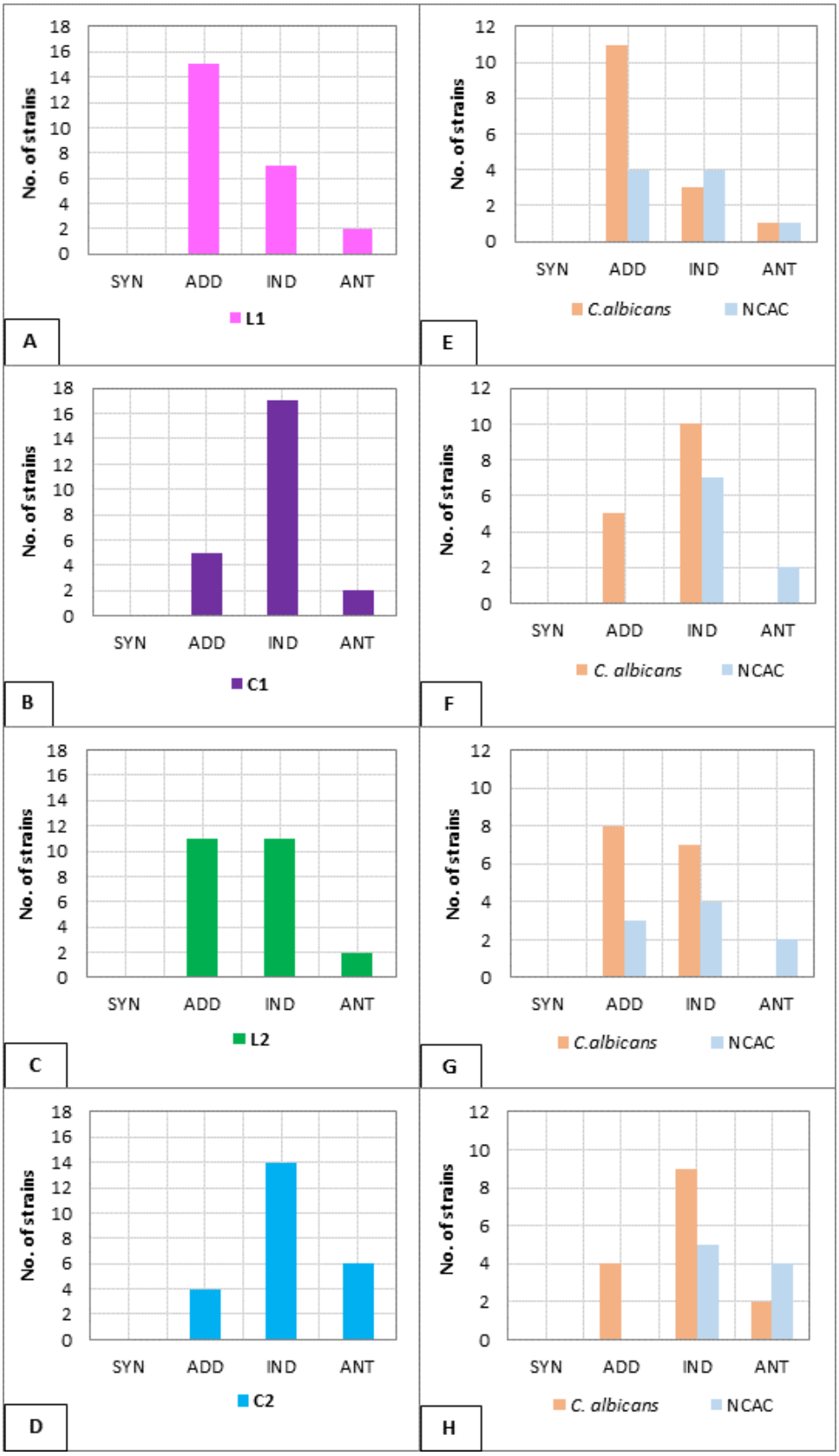


Figure 3. Interpretation of the determined FIC indices (SYN—synergy, ADD—addition, IND—indifference, ANT—antagonism): (**A**) L1; (**B**) C1; (**C**) L2; (**D**) C2; and with differentiation between *C. albicans* and NCAC isolates: (**E**) L1; (**F**) C1; (**G**) L2; (**H**) C2.

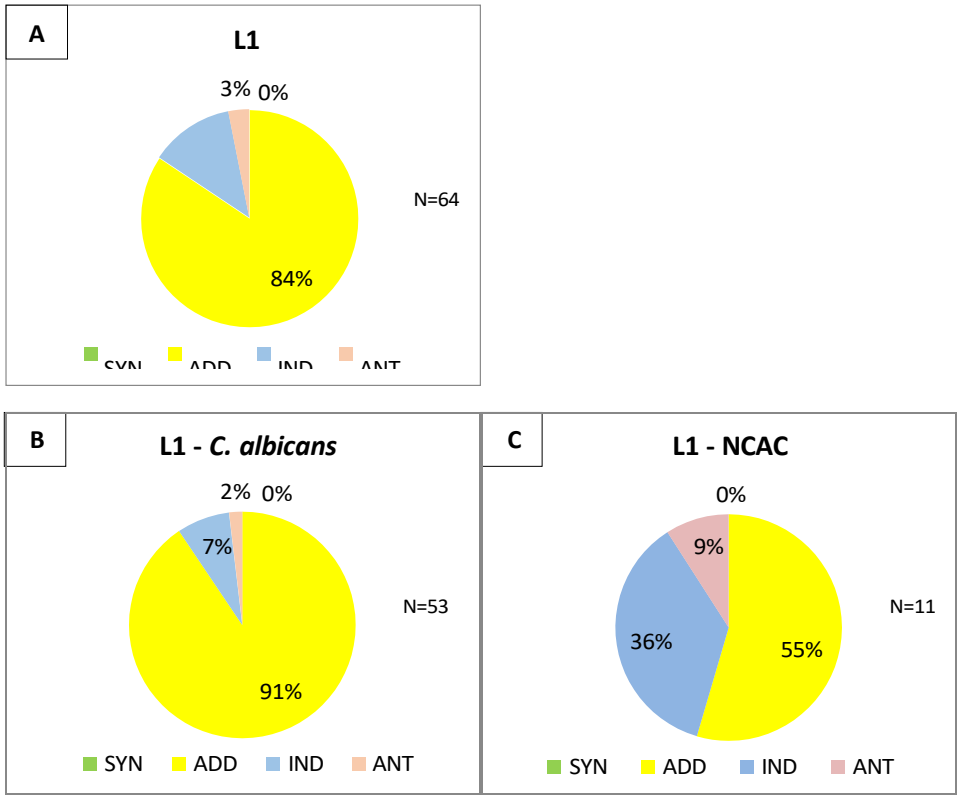


Figure 4. Distribution of FIC indices for combination of fluconazole–L1: (**A**) total—64 strains; (**B**) *C. albicans*—53 strains; (**C**) NCAC—11 strains.

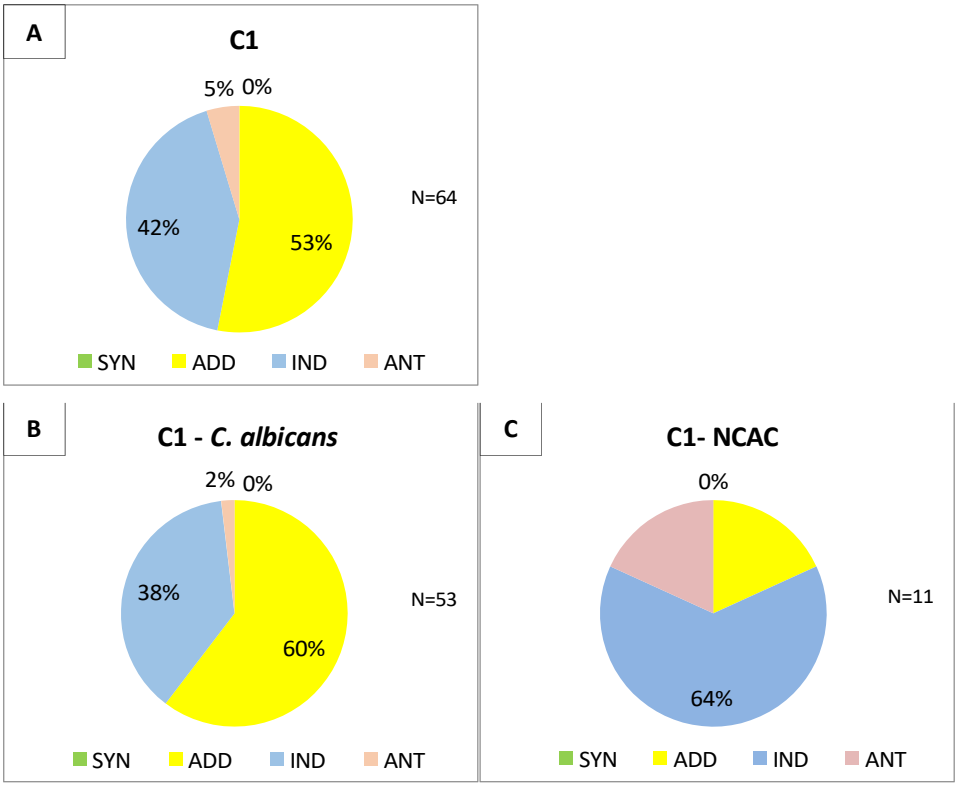


Figure 5. Distribution of FIC indices for the combination of fluconazole–C2; (**A**) total-64 strains; (**B**) *C. albicans*-53 strains; (**C**) NCAC-11 strains.

Interestingly, the FIC index itself is an arithmetic mean of eight obtained individual FIC values. When searching for the most advantageous combination of fluconazole–lipopeptide concentrations, it is the single FIC values (and the corresponding concentrations) that should be taken into account and interpreted. For the vast majority of strains and both USCLs–azole pairs, a synergistic effect could be observed (FIC ≤ 0.5). This applies in particular to the linear lipopeptide L1. An analysis of the most beneficial (the lowest possible) FIC values of fluconazole–L1 showed that synergy between these two compounds is achieved against ≈72% (46/64) of the strains. Consistent results were obtained for *C. albicans* isolates (synergistic effect against 40/53, ≈75% of strains). Synergy was also the most common among NCAC fungi (6/11, ≈55%). In the case of cyclic USCL, synergistic effect occurred less often when single FIC values were analyzed, with the additive effect being dominant. For the fluconazole–C1 combination, synergism was present in ≈23% (15/64) of cases, including in ≈26% (14/53) concerned *C. albicans* and only ≈10% (1/11) NCAC. At the same time, additive effect was achieved in ≈61% (39/64)- for *C. albicans* and in ≈55% (6/11) of the remaining strains. Lipopeptide concentrations corresponding to FIC values indicating synergy were at least four times lower than MICs obtained for these strains. For example, when the MIC value of L1 was 64 µg/mL, a synergistic effect was observed when a combination of 8 µg/mL of lipopeptide and 0.031 µg/mL of fluconazole was used. The concentrations of the discussed fluconazole–L1 pair corresponding to synergistic effect, compared to the obtained MIC values, are shown in Table 2. The corresponding data for the fluconazole–C1 combination were included in the supplementary material as Supplementary Table S1.

Table 2. Concentrations of fluconazole–L1 combination exhibited a synergistic effect against 46 isolates of *Candida* strains.

	FIC		
MIC of L1 [µg/mL]	**L1 [µg/mL]**	**Fluconazole [µg/mL] (Random Order)**	**No. of Strains Against Which This Combination was Effective**
16	2	0.002 or 0.5	2 × *C. albicans* 1 × *C. glabrata*
16	4	0.002 or 0.031 or 0.5	5 × *C. albicans*
32	8	0.002 or 0.031	8 × *C. albicans*
32	4	0.002 or 0.004 or 0.5	6 × *C. albicans* 1 × *C. glabrata*
32	2	0.002 or 0.063 or 1	5 × *C. albicans* 3 × *C. glabrata*
64	16	0.002 or 0.016	3 × *C. albicans*
64	8	0.002 or 0.031	6 × *C. albicans*
64	4	0.002 or 0.004	4 × *C. albicans*
64	2	0.004 or 1	1 × *C. albicans* 1 × *C. lusitaniae*

4. Discussion

Being a local infection not associated with mortality, Vulvovaginal candidiasis (VVC) is a clinical problem that is relatively often underestimated in comparison to other types of infections, including other candidosis [6,10]. With the pathomechanism of VVC still not fully understood and a possibility that biofilm formation could be one of the most crucial virulence factors of *Candida* in the development of this condition, the search for new antimicrobial agents also focuses on anti-biofilm activity [3,5,10,48].

Antimicrobial peptides (AMPs), including lipopeptides, with a broad spectrum of activity and a different mechanism of action compared to traditional antibiotics, are capable of eradication of fungal biofilms [16–18,21]. Based on our previous study, ultrashort

cationic lipopeptides (USCLs) with the most potent antifungal and antibiofilm activities were selected. Two with linear structure, one modified by replacing one lysine residue (K) with an arginine residue (R)—C_{16}-KKKK-NH_2 (L1) and C_{16}-KRKK-NH_2 (L2), as well as two cyclic analogs—C_{16}-CKKKKC-NH_2 (C1) and C_{16}-CKRKKC-NH_2 (C2) (C-L-cysteine residue) [16].

The literature indicates the possibility of a beneficial effect of combinations of fluconazole with various AMPs against e.g., yeast-like fungi, most likely due to the different mechanisms of action of these two groups of compounds [30,37–42]. Meanwhile, to the best of our knowledge, no such experiments have been performed using USCLs against *Candida* isolated from VVC.

With one exception of a single *C. lusitaniae* isolate, all tested strains were found to be susceptible to fluconazole (Figure 1A). This is not an unusual situation; more surprising is the fact of such frequent clinical therapeutic failures with this mycostatic. Considering the multifactorial pathomechanism of vaginal invasion by yeast-like fungi, their ability to form a highly resistant biofilm structure may be the major cause of the ineffectiveness of conventionally used azoles [11,49–51]. All four analyzed ultrashort lipopeptides showed activity against *Candida* strains. In the case of cyclic analogs, the achieved concentrations inhibiting the growth of planktonic cells are 2–3 times lower than those of their linear counterparts (Figure 1B,C). On the other hand, when comparing the activity of USCLs consisting of only lysine residues with lipopeptides with one arginine residue, the differences still seem to depend on the cyclic/linear structure of the compared compounds. In the case of cyclic analogs, the differences between MICs for C1 and C2 are virtually unnoticeable, while the antimicrobial activity of linear L2 exceeds that of a lipopeptide consisting exclusively of lysine (L1). This finding is consistent with those of our earlier study in which activity against different *Candida* reference strains was analyzed [16]. Similarly to our previous reports, disulfide-cyclized lipopeptides were substantially more active against both biofilm and planktonic cultures of yeast-like fungi than the corresponding parent molecules. There is an unconfirmed hypothesis that disulfide cyclized USCLs are transported inside the fungal cell, causing degradation of the cell membrane and its interior and leading to cell death [52]. Also, the potential advantage of linear analogs with the arginine residue, observed both by Neubauer and in this study, may support the first reports of the accumulation of protamine (salmon) rich in cationic arginine as necessary for anti-*Candida* activity [52]. The determined SIs (Table 1) of cyclic lipopeptides (5.50 and 8.83) are much higher than those of their linear counterparts (0.28 and 0.89). Those results are in agreement with the literature. It has been shown that linear short cationic lipopeptides with hexadecanoic acid chain exhibited no selectivity between pathogens and normal human cells [16,18,21,53]. It is worth mentioning that for similar USCL, consisting of only two lysine residues (C_{16}-KK-NH_2), antifungal activity was already demonstrated, e.g., against *Cryptococcus neoformans* and dermatophytes [43,54].

Similar conclusions are provided by the analysis of the obtained concentrations of biofilm eradication. While fluconazole failed to deal with *Candida* biofilm (Figure 2A), all lipopeptides tested were capable of eradicating this structure (Figure 2B,C). Again, cyclic analogs exhibited more enhanced antibiofilm activity than linear parent molecules. However, there are no substantial differences between the values of eradicating concentrations obtained for analogs composed only of lysine residues versus compounds enriched with an arginine residue (Figure 1B,C). The calculated GM_MBECs support this thesis. The results of research on biofilm are also consistent with our previous reports [16]. Higher minimum concentrations of compounds obtained for the biofilm structure compared to planktonic cells are not surprising. Biofilms, both bacterial and fungal, are characterized by a much higher resistance to antimicrobial agents than planktonic cells and can be associated with therapeutic failure [9,51].

The present results showed that USCLs in combination with fluconazole can give various effects. The FIC indices obtained in the checkerboard method indicate that an additive antifungal effect was obtained more frequently for a combination of fluconazole

and linear lipopeptides than for cyclic ones. Preliminary experiments on 24 random *Candida* strains and combinations of all four USCLs with the tested azole showed that the indifferent antifungal effect against vaginally isolated fungi was predominantly observed with cyclic USCLs (Figure 3B,D)). In the case of the L2 lipopeptide, the additive effect with fluconazole occurred more often than for its cyclic analog (≈46% vs. ≈17%). Moreover, an additive effect was the most frequent with L1 (≈63%). Analyzing the above data with regard to the *Candida* species (15 isolates of *C. albicans* vs. 9 NCAC), the obtained results for cyclic lipopeptides are very similar, both *C. albicans* and other species represented an indifferent effect, while for strains from the NCAC group no additive effect was observed. A comparison of the effect of linear USCLs against *C. albicans* indicates the advantage of the analog consisting of four lysine residues, for which a vast majority has an additive effect (73%). Studies on the linear compound enriched with arginine and combined with fluconazole revealed that it had an additive and indifferent effect on a similar percentage of strains (53% and 47%, respectively). Due to the small number of isolates from the NCAC group, a detailed analysis of the distribution of the obtained results seems unreliable.

Lipopeptide L1 was selected for further studies with fluconazole owing to promising results in the preliminary results discussed above. The cyclic analog was included in this study to learn how different structures of USCLs in combination with fluconazole can affect antifungal activity against strains derived from VVC. Although in the case of both tested cyclic compounds the indifference was definitely the dominant effect, in the case of C1, a negative (antagonistic) effect was observed less frequently than for C2 (8% and 25%, respectively). Hence, testing of the remaining pool of 40 strains was performed on a pair of USCLs composed only of lysine residues. The overall FIC index analysis for the entire pool of 62 isolates and 2 reference strains confirmed the predominant additive effect of the fluconazole–L1 combination: 84% in total, 91% including *C. albicans* strains, and 55% of NCAC, although in this case, the relatively small number of isolates (11) should still be kept in mind. Interestingly, similar results were obtained for the cyclic lipopeptide, for which an additive effect was observed in 53% of cases, of which 60% were against *C. albicans*. In the NCAC group, indifference remained the most frequent result (64%). The literature has described the possibility of a favorable antifungal effect due to combinations of fluconazole with various AMPs [37–42]. The use of compounds with different mechanisms of action is generally recommended. Combined antifungal therapy has many potential benefits, such as enhancement of the fungicidal effect and broadening the spectrum of activity, which enable to fight polymicrobial infections, reduce the dose of the compounds, and thus also reduce dose-dependent toxicity as well as overcome the resistance of microorganisms [55,56]. It is noted that antimicrobial peptides interacting with the membrane bilayers could, in a way, sensitize *Candida* cells to fluconazole by increasing azole penetration into the cell, where its molecular target—14α-lanosterol demethylase (Erg enzyme)—is located and involved in ergosterol synthesis. In effect, the composition of the cell membrane changes; it liquefies and increases the permeability for K^+ and ATP, causing a fungistatic effect [57]. On the other hand, the interaction of fluconazole with the cell membrane may enhance its permeabilization by various AMPs, including lipopeptides, and enhance their fungicidal activity [55,58,59]. Other mechanisms that may be responsible for the synergistic effect of combining triazoles with compounds with a different mechanism of action include sequential inhibition of different stages in the mutual biochemical pathway or simultaneous interaction with the *Candida* cell wall and/or membrane [55]. However, this hypothesis remains unconfirmed as of today.

Last but not least, there is one more interesting aspect of research using the checkerboard method. Knowing the general nature of the interaction of the combination of fluconazole with the lipopeptide against *Candida*, the next step is to select the most favorable and effective concentrations of both compounds to combat fungi. For this purpose, the individual FIC (as the FIC index is the arithmetic mean of eight different FIC values) obtained for a given pair of compounds should be interpreted and the corresponding concentrations selected. In this way, the most favorable (the lowest) FIC for each of the

isolates was analyzed in both fluconazole–L1 and fluconazole–C1 combinations. In 72% of strains (75% of *C. albicans*, and 55% of NCAC), there was such a combination of concentrations of linear lipopeptide and FLC for which FIC indicated a synergistic effect (FIC ≤ 0.5). The most beneficial FICs for the cyclic lipopeptide still showed a predominant additive effect (61%), although a synergistic effect was also observed (23%). A detailed analysis revealed that the concentration of L1 in combination with fluconazole that results in synergy is up to four-fold lower (2 vs. 16 μg/mL) than when lipopeptide is used separately (MIC value, Table 2). The literature contains an increasing body of reports about the results of similar studies of interactions of different compounds, not always having any activity against *Candida* alone, indicating a synergistic effect of their combinations with a number of antimycotics, including triazoles. There is a great interest in research on amphiphilic Lactoferrin (LF and its derivatives, cationic peptides), the use of which together with fluconazole (and not only) results in a significant increase in fungistatic activity and a decrease in MIC values [60–63]. Although the mechanisms responsible for this phenomenon remain unexplained, attention is drawn to the beneficial effects of cationic compounds, such as LF, which may enhance the hydrophobicity of the surface of microbial cells and potentiate the antifungal activity of other compounds [61]. Another example is the advantageous synergistic effect of combinations with fluconazole of such positively charged compounds as microbicidal cationic oligomers, styrylpyridinium compounds, and novel antimicrobial peptides such as KABT-AMP derivatives or ToAP2 [55,59,64,65]. Recently, the synergism of fluconazole with surfactin (SU) against *C. albicans* has been described in more detail. Suchodolski et al. showed that SU binds to chitin and β-glucan on the surface of fungal cells, exposing it to the components of the host's immune system. However, to achieve the necessary effect, there seems to be required a reduction or complete lack of ergosterol, resulting in the corresponding changes in cell membrane, and this is ensured by the presence of fluconazole [30]. Derivatives of quaternary ammonium compounds (QAC) are other compounds whose activity is similar to that of cationic surfactants and which at the same time have a structure similar to lipopeptides (positive charge, presence of a lipid chain). One representative of this group, compound K21, was recently tested for antifungal activity for the first time. It seems to be an effective alternative to fluconazole against *Candida* strains resistant to this mycostatic. K21 also shows synergism with triazoles towards NCAC, including *C. dubliniensis* and *C. tropicalis*, but no such effect was observed for *C. albicans* [66]. Meanwhile, a combination of another quaternary ammonium compound, domiphen bromide, with miconazole (imidazole) showed a synergistic effect against not only *Candida* planktonic cells, but also a biofilm, although this effect did not occur in the case of triazoles, including fluconazole [67]. On the other hand, the mechanism of action of benzimidazolium-based QAC gemini surfactants was identified as an influence on ergosterol synthesis in a manner similar to that of triazoles. Nevertheless, benzimidazolium-based QACs were more effective and their combination with fluconazole results in a synergistic effect against various *Candida* species [68]. There are also reports in the literature about the synergistic effect of USCL with a structure similar to L1 and L2 with fluconazole and other triazoles, as well as with amphotericin B or terbinafine against *Cryptococcus neoformans* fungi and various representatives of dermatophytes [43,54]. Recently, there have also been reports of the possible synergistic effect between a CEO (citronella essential oil) and ZnO NPs (films based on chitosan with zinc oxide, ZnO, and-nanoparticles, NPs) [69]. It is also postulated that the combination of dietary flavonoid, quercetin, with fluconazole is effective against *C. albicans*, including the biofilm created by these strains on the vaginal mucosa in murine Vulvovaginal candidiasis model [70]. However, the mechanism of interaction of the above-mentioned compounds alone and in combination against fungi remains unclear. The literature and our results together clearly demonstrate the enormous potential of ultrashort cationic lipopeptides as compounds enhancing the activity of the existing antimycotics.

Therefore, the results achieved in this work constitute another important premise in the search for antifungal compounds and their combinations with conventional mycobiotics.

Moreover, our findings can contribute to the broadening of knowledge in the search for mechanisms involved in the interactions of various cationic compounds with target cells and other antimicrobial compounds. Importantly, the most serious problems to be solved before the actual use of USCLs in the treatment of fungal infections are their relatively high toxicity and unsatisfactory selectivity between microorganisms and human cells [18,21]. The use of combination therapy has a potential to significantly reduce the concentration of lipopeptides effectively against *Candida* and to reduce toxicity towards human cells.

5. Conclusions

The results of this study performed with clinical isolates of *Candida* species confirmed high antifungal potency of USCLs, which have previously been studied only with reference yeast strains. Among the four tested lipopeptides, the cyclic compounds C1 and C2 (C_{16}-CKKKKC-NH_2 and C_{16}-CKRKKC-NH_2) showed higher activity against planktonic cells and biofilm of *Candida* isolated from VVC than their linear analogs, L1 and L2 (C_{16}-KKKK-NH_2 and C_{16}-KRKK-NH_2). Both cyclic counterparts were also more selective to pathogens over human cells, as demonstrated by SIs. Although the linear lipopeptide with a single arginine residue appeared to be more active against planktonic cells than the USCL consisting of only four lysine residues, no similar relationship was observed for biofilm-eradicating concentrations. With regard to VVC, it would be undoubtedly worthwhile to conduct studies on the toxicity of these compounds towards vaginal epithelium cell lines and to take into account a larger number of strains from the NCAC group. The study on the interactions of fluconazole combined with lipopeptides showed the advantage of linear USCLs over cyclic ones, especially C_{16}-KKKK-NH_2. The concentrations of the linear lipopeptides causing a synergistic effect against *Candida* species turned out to be at least four-fold lower than when lipopeptides were used separately. Presumably, it would be possible to use a combination therapy, achieving beneficial fungicidal effects owing to the use of compounds with different mechanisms of action, against which the development of resistance would be significantly impeded, at low non-toxic and therefore safer concentrations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pharmaceutics13101589/s1, Table S1. Concentrations of fluconazole–C1 combination exhibited a synergistic effect against 15 isolates of *Candida* strains.

Author Contributions: Conceptualization, P.C., J.N. and G.G.; methodology, P.C.; formal analysis,P.C. and D.N.; data curation, P.C., J.N. and D.N.; writing—original draft preparation, P.C.; writing— review and editing, D.N. and J.N.; supervision, G.G. and W.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subvention according to number of STM.A130.20.102 from the IT Simple system of Wroclaw Medical University.

Institutional Review Board Statement: The study protocol was approved by the local Bioethics Committee of Wrocław Medical University (No. 774/2018, approval date: 27 December 2018). All experiments were performed in accordance with relevant guidelines and regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no competing interests.

References

1. Sobel, J.D. Vulvovaginal candidosis. *Lancet* 2007, *369*, 1961–1971. [CrossRef]
2. Gonçalves, B.; Ferreira, C.; Alves, C.T.; Henriques, M.; Azeredo, J.; Silva, S. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit. Rev. Microbiol.* 2016, *42*, 905–927. [CrossRef] [PubMed]
3. Muzny, C.A.; Schwebke, J.R. Biofilms: An underappreciated mechanism of treatment failure and recurrence in vaginal infections.*Clin. Infect. Dis.* 2015, *61*, 601–606. [CrossRef] [PubMed]
4. Brandolt, T.M.; Klafke, G.B.; Gonçalves, C.V.; Bitencourt, L.R.; de Martinez, A.M.B.; Mendes, J.F.; Meireles, M.C.A.; Xavier, M.O. Prevalence of Candida spp. in cervical-vaginal samples and the in vitro susceptibility of isolates. *Braz. J. Microbiol.* 2017, *48*, 145–150. [CrossRef] [PubMed]
5. Hacioglu, M.; Guzel, C.B.; Savage, P.B.; Seher Birteksoz Tan, A. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against Candida spp. Isolated from vulvovaginal candidiasis. *Med. Mycol.* 2019, *57*, 291–299. [CrossRef] [PubMed]
6. Sherry, L.; Kean, R.; McKloud, E.; O'Donnell, L.E.; Metcalfe, R.; Jones, B.L.; Ramage, G. Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole. *Antimicrob. Agents Chemother.* 2017, *61*, 1–4. [CrossRef]
7. Naglik, J.R.; Rodgers, C.A.; Shirlaw, P.J.; Dobbie, J.L.; Fernandes-Naglik, L.L.; Greenspan, D.; Agabian, N.; Challacombe, S.J. Differential Expression of Candida albicans Secreted Aspartyl Proteinase and Phospholipase B Genes in Humans Correlates with Active Oral and Vaginal Infections. *J. Infect. Dis.* 2003, *188*, 469–479. [CrossRef]
8. Swidsinski, A.; Guschin, A.; Tang, Q.; Dörffel, Y.; Verstraelen, H.; Tertychnyy, A.; Khayrullina, G.; Luo, X.; Sobel, J.D.; Jiang, X. Vulvovaginal candidiasis: Histologic lesions are primarily polymicrobial and invasive and do not contain biofilms. *Am. J. Obstet. Gynecol.* 2019, *220*, 91.e1–91.e8. [CrossRef]
9. Harriott, M.M.; Lilly, E.A.; Rodriguez, T.E.; Fidel, P.L.; Noverr, M.C. Candida albicans forms biofilms on the vaginal mucosa.*Microbiology* 2010, *156*, 3635–3644. [CrossRef]
10. Paiva, L.C.F.; Vidigal, P.G.; Donatti, L.; Svidzinski, T.I.E.; Consolaro, M.E.L. Assessment of in vitro biofilm formation by Candida species isolates from vulvovaginal candidiasis and ultrastructural characteristics. *Micron* 2012, *43*, 497–502. [CrossRef]
11. Rodríguez-Cerdeira, C.; Gregorio, M.C.; Molares-Vila, A.; López-Barcenas, A.; Fabbrocini, G.; Bardhi, B.; Sinani, A.; Sánchez- Blanco, E.; Arenas-Guzmán, R.; Hernandez-Castro, R. Biofilms and vulvovaginal candidiasis. *Colloids Surf. B Biointerfaces* 2019, *174*, 110–125. [CrossRef]
12. Harriott, M.M.; Noverr, M.C. Importance of Candida-bacterial polymicrobial biofilms in disease. *Trends Microbiol.* 2011, *19*,557–563. [CrossRef]
13. Tsui, C.; Kong, E.F.; Jabra-Rizk, M.A. Pathogenesis of Candida albicans biofilm. *Pathog. Dis.* 2016, *74*, ftw018. [CrossRef]
14. Khan, F.; Bamunuarachchi, N.I.; Pham, D.T.N.; Tabassum, N.; Khan, M.S.A.; Kim, Y.M. Mixed biofilms of pathogenic Candida- bacteria: Regulation mechanisms and treatment strategies. *Crit. Rev. Microbiol.* 2021, 1–29. [CrossRef]
15. Dawgul, M.; Barańska-Rybak, W.; Bielińska, S.; Nowicki, R.; Kamysz, W. Wpływ peptydów przeciwdrobnoustrojowych na biofilm Candida. *Alerg. Astma Immunol.* 2010, *15*, 220–225.
16. Neubauer, D.; Jaś´kiewicz, M.; Sikorska, E.; Bartoszewska, S.; Bauer, M.; Kapusta, M.; Narajczyk, M.; Kamysz, W. Effect of disulfide cyclization of ultrashort cationic lipopeptides on antimicrobial activity and cytotoxicity. *Int. J. Mol. Sci.* 2020, *21*, 7208. [CrossRef]
17. Czechowicz, P.; Nowicka, J. Antimicrobial activity of lipopeptides. *Postep. Mikrobiol.* 2018, *57*, 213–227.
18. Mangoni, M.L.; Shai, Y. Short native antimicrobial peptides and engineered ultrashort lipopeptides: Similarities and differences in cell specificities and modes of action. *Cell. Mol. Life Sci.* 2011, *68*, 2267–2280. [CrossRef] [PubMed]
19. Jones, L.; Hobden, C.; O'Shea, P. Use of a real-time fluorescent probe to study the electrostatic properties of the cell surface of Candida albicans. *Mycol. Res.* 1995, *99*, 969–976. [CrossRef]
20. Lohan, S.; Cameotra, S.S.; Bisht, G.S. Systematic study of non-natural short cationic lipopeptides as novel broad-spectrum antimicrobial agents. *Chem. Biol. Drug Des.* 2013, *82*, 557–566. [CrossRef]
21. Makovitzki, A.; Avrahami, D.; Shai, Y. Ultrashort antibacterial and antifungal lipopeptides. *Proc. Natl. Acad. Sci. USA* 2006, *103*,15997–16002. [CrossRef]
22. Greber, K.E. Synthesis and Surface Activity of Cationic Amino Acid-Based Surfactants in Aqueous Solution. *J. Surfactants Deterg.*2017, *20*, 1189–1196. [CrossRef]
23. Serrano, G.N.; Zhanel, G.G.; Schweizer, F. Antibacterial activity of ultrashort cationic lipo-β-peptides. *Antimicrob. Agents Chemother.* 2009, *53*, 2215–2217. [CrossRef]
24. Hensel, B.; Jakop, U.; Scheinpflug, K.; Mühldorfer, K.; Schröter, F.; Schäfer, J.; Greber, K.; Jung, M.; Schulze, M. Low temperature preservation of porcine semen: Influence of short antimicrobial lipopeptides on sperm quality and bacterial load. *Sci. Rep.* 2020, *10*, 1–12. [CrossRef] [PubMed]
25. Ciandrini, E.; Morroni, G.; Arzeni, D.; Kamysz, W.; Neubauer, D.; Kamysz, E.; Cirioni, O.; Brescini, L.; Baffone, W.; Campana, R. Antimicrobial Activity of Different Antimicrobial Peptides (AMPs) Against Clinical

Methicillin-resistant Staphylococcus aureus (MRSA). *Curr. Top. Med. Chem.* 2018, *18*, 2116–2126. [CrossRef] [PubMed]
26. Greber, K.E.; Dawgul, M.; Kamysz, W.; Sawicki, W. Cationic net charge and counter ion type as antimicrobial activity determinant factors of short lipopeptides. *Front. Microbiol.* 2017, *8*, 1–10. [CrossRef] [PubMed]
27. Sikorska, E.; Dawgul, M.; Greber, K.; Iłowska, E.; Pogorzelska, A.; Kamysz, W. Self-assembly and interactions of short antimicrobial cationic lipopeptides with membrane lipids: ITC, FTIR and molecular dynamics studies. *Biochim. Biophys. Acta Biomembr.* 2014, *1838*, 2625–2634. [CrossRef] [PubMed]
28. Desmyttere, H.; Deweer, C.; Muchembled, J.; Sahmer, K.; Jacquin, J.; Coutte, F.; Jacques, P. Antifungal activities of bacillus subtilis lipopeptides to two venturia inaequalis strains possessing different tebuconazole sensitivity. *Front. Microbiol.* 2019, *10*, 1–10. [CrossRef]
29. Ceresa, C.; Rinaldi, M.; Fracchia, L. Synergistic activity of antifungal drugs and lipopeptide AC7 against *Candida albicans* biofilm on silicone. *AIMS Bioeng.* 2017, *4*, 318–334. [CrossRef]
30. Suchodolski, J.; Derkacz, D.; Muraszko, J.; Panek, J.J.; Jezierska, A.; Łukaszewicz, M.; Krasowska, A. Fluconazole and lipopeptide surfactin interplay during Candida albicans plasma membrane and cell wall remodeling increases fungal immune system exposure. *Pharmaceutics* 2020, *12*, 314. [CrossRef]
31. Liu, X.; Ren, B.; Gao, H.; Liu, M.; Dai, H.; Song, F.; Yu, Z.; Wang, S.; Hu, J.; Kokare, C.R.; et al. Optimization for the production of surfactin with a new synergistic antifungal activity. *PLoS ONE* 2012, *7*, 1–9. [CrossRef] [PubMed]
32. Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochimie* 1992, *74*, 1047–1051. [CrossRef]
33. Tabbene, O.; Di Grazia, A.; Azaiez, S.; Ben Slimene, I.; Elkahoui, S.; Alfeddy, M.N.a.; Casciaro, B.; Luca, V.; Limam, F.; Mangoni,M.L. Synergistic fungicidal activity of the lipopeptide bacillomycin D with amphotericin B against pathogenic Candida species.*FEMS Yeast Res.* 2015, *15*, fov022. [CrossRef]
34. Ramachandran, R.; Shrivastava, M.; Narayanan, N.N.; Thakur, R.L.; Chakrabarti, A.; Roy, U. Evaluation of antifungal efficacy of three new cyclic lipopeptides of the class bacillomycin from Bacillus subtilis RLID 12.1. *Antimicrob. Agents Chemother.* 2018, *62*, 1–11. [CrossRef]
35. Kai, H.; Yamashita, M.; Nakamura, I.; Yoshikawa, K.; Nitta, K.; Watanabe, M.; Inamura, N.; Fujie, A. Synergistic antifungal activity of KB425796-C in combination with micafungin against Aspergillus fumigatus and its efficacy in murine infection models.*J. Antibiot.* 2013, *66*, 479–484. [CrossRef]
36. Dimkic´, I.; Beric´, T.; Stevic´, T.; Pavlovic´, S.; Šavikin, K.; Fira, D.; Stankovic´, S. Additive and synergistic effects of Bacillus spp. isolates and essential oils on the control of phytopathogenic and saprophytic fungi from medicinal plants and marigold seeds. *Biol. Control.* 2015, *87*, 6–13. [CrossRef]
37. Sun, W.; Zhang, L.; Lu, X.; Feng, L.; Sun, S. The synergistic antifungal effects of sodium phenylbutyrate combined with azoles against Candida albicans via the regulation of the Ras-cAMP-PKA signalling pathway and virulence. *Can. J. Microbiol. Can.* 2019, *65*, 105–115. [CrossRef] [PubMed]
38. Taveira, G.B.; Carvalho, A.O.; Rodrigues, R.; Trindade, F.G.; Da Cunha, M.; Gomes, V.M. Thionin-like peptide from Capsicum annuum fruits: Mechanism of action and synergism with fluconazole against Candida species. *BMC Microbiol.* 2016, *16*, 1–13. [CrossRef]
39. De Aguiar, F.L.L.; Cavalcante, C.; dos Santos Fontenelle, R.O.; Falcão, C.B.; Andreu, D.; Rádis-Baptista, G. The antiproliferative peptide Ctn[15–34] is active against multidrug-resistant yeasts Candida albicans and Cryptococcus neoformans. *J. Appl. Microbiol.* 2020, *128*, 414–425. [CrossRef]
40. Maurya, I.K.; Thota, C.K.; Sharma, J.; Tupe, S.G.; Chaudhary, P.; Singh, M.K.; Thakur, I.S.; Deshpande, M.; Prasad, R.; Chauhan,V.S. Mechanism of action of novel synthetic dodecapeptides against Candida albicans. *Biochim. Biophys. Acta Gen. Subj.* 2013,*1830*, 5193–5203. [CrossRef]
41. Mora-Navarro, C.; Caraballo-Leõn, J.; Torres-Lugo, M.; Ortiz-Bermúdez, P. Synthetic antimicrobial β-peptide in dual-treatment with fluconazole or ketoconazole enhances the in vitro inhibition of planktonic and biofilm Candida albicans. *J. Pept. Sci.* 2015, *21*, 853–861. [CrossRef]
42. Maurya, I.K.; Pathak, S.; Sharma, M.; Sanwal, H.; Chaudhary, P.; Tupe, S.; Deshpande, M.; Chauhan, V.S.; Prasad, R. Antifungal activity of novel synthetic peptides by accumulation of reactive oxygen species (ROS) and disruption of cell wall against Candida albicans. *Peptides* 2011, *32*, 1732–1740. [CrossRef] [PubMed]
43. Simonetti, O.; Arzeni, D.; Ganzetti, G.; Silvestri, C.; Cirioni, O.; Gabrielli, E.; Castelletti, S.; Kamysz, W.; Kamysz, E.; Scalise, G.; et al. In vitro activity of the lipopeptide derivative (Pal-lys-lys-NH 2), alone and in combination with antifungal agents, against clinical isolates of dermatophytes. *Br. J. Dermatol.* 2009, *161*, 249–252. [CrossRef]
44. Pfaller, M.A.; Haturvedi, V.; Espinel-Ingroff, A.; Ghannoum, M.A.; Gosey, L.L.; Odds, F.C.; Rex, J.H.; Rinaldi, M.G.; Sheehan, D.J.; Walsh, T.J.; et al. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition Serving The World' S Medical Science Community Through Voluntary Consensus*; NCCLS: Wayne, PA, USA, 2002; Volume 22, ISBN 1562384694.
45. Krom, B.P.; Cohen, J.B.; McElhaney-Feser, G.; Busscher, H.J.; van der Mei, H.C.; Cihlar, R.L. Conditions for Optimal Candida Biofilm Development in Microtiter Plates. In *Candida Albicans Methods and Protocols*;

Humana Press: Totowa, NJ, USA, 2009; ISBN 9781626239777.
46. Nilius, A.M. *Antimicrobial Pharmacodynamics in Theory and Clinical Practice*; Informa Healthcare USA, Inc.: New York, NY, USA, 2002; Volume 17, ISBN 9780824729257.
47. EUCAST Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents.*Clin. Microbiol. Infect.* 2000, *6*, 503–508. [CrossRef]
48. Tulasidas, S.; Rao, P.; Bhat, S.; Manipura, R. A study on biofilm production and antifungal drug resistance among Candida species from vulvovaginal and bloodstream infections. *Infect. Drug Resist.* 2018, *11*, 2443–2448. [CrossRef] [PubMed]
49. Capoci, I.R.G.; Bonfim-Mendonça, P.D.S.; Arita, G.S.; Pereira, R.R.D.A.; Consolaro, M.E.L.; Bruschi, M.L.; Negri, M.; Svidzinski,T.I.E. Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal *Candida albicans*. *Evid. Based Complement. Altern. Med.* 2015, *2015*. [CrossRef]
50. Consolaro, M.E.L.; Albertoni, T.A.; Svidzinski, A.E.; Peralta, R.M.; Svidzinski, T.I.E. Vulvovaginal candidiasis is associated with the production of germ tubes by *Candida albicans*. *Mycopathologia* 2005, *159*, 501–507. [CrossRef]
51. Scarsini, M.; Tomasinsig, L.; Arzese, A.; D'Este, F.; Oro, D.; Skerlavaj, B. Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of Candida species isolated from vaginal infections. *Peptides* 2015, *71*, 211–221. [CrossRef]
52. Nagao, J.; Cho, T.; Mitarai, M.; Iohara, K.; Hayama, K.; Abe, S.; Tanaka, Y. Antifungal activity in vitro and in vivo of a salmon protamine peptide and its derived cyclic peptide against Candida albicans. *FEMS Yeast Res.* 2016, *17*, fow099. [CrossRef] [PubMed]
53. Dawgul, M.A.; Greber, K.E.; Bartoszewska, S.; Baranska-Rybak, W.; Sawicki, W.; Kamysz, W. In vitro evaluation of cytotoxicity and permeation study on lysine- and arginine-based lipopeptides with proven antimicrobial activity. *Molecules* 2017, *22*, 2173. [CrossRef] [PubMed]
54. Barchiesi, F.; Giacometti, A.; Cirioni, O.; Arzeni, D.; Silvestri, C.; Kamysz, W.; Abbruzzetti, A.; Riva, A.; Kamysz, E.; Scalise, G. In vitro activity of the synthetic lipopeptide PAL-Lys-Lys-NH2 alone and in combination with antifungal agents against clinical isolates of *Cryptococcus neoformans*. *Peptides* 2007, *28*, 1509–1513. [CrossRef]
55. Yuan, Y.; Zhou, F.; Su, H.; Zhang, Y. Structural design of microbicidal cationic oligomers and their synergistic interaction with azoles against Candida albicans. *Sci. Rep.* 2019, *9*, 1–11. [CrossRef]
56. Bozkurt-Guzel, C.; Hacioglu, M.; Savage, P.B. Investigation of the in vitro antifungal and antibiofilm activities of ceragenins CSA-8, CSA-13, CSA-44, CSA-131, and CSA-138 against Candida species. *Diagn. Microbiol. Infect. Dis.* 2018, *91*, 324–330. [CrossRef]
57. Venisse, N.; Grégoire, N.; Marliat, M.; Couet, W. Mechanism-based pharmacokinetic-pharmacodynamic models of in vitro fungistatic and fungicidal effects against *Candida albicans*. *Antimicrob. Agents Chemother.* 2008, *52*, 937–943. [CrossRef]
58. Czechowicz, P.; Jas´kiewicz, M.; Neubauer, D.; Gos´ciniak, G.; Kamysz, W. Anticandidal Activity of Omiganan and Its Retro Analog Alone and in Combination with Fluconazole. *Probiotics Antimicrob. Proteins* 2021, *13*, 1173–1182. [CrossRef] [PubMed]
59. Do Nascimento Dias, J.; de Souza Silva, C.; de Araújo, A.R.; Souza, J.M.T.; de Holanda Veloso Júnior, P.H.; Cabral, W.F.; da Glóriada Silva, M.; Eaton, P.; de Souza de Almeida Leite, J.R.; Nicola, A.M.; et al. Mechanisms of action of antimicrobial peptides ToAP2 and NDBP-5.7 against Candida albicans planktonic and biofilm cells. *Sci. Rep.* 2020, *10*, 1–14. [CrossRef] [PubMed]
60. Fernandes, K.E.; Carter, D.A. The antifungal activity of lactoferrin and its derived peptides: Mechanisms of action and synergy with drugs against fungal pathogens. *Front. Microbiol.* 2017, *8*, 1–10. [CrossRef] [PubMed]
61. Kobayashi, T.; Kakeya, H.; Miyazaki, T.; Izumikawa, K.; Yanagihara, K.; Ohno, H.; Yamamoto, Y.; Tashiro, T.; Kohno, S. Synergistic antifungal effect of lactoferrin with azole antifungals against candida albicans and a proposal for a new treatment method for invasive candidiasis. *Jpn. J. Infect. Dis.* 2011, *64*, 292–296. [PubMed]
62. Lupetti, A.; Paulusma-Annema, A.; Welling, M.M.; Dogterom-Ballering, H.; Brouwer, C.P.J.M.; Senesi, S.; Van Dissel, J.T.; Nibbering, P.H. Synergistic activity of the N-terminal peptide of human lactoferrin and fluconazole against Candida species. *Antimicrob. Agents Chemother.* 2003, *47*, 262–267. [CrossRef] [PubMed]
63. Bondaryk, M.; Staniszewska, M.; Zielińska, P.; Urbańczyk-Lipkowska, Z. Natural antimicrobial peptides as inspiration for design of a new generation antifungal compounds. *J. Fungi* 2017, *3*, 46. [CrossRef] [PubMed]
64. Vaitkiene˙, S.; Kuliešiene˙, N.; Sakalauskaite˙, S.; Bekere, L.; Krasnova, L.; Vigante, B.; Duburs, G.; Daugelavic˘ius, R. Antifungal activity of styrylpyridinium compounds against Candida albicans. *Chem. Biol. Drug Des.* 2021, *97*, 253–265. [CrossRef] [PubMed]
65. Lum, K.Y.; Tay, S.T.; Le, C.F.; Lee, V.S.; Sabri, N.H.; Velayuthan, R.D.; Hassan, H.; Sekaran, S.D. Activity of novel synthetic peptides against *Candida albicans*. *Sci. Rep.* 2015, *5*. [CrossRef] [PubMed]

66. John, C.N.; Abrantes, P.M.D.S.; Prusty, B.K.; Ablashi, D.V.; Africa, C.W.J. K21 compound, a potent antifungal agent: Implications for the treatment of fluconazole-resistant HIV-associated Candida species. *Front. Microbiol.* 2019, *10*, 1021. [CrossRef] [PubMed]
67. Tits, J.; Cools, F.; de Cremer, K.; de Brucker, K.; Berman, J.; Verbruggen, K.; Gevaert, B.; Cos, P.; Cammue, B.P.A.; Thevissen, K. Combination of miconazole and domiphen bromide is fungicidal against biofilms of resistant candida spp. *Antimicrob. Agents Chemother.* 2020, *64*, e01296-20. [CrossRef] [PubMed]
68. Wani, F.A.; Amaduddin; Aneja, B.; Sheehan, G.; Kavanagh, K.; Ahmad, R.; Abid, M.; Patel, R. Synthesis of Novel Benzimidazolium Gemini Surfactants and Evaluation of Their Anti-Candida Activity. *ACS Omega* 2019, *4*, 11871–11879. [CrossRef]
69. Motelica, L.; Ficai, D.; Ficai, A.; Ilie, C.; Oprea, O.; Andronescu, E. NPs/Citronella Essential Oil Nanocomposite—Potential Coating for Grapes. *Foods* 2020, *9*, 1801. [CrossRef] [PubMed]
70. Khan, F.; Bamunuarachchi, N.I.; Tabassum, N.; Jo, D.M.; Khan, M.M.; Kim, Y.M. Suppression of hyphal formation and virulence of Candida albicans by natural and synthetic compounds. *Biofouling* 2021, *37*, 626–655. [CrossRef] [PubMed]

10.3. Artykuł nr 3

International Journal of
Molecular Sciences

MDPI

Article

Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of *Candida albicans* Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study

Paulina Czechowicz [1,*], Joanna Nowicka [1], Damian Neubauer [2], Grzegorz Chodaczek [3], Paweł Krzyżek [1] and Grażyna Gościniak [1]

[1] Department of Microbiology, Faculty of Medicine, Wrocław Medical University, 50-368 Wrocław, Poland

[2] Department of Inorganic Chemistry, Faculty of Pharmacy, Medical University of Gdansk, 80-416 Gdansk, Poland

[3] Bioimaging Laboratory, Łukasiewicz Research Network–PORT Polish Center for Technology Development, 54-066 Wrocław, Poland

* Correspondence: paulina.czechowicz.umedwroc@gmail.com; Tel.: +48-71-784-13-01

Abstract: In recent years, clinicians and doctors have become increasingly interested in fungal infections, including those affecting the mucous membranes. Vulvovaginal candidiasis (VVC) is no exception. The etiology of this infection remains unexplained to this day, as well as the role and significance of asymptomatic vaginal *Candida* colonization. There are also indications that in the case of VVC, standard methods of determining drug susceptibility to antifungal drugs may not have a real impact on their clinical effectiveness—which would explain, among other things, treatment failures and relapse rates. The aim of the study was to verify the promising results obtained previously in vitro using standard methods, in a newly developed ex vivo model, using tissue fragments of the mouse vagina. The main goal of the study was to determine whether the selected ultrashort cyclic lipopeptides (USCLs) and their combinations with fluconazole at specific concentrations are equally effective against *Candida* forming a biofilm directly on the surface of the vaginal epithelium. In addition, the verification was also performed with the use of another model for the study of microorganisms (biofilms) in vitro—the BioFlux system, under microfluidic conditions. The obtained results indicate the ineffectiveness of the tested substances ex vivo at concentrations eradicating biofilm in vitro. Nevertheless, the relatively most favorable and promising results were still obtained in the case of combination therapy—a combination of low concentrations of lipopeptides (mainly linear analogs) with mycostatic fluconazole. Additionally, using BioFlux, it was not possible to confirm the previously obtained results. However, an inhibiting effect of the tested lipopeptides on the development of biofilm under microfluidic conditions was demonstrated. There is an incompatibility between the classic in vitro methods, the newer BioFlux method of biofilm testing, offering many advantages postulated elsewhere, and the ex vivo method. This incompatibility is another argument for the need, on the one hand, to intensify research on the pathomechanism of VVC, and, on the other hand, to verify and maybe modify the standard methods used in the determination of *Candida* susceptibility.

Keywords: *Candida*; biofilm; BioFlux; ex vivo model; ultrashort cyclic lipopeptides; vulvovaginal candidiasis

check for updates

Citation: Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyzek, P.; Gosciniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of *Candida albicans* Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. *Int. J. Mol. Sci.* **2022**, *23*, 14453. https://doi.org/10.3390/ijms232214453

Academic Editor: Honghua Hu

Received: 13 September 2022

Accepted: 18 November 2022

Published: 21 November 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Vaginal infections, including vulvovaginal candidiasis (VVC), have gained increased relevance in everyday medical practice, which is also reflected in the scientific literature. Being the second most commonly diagnosed gynecological infection, VVC affects over 75% of women of reproductive age worldwide and is often associated with therapeutic failures and the risk of recurrence. The most common and relatively easily recognized etiological factors of this type of infection are yeast-like fungi of the genus *Candida*. Physicians and scientists have been paying more and more attention to mucosal candidiasis—including

vulvovaginal candidiasis. Since it is not an infection that requires hospitalization, and its symptoms, as well as possible complications and relapses, are classified as relatively mild, it can be easily underestimated. In recent years, there has been a growing interest in VVC among clinicians and scientists, as well as among patients themselves [1–6]. However, despite the increasing amount of research conducted on the pathogenesis of VVC and its relapsed form, as well as on effective treatment, it turns out that more remains unknown than known. Given the difficulties associated with establishing the detailed mechanism of the development of fungal infection on the vaginal mucosa, it becomes virtually impossible to identify a specific factor as a target for potential new antimicrobials [6–10]. It is, therefore, not surprising that, on the one hand, intensive research is being carried out to identify the specific pathogenesis of these infections (which would facilitate the fight against and prevention of these infections [2,3,7]), and on the other hand, many research studies have focused on the search for new substances or therapeutic regimens with potential antifungal activity in the vagina. Because it has been known for years that *Candida* biofilm plays a role (if not the key role) in treatment resistance and VVC relapses, the newly synthesized agents are also tested for anti-biofilm activity [2,3,7,8,11–13].

Short cationic compounds are probably the largest group among the compounds that can be an alternative to mycobiotics conventionally used against yeast-like fungi. Among them, ultrashort cationic lipopeptides (USCLs) deserve special attention, as they are especially active against fungi. Their structure consists of short positively charged peptides (seven amino acid residues at most), mainly due to lysine (K) or arginine (R) residues, linked to a fatty acid chain [14–17]. As a result, these compounds are amphiphilic and easily react with the negatively charged components of the *Candida* cell membrane—sialic acid and phosphatidylinositol [18,19]. All of this results in the permeabilization of these membranes and, in effect, cell death [15–17,20]. In addition to antifungal activity, USCLs also show many other properties as antimicrobial and anti-adhesive activity, translating to their potent anti-biofilm effect [21].

In recent years, commonly used laboratory methods to determine the effect of such compounds have been considered a significant obstacle in the research on new antifungal and antibiofilm agents. Researchers often point out that in vitro methods, which primarily determine the MIC (minimum inhibitory concentration) and MBEC (minimum biofilm eradication concentration) values, may turn out to be inadequate in relation to the actual in vivo activity of the substance. Hence, various models are increasingly used, including in vivo and ex vivo models, which are mainly animal (mouse and rat) models [12,22]. So far, the uniqueness of the vaginal microenvironment and the crucial importance of the as yet unexplained (but definitely existing) interactions between *Candida* and vaginal epithelium cells (VECs) have been proven. While there are already relatively well-described models of mucosal candidiasis in the oral cavity or the gastrointestinal tract in research, a reliable and universal research model for VVC remains a challenge [12,22]. In 2010, Harriot et al. were the first to obtain *Candida* biofilm on the surface of vaginal tissue in in vivo and ex vivo animal models. Their comparison showed that in both types of models, the kinetics of biofilm formation and its structure are the same and could be used in further research, as well as that they most likely correspond to biofilms obtained in vitro, e.g., on silicone disks [12]. In 2016, Krom and Willems also compared other methods of testing *Candida* biofilm and its drug susceptibility in vitro, indicating an interesting method for studying this structure under dynamic and realistic microfluidic conditions using the BioFlux-based system [23]. The BioFlux system has many advantages, including the most important two: observing the behavior of microorganisms (biofilms) under microfluidic conditions, thanks to the performance of all experiments in the microcapillaries of this system and the possibility of real-time observation of the processes taking place. The study of fungal biofilm in the flow may have some practical impact depending on the site of infection—it is obvious that this model will be much more useful for imitating conditions prevailing in, for example, blood vessels than in mucosal infections. Referring to the strains isolated from VVC, BioFlux enables continuous 24-hour observation of the kinetics of biofilm

development, including *Candida* adhesion and germination, the production of germ tubes and filaments, as well as the subsequent detachment of fragments of the mature structure and its "migration" further. The advantage of BioFlux over standard in vitro methods in drug susceptibility testing is manifested in the assessment of the results—the possibility of observing and assessing the formation of microbial aggregates, their developmental form, and increasing or decreasing coverage of microcapillaries—without the need to take into account potential errors and problems resulting, for example, from manual handling of microscope slides or rinsing of the biofilm. Apart from the evaluation of dynamic processes, BioFlux also enables molecular mechanisms, and in the adjacent microcapillaries, one can simultaneously conduct many other experiments [23–25].

In our previous work, we compared the antifungal activity of two pairs of cationic lipopeptides against *Candida* strains isolated from vulvovaginal candidiasis. These compounds effectively eradicated the mature structure of the biofilm [26]. In in vitro studies using polystyrene plates, we have demonstrated the advantage of the newly synthesized cyclic analogs C_{16}-CKKKKC-NH_2 and C_{16}-CKRKKC-NH_2 over their linear counterparts in the antimicrobial activity against *Candida* in planktonic and biofilm form [26]. At the same time, we have already proven that the use of combination therapy in the form of the simultaneous action of antifungal fluconazole that is conventionally used in VVC and the tested lipopeptides could be equally promising—this time indicating the advantage of the linear compounds C_{16}-KKKK-NH_2 and C_{16}-KRKK-NH_2 [26]. As we have mentioned before, such an approach could solve the problem of the relatively high toxicity of this type of compound in relation to eukaryotic cells, because the combination of USCLs with fluconazole turned out to be effective at concentrations several times lower than when these substances were used separately [26].

The aim of the study was to verify the results obtained previously in classic in vitro tests using the ex vivo VVC mouse model, and in the in vitro biofilm model under microflu- idic conditions with the BioFlux-based system. The most promising pair of lipopeptides selected on the basis of previous work were used: linear C_{16}-KKKK-NH_2 and cyclic C_{16}- CKKKKC-NH_2. Their effect on mature fungal biofilm was tested against two selected clinical strains of *Candida* albicans isolated from VVC, for which the drug susceptibility of the biofilm was previously determined. Using effective in vitro concentrations, the activity of the tested lipopeptides was verified both directly on the mouse vaginal tissue (ex vivo model) and under microfluidic conditions (BioFlux model).

2. Results

2.1 Ex Vivo Animal Model

In all cases, it was possible to obtain 24-h *Candida* biofilm on the surface of the mouse vaginal tissue for both tested isolates. This was confirmed by the results obtained during the homogenization of tissues, as well as by the microscopic photos showing the characteristic long filaments and blastospores of *C. albicans*. The mean CFU per g of tissue for biofilm unexposed to any compounds (positive controls) was $1.93 \pm 1.37 \times 10^8$ for the CA1 strain and $2.02 \pm 1.21 \times 10^8$ for the CA2 strain.

Because our previous studies have shown that fluconazole is not effective in the eradication of *Candida* biofilms [26], high concentrations of amphotericin B (50 µg/mL) were used to control the eradication of this structure from tissues. This compound is not used in the treatment of VVC and shows relatively high toxicity; hence, its use was only intended as a control in our ex vivo model. The mean CFU per g for CA1 treated with AMB was $1.03 \pm 1.11 \times 10^7$ and $1.08 \times 10^7 \pm 7.91 \times 10^6$ for CA2. This results were statistically significant ($p < 0.05$), which can be clearly seen in the Supplementary Materials in Figures S1–S4.

Representative microscopic images corresponding to the described results are shown in Figure 1.

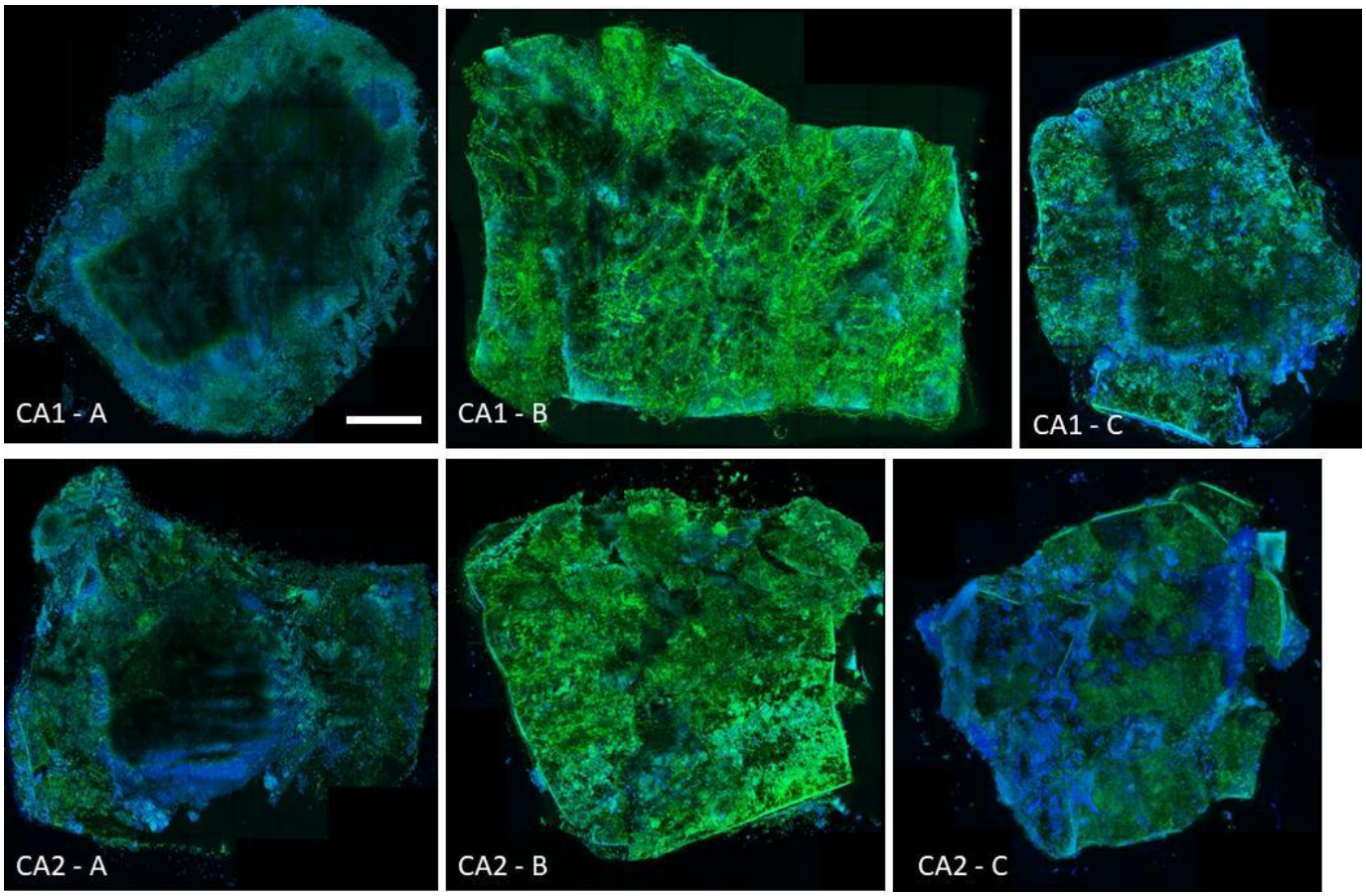


Figure 1. Mature 24-h biofilm of *Candida* albicans strains CA1 and CA2 isolated from VVC on mouse vaginal epithelium tissue (ex vivo model) under the confocal microscopy. Green color shows *C. albicans* based on Calcofluor staining and blue labels the vaginal epithelium based on Evans blue dye. CA**1–A** and CA**2–A**—negative controls (tissue without *Candida*); CA**1–B** and CA**2–B**—biofilm of strain number 1 and strain number 2, respectively; CA**1–C** and CA**2–C**—biofilms treated with amphotericin B (50 μg/mL). Scale bar = 1 mm.

The experiments showed that neither of the tested L1 and C1 lipopeptides is effective in eradicating *C. albicans* biofilm from the surface of the vaginal tissue of mice at concentrations equal to MBECs and 1/2 MBECs (Figure 2).

In the case of eradicating concentrations (MBECs) for the linear L1 compound, the average was $2.14 \pm 1.86 \times 10^8$ and $2.07 \pm 1.09 \times 10^8$ CFU/g of tissue for CA1 and CA2, respectively. Similar values were obtained for the C1 cyclic lipopeptide. For CA1, CFU/g of tissue averaged $2.33 \times 10^8 \pm 8.11 \times 10^7$, and for CA2, the mean CFU/g was $1.70 \pm 1.06 \times 10^8$, which would also indicate a decrease relative to the biofilm not exposed to any compounds. Statistical analysis showed no significance ($p > 0.05$) in the described results (Figures S1–S4 in Supplementary Materials).

When sub-inhibitory concentrations of lipopeptides (1/2 MBECs) were used, the results were slightly more optimistic. For USCL L1, the mean values of obtained CFU/g indicated the eradicating effect of this lipopeptide for both strains—$1.42 \pm 1.26 \times 10^8$ for CA1 and $1.54 \pm 1.02 \times 10^8$ for CA2. The eradicating effect of the C1 analog was indicated by the mean CFU/g tissue result obtained in the case of CA2–$1.21 \times 10^8 \pm 8.48 \times 10^7$. For strain CA1, the mean value was $2.05 \times 10^8 \pm 9.88 \times 10^7$ CFU/g. In this case, the described differences also turned out to be statistically insignificant ($p > 0.05$) (Figures S1–S4 in Supplementary Materials).

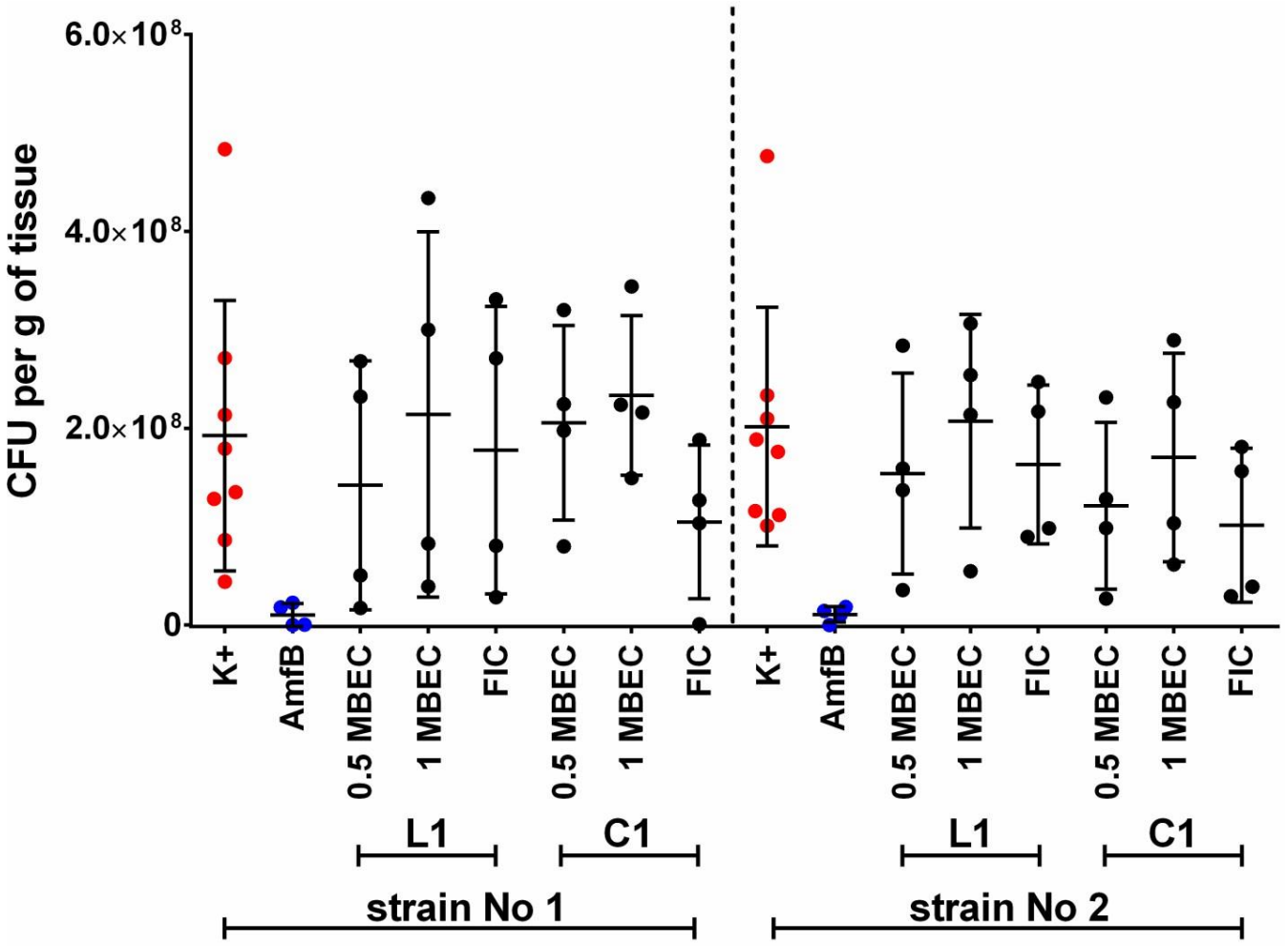


Figure 2. Distribution of CFU per gram of tissue values obtained for strains CA1 and CA2 in all courses of experiments. K(+) is referred to as a positive control (untreated biofilm of *C. albicans*). Biofilms were treated with: AMB—amphotericin B (50 μg/mL), lipopeptides L1 and C1 at a concentra- tion equal to MBEC, 1/2 MBEC, and FIC values (MBEC—minimum biofilm eradication concentration; FIC—fractional inhibitory concentration).

Compound L1 with FLC shows that the use of combinations of fluconazole with the tested lipopeptides, at concentrations equal to FICs, eradicated the *C. albicans* biofilm of both strains used (Figure 2). For compound L1 with FLC, a decrease in CFU/g to $1.78 \pm 1.46 \times 10^8$ and $1.63 \times 10^8 \pm 8.07 \times 10^7$ for CA1 and CA2, respectively, was obtained. Similarly, in the case of the cyclic C1 analog with FLC for strain CA1, the CFU/g value was $1.05 \times 10^8 \pm 7.80 \times 10^7$ and $1.01 \times 10^8 \pm 7.84 \times 10^7$ for CA2. However, again, statis- tically significant differences between the obtained results could not be proven ($p > 0.05$) (Figures S1–S4 in Supplementary Materials).

For the negative controls (tissues without any strain suspension) no growth was observed in all courses of the experiments (CFU/g was 0).

The described results combined together in one graph with standard deviations are shown in Figure 2.

A representative set of experiments in confocal microscopy is presented in Figure 3. As there is always a risk of poorly reflecting quantitative results (such as CFU/g) in microscopy when presenting fragments of photos, it was decided to use a technique that allows for microscopic mapping of the whole of the analyzed tissue. As a result, the overall coverage of the epithelium by the *Candida* biofilm, and especially certain areas of greater filament densities, can be seen in the attached figures. It can also be seen that the surface of this

tissue is not completely flat. When comparing the quantitative values with images from confocal microscopy, it is worth noting that the application (according to the methodology of Harriot et al.) of the Calcofluor dye, along with Evans blue, also stains tissue cells (blue color), not only fungal cells (green color). Importantly, however, the significant quantitative differences, expressed as CFU/g and calculated in Figure 2, are clearly visible in Figure 3 as the difference between the negative and positive controls and the eradicating effect of amphotericin B. More subtle differences in CFU/g, which are described above, are virtually impossible to observe in CM, and the amount of green-stained *Candida* biofilm filaments on tissues treated with lipopeptide in the concentrations of MBEC, 1/2 MBEC, and FIC is very difficult to compare on the basis of microscopic observation alone.

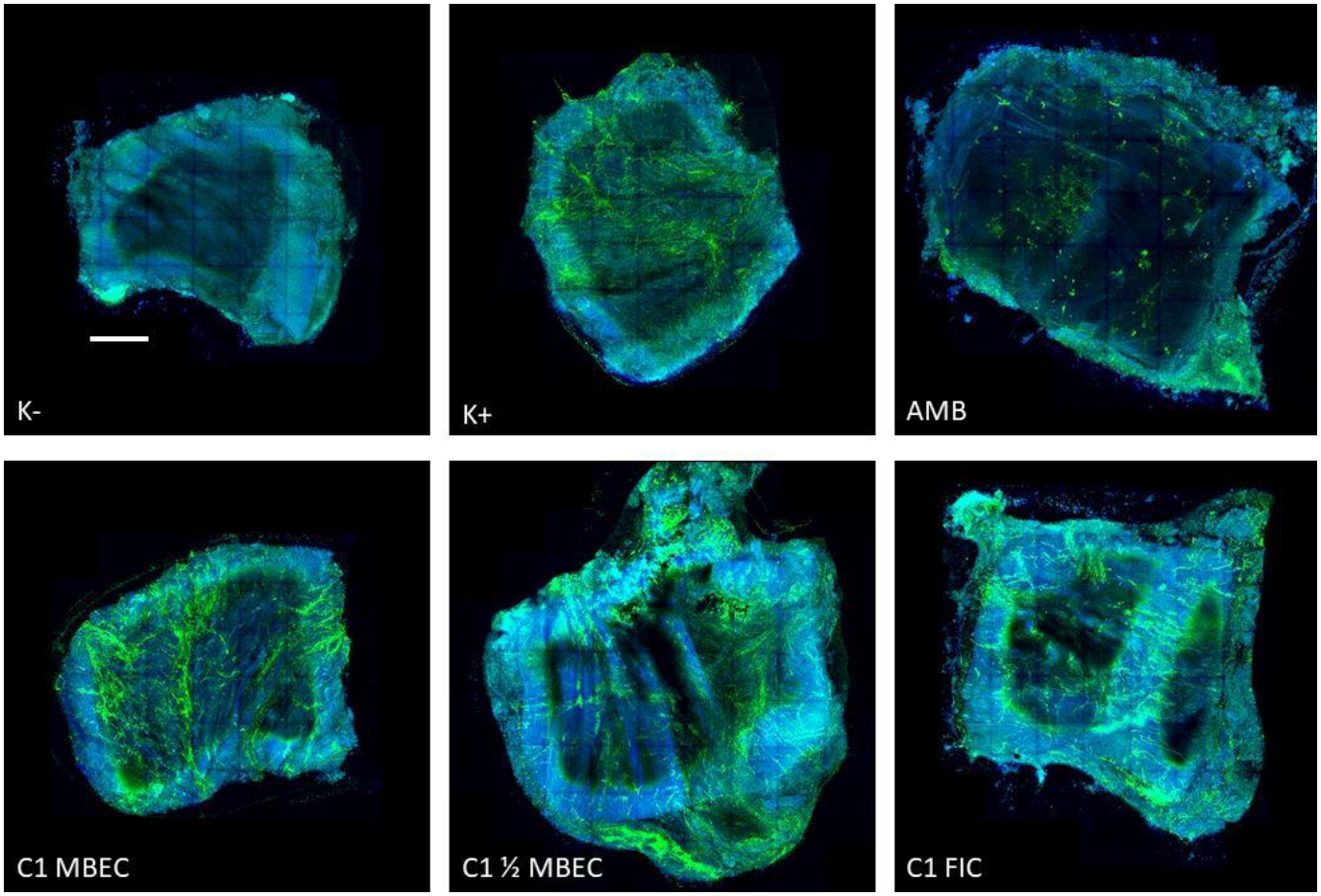


Figure 3. An exemplary set of experiments in confocal microscopy. Green color shows *C. albicans* based on Calcofluor staining, and blue labels the vaginal epithelium based on Evans Blue dye. Mature biofilm of *C. albicans* CA2 treated with: AMB—amphotericin B (50 µg/mL) and cyclic lipopeptide C1 at a concentration equal to MBEC, 1/2 MBEC, and FIC. K(−) and K(+) are referred to as negative and positive controls (MBEC—minimum biofilm eradication concentration; FIC—fractional inhibitory concentration). Scale bar = 1 mm.

2.2 BioFlux Biofilm Model

By applying the BioFlux equipment, it was also possible to obtain mature, 24-h biofilms in microfluidic conditions for both *Candida* strains. Thanks to the possibility of using real-time monitoring of the experiments, the time-lapse series microscopic photos were taken every 1 h for 24 h. These photos clearly show the presence of *Candida* blastospores, which, after 2 h of incubation in RPMI 1640, begin to form germ tubes and then long filaments. From hour 10 to hour 11 of incubation, no further changes in the created biofilm structure could be captured in the photographs. Representative images of biofilm formation over time

for strain number 1 are shown in Figure 4. In Supplementary Materials, Videos S1 and S2 provide animations of all time-lapse microscopic shots captured over the 24 h of incubation of strain CA1 and CA2, respectively.

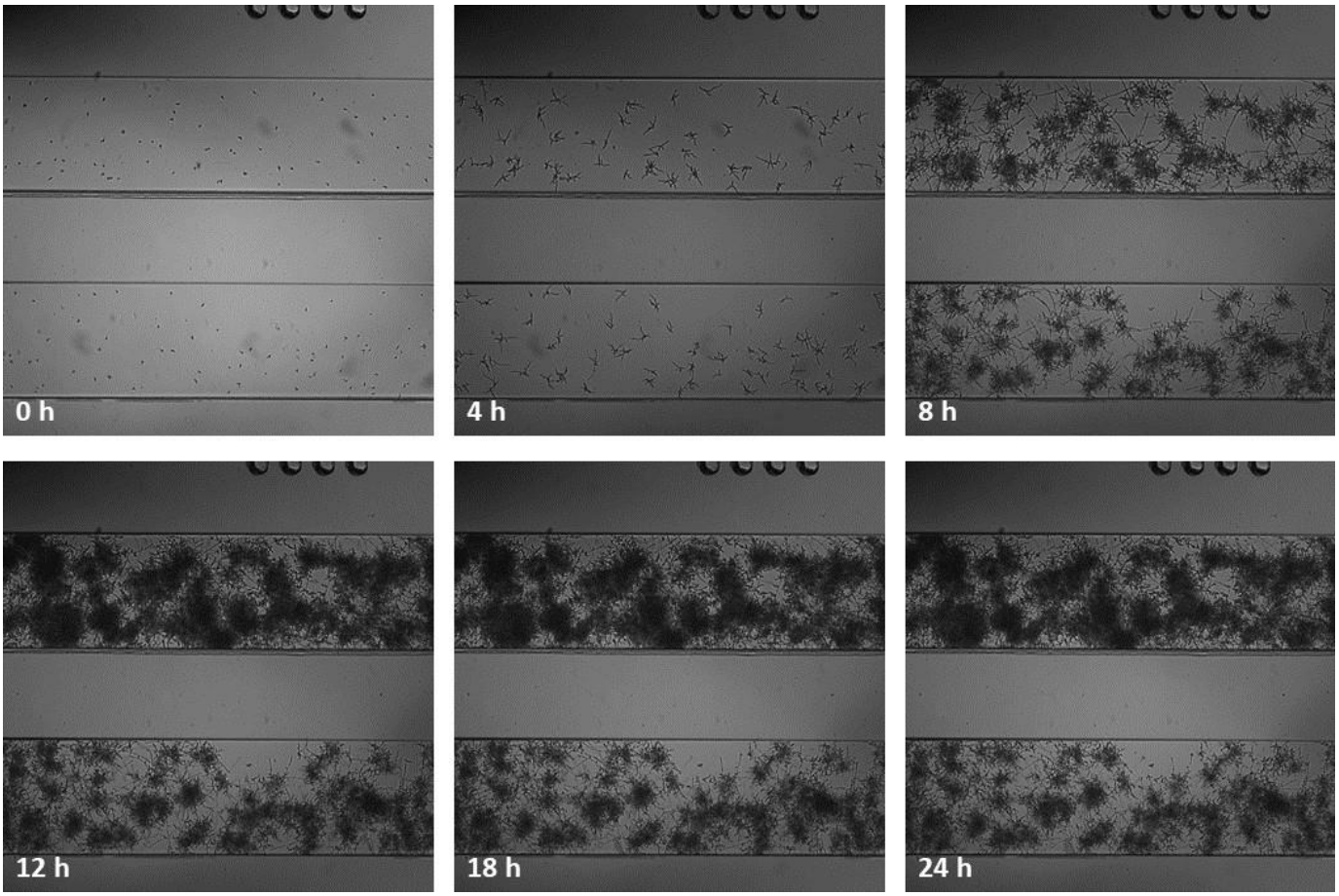
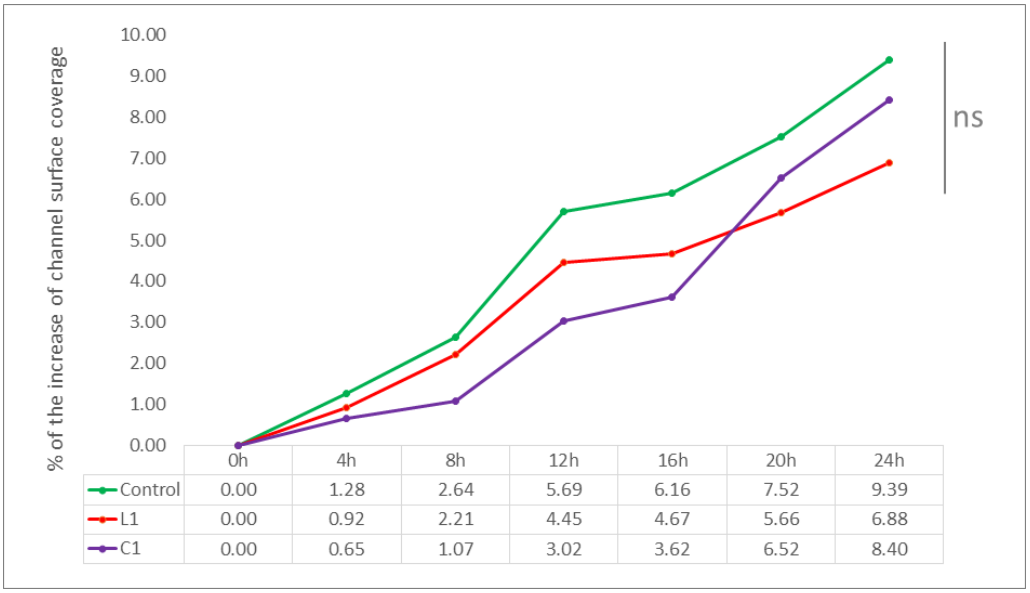


Figure 4. Biofilm formation of *C. albicans* strain CA1 in two separate channels (biofilm formation phase), in a time-lapse series taking images every 1 h over 24 h of incubation in microfluidic condi- tions with RPMI 1640 medium (without additional compounds), using BioFlux Z1000 system. The presented photographs were taken at the start of the experiment (0 h), and after 4, 8, 12, 18, and 24 h.

In the course of experiments using the BioFlux Z1000 system, the influence of both tested lipopeptides L1 and C1 at concentrations equal to MBECs was analyzed. By com- paring the average percentage of increase in biofilm biomass over 24 h of incubation, the inhibitory effect of both compounds in relation to the control samples was visible, although the observed differences turned out to be statistically insignificant ($p > 0.05$). What can be noted is that the influence of the linear L1 analog was found to be the most favorable, especially for strain CA2—in the 8th hour of incubation, the increase was 0.29%, while in the control, it was 2.45%. The final (24 h) increase in biofilm biomass of CA2 for this USCL was slightly more than a half of the value obtained in the control (4.35% vs. 8.60%). The analogous gain for CA1 was 6.88% in the presence of compound L1, compared to 9.39% for the control. The inhibitory effect of the cyclic C1 analog was also seen for both strains and, again, a more favorable result was obtained with CA2. The increase for this strain was 6.18% in the presence of C1 vs. 8.60% for the untreated biofilm. For CA1, the difference in biofilm growth observed was smaller, whereas in the control, it was 9.39%. Treatment with compound C1 lowered this value to 8.40% of channel coverage. On the other hand, for CA1, the increase in channel coverage in the presence of C1 was slightly smaller than in the positive control (8.40% vs. 9.39%). Nevertheless, according to the statistical analysis,

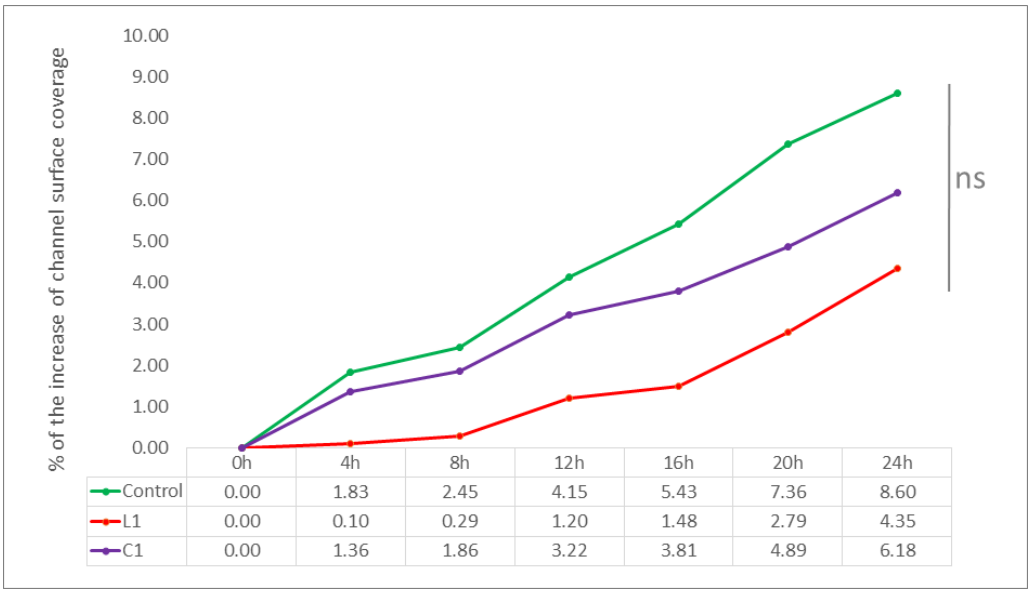
the eradicating effect of the tested USCLs at concentrations equal to MBEC against mature *Candida* biofilm could not be obtained in the Bioflux biofilm model.

The reported results, together with the exact values obtained at certain time points, are shown in Figure 5 for CA1 and in Figure 6 for CA2.



	0h	4h	8h	12h	16h	20h	24h
Control	0.00	1.28	2.64	5.69	6.16	7.52	9.39
L1	0.00	0.92	2.21	4.45	4.67	5.66	6.88
C1	0.00	0.65	1.07	3.02	3.62	6.52	8.40

Figure 5. Growth dynamics of biofilm biomass of *C. albicans* strain CA1 over 24 h in the presence of lipopeptides L1 and C1, expressed as a percentage of channel coverage in the BioFlux Z1000 system. For all tested samples, the biofilm obtained after 24 h of growth in the absence of any substances (a biofilm development phase) was taken as the starting point. All experiments were repeated in triplicate. NS—non-significant.



	0h	4h	8h	12h	16h	20h	24h
Control	0.00	1.83	2.45	4.15	5.43	7.36	8.60
L1	0.00	0.10	0.29	1.20	1.48	2.79	4.35
C1	0.00	1.36	1.86	3.22	3.81	4.89	6.18

Figure 6. Growth dynamics of biofilm biomass of *C. albicans* CA2 over 24 h in the presence of lipopeptides L1 and C1, expressed as a percentage of channel coverage in the BioFlux Z1000 system. For all tested samples, the biofilm obtained after 24 h of growth in the absence of any substances (a biofilm development phase) was taken as the starting point. All experiments were repeated in triplicate. NS—non-significant.

Exemplary set of microscopic images of the CA1 strain biofilm formation phase (first 24 h) and eradication phase (next 24 h), with L1 and C1 visible in Figure 7 below.

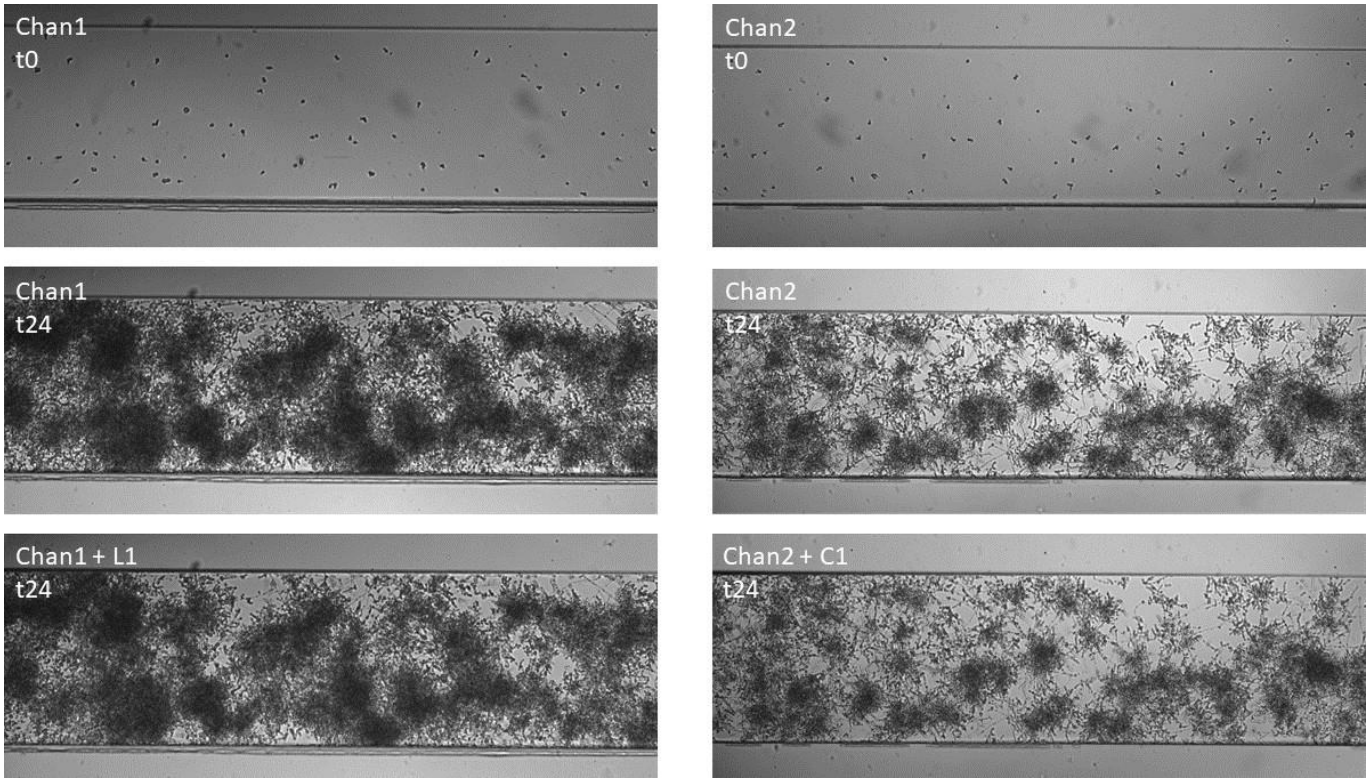


Figure 7. *C. albicans* CA1 biofilm formation and eradication, incubated in RPMI1640 medium under microfluidic conditions, in two separate channels (Chan1 and Chan2) of the BioFlux Z1000 system. t0—start of experiments, CA1 blastospores; t24—formed 24-h mature CA1 biofilm; L1/C1 t24—24-h eradication of CA1 biofilm with lipopeptides L1 or C1, respectively.

3. Discussion

Lipopeptides are a group of substances with a wide range of antimicrobial activity already known to researchers. In our previous work, using classical in vitro methods (determination of MIC, MBEC, and FIC values), we proved the effectiveness of the newly synthesized ultra-short cyclic lipopeptides (USCLs) against yeast-like fungi, both in planktonic and biofilm form [15,26]. Among other properties, USCLs have recognized anti-adhesive properties and, thus, anti-biofilm and antimicrobial action, including against *Candida* [14–17]. For this reason, we are investigating the effect of these compounds on *Candida* strains isolated from vulvovaginal candidiasis (VVC). Both in the scientific literature and in everyday clinical practice, attention is increasingly being paid to this infection for several reasons, including its extremely common occurrence, the still unknown etiology, and the increasingly frequent and difficult to explain treatment failures and recurrences of VVC. Based on our previous research, two (linear and cyclic) of the most promising USCLs were selected for further study. Their structure consists of a palmitic acid residue (C16, hexadecenoic acid) conjugated to a positively charged peptide with L-lysine residues and C-terminal amide. In the case of the cyclic analog, cyclization was achieved with two cysteine (C-cysteine) residues linked by a disulfide bridge [16,19]. In our study, the compounds C_{16}-KKKK-NH_2 (L1) and C_{16}-CKKKKC-NH_2 (C1) were able to inhibit the plankton growth of various *Candida* strains isolated from VVC, as well as to eradicate the mature biofilm structure formed by these isolates at lower concentrations than the other tested USCLs [26]. At the same time, they showed relatively low toxicity towards HaCaT and the lowest hemolytic capacity [15]. In addition, they showed the most favorable (synergistic or additive) effect when using combination therapy—the simultaneous use of these lipopeptides in combination with antifungal fluconazole, which is conventionally used in VVC [26].

The aim of this study was to verify the results obtained previously using standard in vitro methods for determining drug susceptibility. This was performed using two other methods of testing the effect of various compounds on *Candida* biofilm—a newly developed ex vivo model, in which we used mouse vaginal tissues, and a biofilm model in microfluidic conditions, obtained by using BioFlux technology. Two *C. albicans* strains isolated from VVC were selected for the study, with MIC, MBEC, and FIC values previously determined for fluconazole as well as for L1 and C1 lipopeptides [26]. The experiments consisted of creating a mature (i.e., 24-h) biofilm using these strains, and then treating this structure with the tested compounds at concentrations considered in the course of previous studies to be the lowest which were effective in eradication. The expected result was at least a notable reduction in biofilm biomass under the influence of L1 and C1 (at concentrations equal to MBEC) and their combination with fluconazole (at concentrations equal to FIC), similarly to the RPMI 1640 microdilution method on polystyrene plates. In addition, it was decided to use sub-inhibitory concentrations of lipopeptides in the model using mouse vaginal tissues. There exist reports on the potential action of certain AMPs (antimicrobial peptides), including cationic lipopeptides, resulting in sensitization of various microorganisms to the effects of the so-called host defense peptides (HDPs) which naturally occur in the body. Although the mechanism of this action is currently unknown, it has already been observed that the addition of low (sub-inhibitory) concentrations of AMPs to, for example, serum may inhibit bacterial growth, most likely enhancing the action of complement proteins, lysozyme, or lactoferrin [16,27,28]. It is known that there is a mucus layer on the vaginal mucosa and in the vagina itself, produced by the VEC, which has antimicrobial properties and contains many substances, including lactoferrin and lysozyme [29,30]. Unfortunately, in vitro, it is almost impossible, or at least extremely difficult, to recreate or obtain this substance in a manner similar to, for example, the collection of saliva or serum. In addition, obtaining a tissue culture of VK2 cells would not reflect the complex mechanisms between VECs, their products, or yeast-like fungi. Hence, it was decided that we would test the hypothesis of the potential supporting action of lipopeptides at sub-inhibitory concentrations against various, including unidentified, HDPs in the vaginal microenvironment using an ex vivo animal model.

The MBECs were identical for both tested *C. albicans*. In the in vitro microdilution method in RPMI 1640 on polystyrene plates, the use of these concentrations resulted in no signal (MTT solution color change) when visualizing the results, which meant that no metabolically active sessile cells were present [26]. In an ex vivo animal model using mouse vaginal tissue fragments, treatment of the 24 h biofilm formed by these strains with the same concentrations of L1 and C1 did not eradicate *Candida*. In confocal microscopy, a thick layer of fungal hyphae stained with Calcofluor White was still visible. The failure of biofilm eradication in this model was demonstrated by the results obtained with tissue homogenization as well. For both the CA1 and CA2 strains, in the presence of L1 equal to MBEC, the mean values of CFU/g of tissue were generally higher than in positive controls—Figure 2 and Figures S1 and S3 in Supplementary Materials; no statistical significance ($p > 0.05$). For strain CA1, when using C1, the value of CFU/g visibly exceeded the result for the positive control. A low decrease in mean value was only obtained for CA2 using C1 (again, without statistical significance). However, it should be kept in mind that the obtained results may be subject to some variations depending on various laboratory factors. Examples of such steps potentially leading to greater uncertainty include manual homogenization of tissue fragments or transfer of tissues with strain suspensions to microscope slides in CM. Furthermore, one should remember the tissue-dependent characteristics, as the tissues were obtained from successive mice. Therefore, the noticeable standard deviations shown in Figure 2 should not be surprising either. Nevertheless, the presence of a relatively large amount of *Candida* (most often exceeding the values obtained in positive controls) is certain, despite the use of eradicating lipopeptide concentrations. In no case was there a decrease in the amount of fungi on the surface of the tissue, which was comparable, for example, to the significantly lower values obtained for high concentrations

of amphotericin B (Figure 2, Figures S1–S4 in Supplementary Materials) ($p < 0.05$). On this basis, it can be concluded that the results obtained with the classical in vitro methods were not confirmed in the ex vivo method. Of course, the question remains as to which of the methods is more reliable. Microdilution methods have been recognized and approved for many years as reliable by all major institutions and scientific and clinical societies; they are repeatable and verifiable. Their translation into the clinical use of the tested compounds is also confirmed and effective [31,32]. However, there are some clinical exceptions, and we believe that vulvovaginal candidiasis should be considered such an exception. This is because, despite establishing the drug susceptibility of *Candida* isolates in VVC therapy, failures still occur [2,3,6,8]. The discussion on what we actually know about the biofilm formed by microorganisms and what the structure which we clinically consider biofilm, and above all, in vitro, really is, has been going on for several years [33]. When considering the interpretation of the results of our work, these issues are slightly less important—it does not seem to matter whether the 24-h structure formed by the suspension of *C. albicans* plankton cells on the surface of the tissue is called biofilm. In the ex vivo model, we have the opportunity to observe how yeast-like fungi behave towards the vaginal epithelium, as well as how various compounds, including the USCLs tested, may act in this environment. In 2010, Harriot et al. conducted a comparative analysis of the *Candida* biofilm itself, and of the kinetics of its formation, in the in vivo, ex vivo model as well as on polystyrene plates in RPMI 1640. They concluded that the dynamics of biofilm formation and its individual stages, as well as the structure and properties of mature (24-h) biofilm, are comparable and almost identical [12]. If so, the reason for the differences in the results of biofilm eradication by L1 and C1 lipopeptides obtained with these methods should be seen in the interaction of fungi with the abiotic and biotic surfaces. On the surface of the vaginal tissue, *Candida* undoubtedly interact with epithelial cells, deriving primarily nutrients from them (no nutrient medium for fungi is used in the ex vivo model) [34]. According to the current knowledge, *C. albicans* has the ability to actively invade tissues thanks to many virulence factors, including filamentation. Thus, the hyphae observed on the epithelium should be considered an invasive form of these strains, displaying, at the same time, tissue-destructive activity [29,35]. It is also known that the proliferating and filamentous *Candida* blastospores also have the ability to produce a large amount of extracellular substance, or mucus (matrix–ECM in accordance with the dynamics of biofilm formation), which is a barrier impermeable to various substances [6,8,12]. On the surface of a polystyrene plate, an abiotic material, yeast-like fungi also multiply intensively and have the ability to germinate and produce mucus. However, this method provides a nutrient medium—RPMI 1640—so these isolates do not have to compete for substances, and have no possibility of further invasion. Perhaps this is the reason why it is "easier" to eradicate yeast-like fungus overnight on a given surface (which we call mature biofilm) in an in vitro model than in an ex vivo tissue model. If so, the method that uses the animal equivalent of the vaginal epithelium, which undoubtedly more closely imitates the real processes of VVC, seems to be much more reliable than the polystyrene plates. If, in the course of further studies, the described observations and results are confirmed, it could indicate the need to drastically change the methods of *Candida* susceptibility testing used in microbiological diagnostics towards VVC.

The use of concentrations corresponding to the lowest FIC also did not result in complete eradication of *C. albicans* biofilm for either tested strain. However, the use of FIC resulted in the lowest mean number of CFU/g of tissue for the entire experiment (no statistical significance, $p > 0.05$). A decrease can be seen for CA1 and for CA2, for both lipopeptide–fluconazole combinations (Figure 2, Figures S1–S4 in Supplementary Materials). It is worth emphasizing that the fact that the classic checkerboard method used by our team in the previous work to determine the FIC and FIC indexes refers to plankton cells and not to biofilm [26] speaks in favor of the presented results. Thus, naturally, one would expect higher MICs of both substances in combination during eradication of this highly resistant structure than against *Candida* plankton. Meanwhile, concentrations many

times lower than MBEC (and MIC) turned out to be partially effective in our ex vivo method. A comparison of the results obtained in our work (Table 1) suggests that the simultaneous use of linear USCL concentrations 16 times (isolate 1) and 32 times (isolate 2) lower (than MBEC), as well as the use of concentrations of cyclic USCL 32 times (both isolates) lower (than MBEC), with the addition of fluconazole at a concentration 64 times lower than the MIC, produces similar or even better results in eradication of *C. albicans* biofilm from the surface of the murine vaginal epithelium [26]. The above may also prove that the combination of substances with different mechanisms of action can actually be more effective, which has been postulated for a long time [36–39]. As we mentioned in our previous work, the most likely explanation of the activity of these compounds against *C. albicans* is the possibility of sensitizing fungal cells by means of USCLs, which is conducted by causing at least partial damage to cell membranes, enabling faster and more effective interaction of fluconazole with its molecular target inside the cell (14 α-ergosterol demetylase). At the same time, the very interaction of this azole with the cell membrane may increase its permeabilization by lipopeptides [26]. Proving the effectiveness of using a combination of lipopeptides with fluconazole at concentrations many times lower than, when these substances are used separately, could solve the two most serious problems that should be taken into account in research on antimicrobial activity. The use of lipopeptides at much lower concentrations could no longer be associated with the potential relatively high toxicity of these compounds, which is increasingly noted. In addition, by treating cells with substances with different mechanisms of action, the acquisition of resistance by microorganisms, including *Candida*, would be significantly hampered, especially since the acquisition of resistance to AMPs, including USCLs, is already considered unlikely [16]. Thus, the results obtained in the course of the presented studies on the eradication of *C. albicans* biofilm from the tissue, using a combination of low concentrations of fluconazole and both lipopeptide analogs, are highly promising and require further confirmation.

Table 1. Concentration values (μg/mL) for both investigated *Candida* strains (CA1 and CA2) for all tested compounds used in this study: lipopeptides L1 (linear), C1 (cyclic), and fluconazole (FLC), determined in our previous study [26], and amphotericin B (AMB). MIC—minimal inhibitory concen- tration, MBEC—minimal biofilm eradication concentration, FIC—fractional inhibitory concentration.

	AMB	MIC			MBEC			FIC			
		FLC	L1	C1	FLC	L1	C1	FLC + L1	Interpretation	FLC + C1	Interpretation
CA1	50	≤0.125	32	4	>512	256	64	0.001953 + 16	0.875 additive effect	0.0625 + 2	1.0 additive effect
CA2	50	≤0.125	32	4	>512	256	64	0.001953 + 8	0.266 synergistic effect	0.0315 + 2	0.75 additive effect

It seems that the most diverse and difficult to interpret results of tissue biofilm eradication were obtained using sub-inhibitory concentrations (1/2 MBEC). Results were, again, statistically insignificant ($p > 0.05$). With the use of L1, some reduction in mean CFU/g values was obtained for both *C. albicans* strains (Figure 2, Figures S1 and S3 in Supplementary Materials). Regarding C1, one value indicates an eradicating effect—a decrease in mean CFU/g for CA2. For CA1, the obtained values exceeded those determined for the positive controls. It can, therefore, be concluded that the sub-inhibitory MBEC concentrations of the linear lipopeptide have a similar eradicating capacity to the use of the combination of this analog with fluconazole. This would be in contrast to the cyclic USCL, which is actually ineffective at one-half of the MBEC concentrations. At present, it is difficult to determine why a more favorable effect of linear analogs at low concentrations could be observed, since when using the lipopeptide alone, cyclic compounds turn out to be much more effec- tive against *Candida* (and their MIC values are much lower). It is worth noting, however, that both lipopeptides showed synergistic or additive activity with fluconazole, and their combinations were comparably effective in eradicating fungal biofilm from the tissue. It is likely that, in some way, a linear analog at low concentrations sensitizes *Candida* cells to the

effects of fluconazole when used in combination therewith, and also analogously enhances the antifungal activity of HDPs and other immune mechanisms present in the vaginal tissue [16,27,28]. If the cyclic compound acts similarly in combination with fluconazole, and if it is indeed possible, for example, to synergize with HDPs, why does C1 not have this ability when L1 does? Any potential explanation must take into account the differences in the structure of both compounds, as well as their interactions with VECs and the cells of the immune system, and with the substances they produce. Even if the aforementioned hypothesis regarding supporting the action of HDPs and/or other immunological components is true, its confirmation requires many verification studies, focused primarily on determining the nature of the interaction of all the substances and components present in the vagina—especially in the presence of *Candida* fungi.

Under the microfluidic conditions obtained using the BioFlux technology, it was possible to obtain a 24-h structure formed by blastospores and hyphae of both tested *C. albicans* strains—which we call biofilm. The microscopic photos of the channels used in BioFlux, taken every hour, clearly showed the next stages of the development of fungal biofilm—especially visible in Videos S1 and S2 animations and in the example in Figure 4. These observations are consistent with the common knowledge on the kinetics of biofilm formation, and (not for the first time) they confirm the equal suitability of the microflow model and in vitro models, as well as of the in vivo and ex vivo animal models, already proven by Harriot et al. [6,13]. BioFlux is currently considered to be another method of testing the biofilm of microorganisms and its drug susceptibility in vitro. Thanks to the use of BioFlux technology, we were able to observe the behavior of the strains in real time. We managed both to confirm the occurrence of all the commonly described stages of fungal biofilm formation, and to visually and quantitatively verify the effect (or lack thereof) of the tested substances on the investigated *C. albicans* isolates. According to the assumptions, if, for example, yeast-like fungi behave similarly/almost identically on polystyrene plates and in BioFlux channels, they should also show at least similar drug sensitivity when exposed to the same eradicating substances [23]. Following these assumptions, we treated the 24-h structure formed by *C. albicans* strains with L1 and C1 lipopeptides at concentrations equal to MBEC—again, expecting to confirm the effectiveness of these compounds in combating biofilm in vitro. Meanwhile, the results obtained by us do not prove the eradication abilities of USCLs, but the slight (and statistically insignificant) possibilities of limiting the further development of fungal biofilm. In Figures 5 and 6 for strains CA1 and CA2, respectively, we can observe lower percentages of increasing coverage of channels in which biofilm was previously formed. Compared to the positive controls, especially for CA2, a relatively markedly lower further increase in the amount of *Candida* on the surface of the channels can be seen. During the first 8 h of incubation with the L1 flow, there was almost no further multiplication of the fungi (value 0.29 vs. 2.45% in K(+)). Ultimately, after one day, the percentage of coverage increase was nearly 50 percent lower than in K(+) (4.35 vs. 8.60%). The analogous results for CA1 also show an insignificant reduction in multiplication only in the first 8 h of incubation with L1 (2.21 vs. 2.64% in K(+)).After 24 h, the final value is lower by almost ^{1} (6.88 vs. 9.39% in K(+))¨In both cases, compound C1 limited biofilm development of both strains in the channels to a much lesser extent—the final values for CA1 were 8.40% (vs 9.39%), and for CA2, 6.18% (vs 8.60%). In both cases, after 12 h, *C. albicans* formed a very thick layer of biofilm in the channels. This physically, above all, significantly hindered the further flow of any substances. Given the fungal ability to produce a large amount of highly impermeable matrices (ECM), also most likely abundantly present in these cases, the relatively poor penetration of L1 and C1 is not surprising [8,33,40]. It should also come as no surprise that action in the first hours was more effective. It is likely that during the first few flows of substances, there was the greatest mechanical effect on the hyphae present in the channels and on their detachment. These, at the same time, could expose the more sensitive cells located in the deeper layers of the biofilm to the effects of USCLs, limiting their multiplication or even their microbiotic effect (percentages are presented for the entire length of the channels

observed under the microscope). The microfluidic model is, perhaps, not the best reflection of *Candida* kinetics and of the interactions with test substances during VVC. However, comparing the results obtained with the use of BioFlux and with polystyrene plates, it can be concluded that for the effective action of lipopeptides against fungal biofilm, constant contact of microorganism cells with antimicrobial substances is necessary, as is the case with polystyrene plates. However, in both models, it is difficult to detect active invasion of *C. albicans* during adhesion to abiotic surfaces, so this in vitro model does not seem to be appropriate for drawing conclusions regarding the behavior of yeast-like fungi on the surface of the vaginal tissue during infection.

4. Materials and Methods

4.1 Lipopeptide Synthesis

The compounds were obtained by using the method reported previously [15]. Briefly, lipopeptides were synthesized manually by solid-phase Fmoc/tBu methodology. Polystyrene resin modified by Rink Amide linker was used as the solid support (loading ca. 1.0 mmol/g; Orpegen Peptide Chemicals GmbH, Heidelberg, Germany). Deprotection of the Fmoc group was performed with a 20% (*v*/*v*) piperidine (Iris Biotech GmbH, Marktredwitz, Germany) solution in DMF (*N*,*N*-dimethylformamide; POCH, Avantor, Gliwice, Poland) for 15 min. Acylation was conducted with a mixture of DIC:OxymaPure:Fmoc-AA-OH (mole ratio 1:1:1; DIC–*N*,*N*'-Diisopropylcarbodiimide) dissolved in DMF:DCM (1:1, *v*/*v*; DCM—dichloromethane, Chempur, Piekary Slaskie, Poland) in four-fold excess based on the resin for 1.5 h (DIC and OxymaPure; Iris Biotech GmbH, Marktredwitz, Germany). Fmoc-L-Lys(Boc)-OH, Fmoc-L-Cys(Trt)-OH (amino acids were purchased from Orpegen Peptide Chemicals GmbH Heidelberg, Germany), and hexadecanoic acid (C16, palmitic acid; Merck, Darmstadt, Germany) were used in coupling reactions. The peptides were cleaved from the resin using one of the mixtures: (A) TFA (trifluoroacetic acid; Apollo Scientific, Denton, UK), EDT (1,2-ethanedithiol; Merck, Darmstadt, Germany), TIS (triisopropylsilane; Iris Biotech GmbH, Marktredwitz, Germany), and deionized water (92.5:2.5:2.5:2.5, *v*/*v*/*v*/*v*); (B) TFA, TIS, and deionized water (95:2.5:2.5, *v*/*v*/*v*). Mixture A was used with C_{16}-CKKKKC-NH_2 (C1), whereas mixture B was used for C16-KKKK-NH2 (L1). Cleavage was accomplished within 1.5 h under stirring. Then, the peptides were precipitated with cooled diethyl ether (POCH, Avantor, Gliwice, Poland) and lyophilized. The crude peptide with cysteine was dissolved in 20% (*v*/*v*) acetic acid (Chempur, Piekary Slaskie, Poland) solution (0.5 g/L) and oxidized with iodine to obtain the peptide with an intramolecular disulfide bridge. The peptides were purified by RP-HPLC. Pure fractions (>95%, HPLC) were collected and lyophilized. The identity of all compounds was confirmed by mass spectrometry (ESI–MS).

4.2 Candida Strains

Microbiological assays were performed on 2 clinical isolates of *Candida albicans* randomly selected from the pool of strains tested in our previous work [26]. Strains (further referred as CA1 and CA2) were originally isolated from the vaginas of women with vulvovaginal candidiasis and were deposited in the Internal Collection of the Department of Microbiology, Wrocław Medical University. The isolates were stored as suspensions in TSB medium enriched with glycerol as described elsewhere, frozen at −80 °C.

The study protocol was approved by the local Bioethics Committee of Wrocław Medical University (No. 774/2018, approval date: 27 December 2018). All experiments were performed in accordance with relevant guidelines and regulations.

4.3 Ex Vivo Animal Model

All experiments were carried out on tissues harvested from female C57BL/6 mice, 8–12 weeks of age, purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed at the Łukasiewicz Research Network—PORT Polish Center for Technology Development in Wrocław, Poland in individually ventilated cages. A 12:12 h light-dark

cycle was established under specific pathogen-free conditions, with water and food avail- able ad libitum. As described by Harriott et al., the mice were euthanized and their vaginae were excised and cut longitudinally to expose the mucosal surface. Each vagina was di- vided into six sections and placed in a 6-well plate with the mucosal side facing up, in 500 µL of phosphate-buffered saline (PBS) (AppliChem GmBH, Darmstadt, Germany) with penicillin (100 U/mL) and streptomycin (100 µg/mL) to prevent growth of normal vaginal bacteria. Then, *C. albicans* suspensions (subcultured for 24 h on Sabouraud Dextrose Agar with chloramphenicol at a concentration of 100 mg/L) in sterile 0.9% NaCl in concentration of 1–5 × 10^6 CFU/mL were added and incubated for 24 h at 37 °C with CO_2 [12]. After this time, fungal biofilms formed on tissues were washed with PBS, and 500 µL of the test compounds was added. Minimum biofilm eradication concentrations (MBECs) of both lipopeptides, as well as fractional inhibitory concentrations (FICs), were determined in our previous study and are presented in Table 1 [26]. Each experiment included a negative control (tissue without *Candida* suspension), positive control (biofilm of *Candida* not exposed to any compound) and four samples treated with the following: high concentration of amphotericin B (50 µg/mL) (referred as AMB), concentration equal to MBEC and 1/2 MBEC of analyzed lipopeptides, and a combination of lipopeptides with fluconazole equal to FIC value. All samples were prepared in duplicate. Tissues were incubated again for 24 h at 37 °C with CO_2. One set of the experiment was dedicated to confocal microscopy, and the other to homogenization and quantification of the tissue's fungal burden. The experiment was repeated four times.

4.4 Homogenization of Tissues

All tissues were weighed and homogenized in 1 mL sterile H_2O using TissueRuptor II (Qiagen, Germantown, MD, USA). Next, 10-fold dilutions in sterile H_2O were prepared and plated to Sabouraud Dextrose Agar, then incubated overnight at 37 °C. The colonies were counted and CFU per mL and CFU per g (of tissue) values were calculated, as well as mean values and standard deviations.

4.5 Microscopy Assay

Vaginal tissues were stained with 1 mg/mL Calcofluor White solution (Fluka) for 20 min at room temperature to visualize yeast and hyphae. Then, the samples were placed with the epithelial side up onto glass microscope slides and covered with a glass coverslip. The slides were examined on a resonant Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a dry 10× objective (NA 0.4). Calcofluor White (labeling *Candida*) was excited with a 405 nm laser line (emission range 410–460 nm), while Evans blue (labeling vaginal epithelium) was simultaneously excited with a 638 nm laser line (emission range 645–720 nm). Whole pieces of tissues were imaged as mosaics, with single tiles being confocal Z stacks with a 7 µm interval and 2 µm pixel size. Imaging of whole tissues was performed in order to eliminate the possibility of subjective selection and assessment of individual fragments of the observed surface. The same exposure settings were used to take images from every round of experiments.

4.6 BioFlux Biofilm Model

BioFlux 1000Z setup (Fluxion Biosciences, San Francisco, CA, USA) with an inverted fluorescence microscope (GmbH, Jena, Germany) was used to generate microfluidic conditions of biofilm growth [23]. Firstly, the channels of the BioFlux 48-well microfluidics plate (Fluxion Biosciences, San Francisco, CA, USA) were filled with RPMI 1640 medium and rinsed with a strong medium flow of (10 dyne/cm^2) for 10 s. Next, 100 µL of each suspension of both *C. albicans* strains, prepared identically as in the microdilution method for MBEC determination (strains subcultured for 24 h on Sabouraud Dextrose Agar with chloramphenicol at a concentration of 100 mg/L, then suspended in RPMI 1640 medium at a concentration of 1–5 × 10^6 CFU/mL), were placed into the outlet wells, and the flow of the medium was opened from the outlet to inlet channels using a speed rate of 5 dyne/cm^2

for 5 s. After this step, fungi were left for 1 h in order to allow them to adhere to the channels' surface. Then, RPMI 1640 was added to inlet wells up to a final volume of 1 mL, and the flow of 0.5 dyne/cm^2 was set for 24 h. Mature biofilms of *Candida* obtained by the aforementioned method were then either treated with the two tested lipopeptides or not exposed to any antimicrobial compound (a positive control). USCLs with a volume of 1 mL and concentrations equal to the MBECs determined in the previous study [26] were added to the inlet wells, and the flow of 0.5 dyne/cm^2 was maintained for 24 h. The experiment included two positive controls, with biofilm of the tested fungi in RPMI 1640 not exposed to any compound. For all tested samples, the biofilm obtained after 24 h of growth in the absence of any substances (a biofilm development phase) was taken as the starting point, and the biofilm increase obtained during the second day of biofilm growth was regarded as final result. A time-lapse series of images was taken every 1 h during the entire experiment. The photos obtained in that manner were then analyzed by the BioFlux Montage software. All experiments were repeated in triplicate.

4.7 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 9 (GraphPad Co., San Diego, CA, USA). The normality of distribution was checked by the Shapiro–Wilk test. As all values were normally distributed, the one-way ANOVA test was further used. The results of statistical analyses were considered significant for values with $p < 0.05$.

5. Conclusions

The results presented in our study contradict the results obtained previously during the in vitro study of the efficacy of C_{16}-KKKK-NH_2 and C_{16}-CKKKKC-NH_2 ultrashort lipopeptides against *Candida* strains isolated from VVC. This is because in no case was it possible to achieve a complete eradication of the biofilm. Both the ex vivo animal model using the murine vaginal epithelium and the microfluidic biofilm model failed to confirm the eradicating effects of L1 and C1 at previously determined concentrations (MBEC). In the method using BioFlux, it is possible to observe some weak effect of limiting the further development of fungal biofilm, which is stronger with the use of a linear analog than a cyclic one. In addition, the action of both USCLs at sub-inhibitory concentrations (1/2 MBEC) ex vivo indicates a potentially more beneficial effect of L1, possibly through active sensitization of *Candida* to the action of substances belonging to, for example, HDPs present in the vagina. However, the interactions in the vaginal microenvironment between lipopeptides and possible HDPs require further detailed research focused on identifying such compounds and their mechanisms of action. As in our previous work, the most favorable results were obtained in an ex vivo model when using the combination of USCLs with fluconazole conventionally used in VVC. The use of concentrations corresponding to the lowest previously determined FIC—i.e., many times lower than when applied separately to both plankton (MIC) and biofilm (MBEC)—resulted, in almost every case, in eradication at a higher level than at other concentrations of L1 and C1. Thus, the advantage of combination therapy using compounds with different mechanisms of action over the use of test substances separately was demonstrated once again. It should be emphasized that routine in vitro susceptibility testing of fungi in clinical practice almost always indicates the susceptibility of fungi grown in VVC. In our study, we proved that these methods most likely do not reflect the behavior of *Candida* in vivo during infection, and that the actual eradication of these microorganisms is much more difficult than it would appear from the mycogram. This is most likely the reason for the frequently observed therapeutic failures and for the increasing rate of recurrence of infections.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232214453/s1; Videos S1 and S2 provide animations of all time-lapse microscopic shots captured during the 24 h of incubation of strain CA1 and CA2, respectively, in microfluidic conditions obtained by the BioFlux model. Figures S1–S4 show results of

Candida biofilm eradication (CFU/g) under the influence of tested substances with statistical analysis, for both strains CA1 and CA2 separately.

Author Contributions: P.C., J.N. and G.C. designed this study. P.C. and G.C. performed the experi- ments. P.K. performed the BioFlux experiments. P.C., J.N., D.N., G.C. and P.K. analyzed the results.P.C. wrote the manuscript and prepared the figures. G.G. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Ministry of Health subventions according to numbers of STM.A130.20.102 and SUBZ.A130.22.010 from the IT Simple system of Wroclaw Medical University and by the National Science Centre (Poland), grant No. 2014/14/E/NZ6/00365. The Bioflux system was funded the National Centre for Research and Development (NCBiR) No. IA/SP/453975/2020.

Institutional Review Board Statement: The study protocol was approved by the local Bioethics Committee of Wrocław Medical University (No. 774/2018, approval date: 27 December 2018). All experiments were performed in accordance with relevant guidelines and regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations

CA1–*C. albicans* strain no 1
CA2–*C. albicans* strain no 2
L1–linear analogue 1 (C_{16}-KKKK-NH_2)
C1–cyclic analogue 1 (C_{16}-CKKKKC-NH_2)
AMB–amphotericin B
FLC–fluconazole
K—negative control
K+–positive control
K–lysine
USCL–ultrashort cyclic lipopeptides
AMPs–antimicrobial peptides
HDPs–host defense peptides
VVC–vulvovaginal candidiasis
CFU–colony forming unit
MIC–minimum inhibitory concentration
MBEC–minimum biofilm eradication concentration
FIC–fractional inhibitory concentration
R–arginine
C16–hexadecenoic acid
NH2–amide residue
VECs–vaginal epithelial cells

References

1. Sobel, J.D. Vulvovaginal candidosis. *Lancet* 2007, *369*, 1961–1971. [CrossRef]
2. Gonçalves, B.; Ferreira, C.; Alves, C.T.; Henriques, M.; Azeredo, J.; Silva, S. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit. Rev. Microbiol.* 2016, *42*, 905–927. [CrossRef] [PubMed]
3. Muzny, C.A.; Schwebke, J.R. Biofilms: An underappreciated mechanism of treatment failure and recurrence in vaginal infections.*Clin. Infect. Dis.* 2015, *61*, 601–606. [CrossRef] [PubMed]
4. Brandolt, T.M.; Klafke, G.B.; Gonçalves, C.V.; Bitencourt, L.R.; de Martinez, A.M.B.; Mendes, J.F.; Meireles, M.C.A.; Xavier, M.O. Prevalence of *Candida* spp. in cervical-vaginal samples and the in vitro susceptibility of isolates. *Braz. J. Microbiol.* 2017, *48*, 145–150. [CrossRef] [PubMed]

5. Sherry, L.; Kean, R.; Mckloud, E.; Donnell, L.E.O.; Metcalfe, R.; Jones, B.L. Biofilms formed by isolates from patients are heterogeneous and insensitive to fluconazole. *Antimicrob. Agents Chemother.* 2017, *61*, e01065-17. [CrossRef]
6. Czechowicz, P.; Nowicka, J.; Gos´ciniak, G. Virulence Factors of *Candida* spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis. *Int. J. Mol. Sci.* 2022, *23*, 5895. [CrossRef]
7. Swidsinski, A.; Guschin, A.; Tang, Q.; Dörffel, Y.; Verstraelen, H.; Tertychnyy, A.; Khayrullina, G.; Luo, X.; Sobel, J.D.; Jiang, X. Vulvovaginal candidiasis: Histologic lesions are primarily polymicrobial and invasive and do not contain biofilms. *Am. J. Obstet. Gynecol.* 2019, *220*, 91.e1–91.e8. [CrossRef]
8. Rodríguez-Cerdeira, C.; Gregorio, M.C.; Molares-Vila, A.; López-Barcenas, A.; Fabbrocini, G.; Bardhi, B.; Sinani, A.; Sánchez- Blanco, E.; Arenas-Guzmán, R.; Hernandez-Castro, R. Biofilms and vulvovaginal candidiasis. *Colloids Surf. B Biointerfaces* 2019, *174*, 110–125. [CrossRef]
9. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D.W.; Azeredo, J. Adherence and biofilm formation of non-*Candida* albicans *Candida* species. *Trends Microbiol.* 2011, *19*, 241–247. [CrossRef]
10. Peters, B.M.; Yano, J.; Noverr, M.C.; Fidel, P.L. *Candida* Vaginitis: When Opportunism Knocks, the Host Responds. *PLoS Pathog.* 2014, *10*, e1003965. [CrossRef]
11. Hacioglu, M.; Guzel, C.B.; Savage, P.B.; Seher Birteksoz Tan, A. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against *Candida* spp. Isolated from vulvovaginal candidiasis. *Med. Mycol.* 2019, *57*, 291–299. [CrossRef] [PubMed]
12. Harriott, M.M.; Lilly, E.A.; Rodriguez, T.E.; Fidel, P.L.; Noverr, M.C. *Candida* albicans forms biofilms on the vaginal mucosa.*Microbiology* 2010, *156*, 3635–3644. [CrossRef] [PubMed]
13. Harriott, M.M.; Noverr, M.C. Importance of *Candida*-bacterial polymicrobial biofilms in disease. *Trends Microbiol.* 2011, *19*, 557–563. [CrossRef] [PubMed]
14. Dawgul, M.; Barańska-Rybak, W.; Bielińska, S.; Nowicki, R.; Kamysz, W. Wpływ peptydów przeciwdrobnoustrojowych na biofilm *Candida*. *Alerg. Astma Immunol.* 2010, *15*, 220–225.
15. Neubauer, D.; Jas´kiewicz, M.; Sikorska, E.; Bartoszewska, S.; Bauer, M.; Kapusta, M.; Narajczyk, M.; Kamysz, W. Effect of disulfide cyclization of ultrashort cationic lipopeptides on antimicrobial activity and cytotoxicity. *Int. J. Mol. Sci.* 2020, *21*, 7208. [CrossRef] [PubMed]
16. Czechowicz, P.; Nowicka, J. Antimicrobial activity of lipopeptides. *Postep. Mikrobiol.* 2018, *57*, 213–227. [CrossRef]
17. Mangoni, M.L.; Shai, Y. Short native antimicrobial peptides and engineered ultrashort lipopeptides: Similarities and differences in cell specificities and modes of action. *Cell. Mol. Life Sci.* 2011, *68*, 2267–2280. [CrossRef]
18. Jones, L.; Hobden, C.; O'Shea, P. Use of a real-time fluorescent probe to study the electrostatic properties of the cell surface of*Candida* albicans. *Mycol. Res.* 1995, *99*, 969–976. [CrossRef]
19. Lohan, S.; Cameotra, S.S.; Bisht, G.S. Systematic study of non-natural short cationic lipopeptides as novel broad-spectrum antimicrobial agents. *Chem. Biol. Drug Des.* 2013, *82*, 557–566. [CrossRef]
20. Makovitzki, A.; Avrahami, D.; Shai, Y. Ultrashort antibacterial and antifungal lipopeptides. *Proc. Natl. Acad. Sci. USA* 2006, *103*,15997–16002. [CrossRef]
21. Greber, K.E. Synthesis and Surface Activity of Cationic Amino Acid-Based Surfactants in Aqueous Solution. *J. Surfactants Deterg.*2017, *20*, 1189–1196. [CrossRef] [PubMed]
22. Naglik, J.R.; Fidel, P.L.; Odds, F.C. Animal models of mucosal *Candida* infection. *FEMS Microbiol. Lett.* 2008, *283*, 129–139. [CrossRef] [PubMed]
23. Krom, B.P.; Willems, H.M. In vitro Models for *Candida* Biofilm Development. In *Candida Species*; Humana Press: New York, NY, USA, 2016; Volume 1356, pp. 95–106. ISBN 978-1-4939-3051-7.
24. Krzyz˙ek, P.; Migdał, P.; Grande, R.; Gos´ciniak, G. Biofilm Formation of Helicobacter pylori in Both Static and Microfluidic Conditions Is Associated With Resistance to Clarithromycin. *Front. Cell. Infect. Microbiol.* 2022, *12*, 351. [CrossRef] [PubMed]
25. Krom, B.P.; Cohen, J.B.; McElhaney-Feser, G.; Busscher, H.J.; van der Mei, H.C.; Cihlar, R.L. Conditions for Optimal *Candida*Biofilm Development in Microtiter Plates. In *Candida Albicans*; Humana Press: Totowa, NJ, USA, 2009; ISBN 9781626239777.
26. Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gos´ciniak, G. Antifungal activity of linear and disulfide-cyclized ultrashort cationic lipopeptides alone and in combination with fluconazole against vulvovaginal *Candida* spp. *Pharmaceutics* 2021, *13*, 1589. [CrossRef]
27. Citterio, L.; Franzyk, H.; Palarasah, Y.; Andersen, T.E.; Mateiu, R.V.; Gram, L. Improved in vitro evaluation of novel antimicrobials: Potential synergy between human plasma and antibacterial peptidomimetics, AMPs and antibiotics against human pathogenic bacteria. *Res. Microbiol.* 2016, *167*, 72–82. [CrossRef]
28. Jammal, J.; Zaknoon, F.; Kaneti, G.; Hershkovits, A.S.; Mor, A. Sensitization of Gram-Negative Bacilli to Host Antibacterial Proteins. *J. Infect. Dis.* 2017, *215*, 1599–1607. [CrossRef]
29. Willems, H.M.E.; Ahmed, S.S.; Liu, J.; Xu, Z.; Peters, B.M. Vulvovaginal candidiasis: A current understanding and burning questions. *J. Fungi* 2020, *6*, 27. [CrossRef]

30. Fidel, P.L. History and update on host defense against vaginal candidiasis. *Am. J. Reprod. Immunol.* 2007, *57*, 2–12. [CrossRef]
31. Arendrup, M.C.; Meletiadis, J.; Mouton, J.W.; Lagrou, K.; Hamal, P.; Guinea, J. (EUCAST), S. on A.S.T. (AFST) of the E.E.C. forA.S.T. In *EUCAST Antifungal MIC Method for Yeasts: Method for the Determination of Broth Dilution Minimum Inhibitory Concentrations of Antifungal Agents for Yeasts*; Eucast: Växjö, Sweden, 2020; pp. 1–21.
32. Arendrup, M.C.; Prakash, A.; Metetiadis, J.; Sharma, C.; Chowdhary, A. Comparison of EUCAST and CLSI Reference Microdilu- tion MICs of Eight and Associated Tentative. *Antimicrob. Agents Chemother.* 2017, *61*, e00485-17. [CrossRef]
33. Kean, R.; Delaney, C.; Rajendran, R.; Sherry, L.; Metcalfe, R.; Thomas, R.; McLean, W.; Williams, C.; Ramage, G. Gaining insights from *Candida* biofilm heterogeneity: One size does not fit all. *J. Fungi* 2018, *4*, 12. [CrossRef]
34. Yano, J.; Peters, B.M.; Noverr, M.C.; Fidel, P.L. Novel Mechanism behind the Immunopathogenesis of Vulvovaginal Candidiasis: "Neutrophil Anergy". *Infect. Immun.* 2018, *86*, e00684-17. [CrossRef] [PubMed]
35. Richardson, J.P.; Willems, H.M.E.; Moyes, D.L.; Shoaie, S.; Barker, K.S.; Tan, L.; Palmer, G.E.; Hube, B.; Naglik, J.R.; Peters, M. Candidalysin Drives Epithelial Signaling, Neutrophil Recruitment, and Immunopathology at the Vaginal Mucosa. *Infect. Immun.* 2018, *86*, e00645-17. [CrossRef] [PubMed]
36. Ceresa, C.; Rinaldi, M.; Fracchia, L. Synergistic activity of antifungal drugs and lipopeptide AC7 against *Candida* albicans biofilm on silicone. *AIMS Bioeng.* 2017, *4*, 318–334. [CrossRef]
37. Suchodolski, J.; Derkacz, D.; Muraszko, J.; Panek, J.J.; Jezierska, A.; Łukaszewicz, M.; Krasowska, A. Fluconazole and lipopeptide surfactin interplay during *Candida* albicans plasma membrane and cell wall remodeling increases fungal immune system exposure. *Pharmaceutics* 2020, *12*, 314. [CrossRef]
38. Liu, X.; Ren, B.; Gao, H.; Liu, M.; Dai, H.; Song, F.; Yu, Z.; Wang, S.; Hu, J.; Kokare, C.R.; et al. Optimization for the production of surfactin with a new synergistic antifungal activity. *PLoS ONE* 2012, *7*, e34430. [CrossRef]
39. Olfa, T.; Antonio, D.G.; Sana, A.; Imen, B.S.; Salem, E.; Najib, A.M.; Bruno, C.; Vincenzo, L.; Ferid, L.; Luisa, M.M. Synergistic fungicidal activity of the lipopeptide bacillomycin D with amphotericin B against pathogenic *Candida* species. *FEMS Yeast Res.* 2015, *15*, fov022. [CrossRef]
40. Nobile, C.J.; Johnson, A.D. *Candida* albicans Biofilms and Human Disease. *Annu. Rev. Microbiol.* 2015, *69*, 71–92. [CrossRef]

11. OŚWIADCZENIA WSPÓŁAUTORÓW

Dr n. med. Joanna Nowicka
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 13.02.23 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Gościniak, G. Virulence Factors of Candida spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis. Int. J. Mol. Sci. 2022, 23, 5895. https://doi.org/10.3390/ijms23115895

mój udział polegał na pomocy w konceptualizacji pracy oraz przygotowaniu części artykułu oraz nadzór i edycja ostatecznej wersji manuskryptu pracy naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
asystent
dr Joanna Nowicka

Prof. dr hab. Grażyna Gościniak
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 6.03.2023

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Gościniak, G. Virulence Factors of Candida spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis. Int. J. Mol. Sci. 2022, 23, 5895. https://doi.org/10.3390/ijms23115895

mój udział polegał na nadzorowaniu przygotowywania pracy oraz edycji ostatecznej wersji manuskryptu publikacji naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
kierownik
Gościniak
prof. dr hab. Grażyna Gościniak

Dr n. med. Joanna Nowicka
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 13.02.23r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gościniak, G. Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal Candida spp. Pharmaceutics 2021, 13, 1589. https://doi.org/10.3390/pharmaceutics13101589

mój udział polegał na pomocy w konceptualizacji pracy oraz przy analizowaniu uzyskanych w toku eksperymentów danych, w tym sposobu ich prezentacji, a także nadzorze i edycji manuskryptu publikacji naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
asystent
dr Joanna Nowicka

Dr n. farm. Damian Neubauer
Katedra Chemii Nieorganicznej, Gdański Uniwersytet Medyczny

Gdańsk, dn. 23.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gościniak, G. Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal Candida spp. Pharmaceutics 2021, 13, 1589. https://doi.org/10.3390/pharmaceutics13101589

mój udział polegał na syntezie badanych lipopeptydów, pomocy przy analizowaniu uzyskanych w toku eksperymentów danych oraz sposobu ich prezentacji, nadzorze i edycji manuskryptu publikacji naukowej.

Damian Neubauer

Podpis

Prof. dr hab. Wojciech Kamysz
Katedra Chemii Nieorganicznej, Gdański Uniwersytet Medyczny

Gdańsk, dn. 23.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gościniak, G. Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal Candida spp. Pharmaceutics 2021, 13, 1589. https://doi.org/10.3390/pharmaceutics13101589

mój udział polegał na nadzorowaniu przebiegu badań z wykorzystaniem lipopeptydów oraz przygotowywaniu ostatecznej wersji manuskryptu publikacji naukowej.

Kamysz

Podpis

Prof. dr hab. Grażyna Gościniak
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 6.03.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Neubauer, D.; Nowicka, J.; Kamysz, W.; Gościniak, G. Antifungal Activity of Linear and Disulfide-Cyclized Ultrashort Cationic Lipopeptides Alone and in Combination with Fluconazole against Vulvovaginal Candida spp. Pharmaceutics 2021, 13, 1589. https://doi.org/10.3390/pharmaceutics13101589

mój udział polegał na nadzorowaniu przebiegu badań oraz pomocy w przygotowywaniu ostatecznej wersji manuskryptu publikacji naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
kierownik
prof. dr hab. Grażyna Gościniak

Dr n. med. Joanna Nowicka
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 13.02.2231.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyżek, P.; Gościniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of Candida albicans Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. Int. J. Mol. Sci. 2022, 23, 14453. https://doi.org/10.3390/ijms232214453

mój udział polegał na pomocy w konceptualizacji pracy oraz przy analizowaniu uzyskanych w toku eksperymentów danych, w tym sposobu ich prezentacji, a także nadzorze i edycji manuskryptu publikacji naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
asystent
dr Joanna Nowicka

Dr n. farm. Damian Neubauer
Katedra Chemii Nieorganicznej, Gdański Uniwersytet Medyczny

Gdańsk, dn. 23.02.2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyżek, P.; Gościniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of Candida albicans Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. Int. J. Mol. Sci. 2022, 23, 14453. https://doi.org/10.3390/ijms232214453

mój udział polegał na syntezie badanych lipopeptydów, pomocy przy analizowaniu uzyskanych w toku eksperymentów danych oraz sposobu ich prezentacji, nadzorze i edycji manuskryptu publikacji naukowej.

Podpis

Wrocław, dn. 14.02.2023

Dr hab. Grzegorz Chodaczek
Sieć Badawcza Łukasiewicz – PORT Polski Ośrodek Rozwoju Technologii

OŚWIADCZENIE

Oświadczam, że w pracy:

Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyżek, P.; Gościniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of Candida albicans Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. Int. J. Mol. Sci. 2022, 23, 14453. https://doi.org/10.3390/ijms232214453

mój udział polegał na przygotowywaniu tkanek zwierzęcych wykorzystywanych w modelu *ex vivo* oraz przeprowadzeniu analiz mikroskopowych w toku zaplanowanych w pracy eksperymentów.

Grzegorz Chodaczek

Dr n. med. Paweł Krzyżek
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 09.02.2023

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyżek, P.; Gościniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of Candida albicans Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. Int. J. Mol. Sci. 2022, 23, 14453. https://doi.org/10.3390/ijms232214453

mój udział polegał na przeprowadzeniu analiz z wykorzystaniem modelu biofilmu w warunkach mikroprzepływowych Bioflux wraz z pomocą w analizowaniu i przedstawianiu uzyskanych danych, a także pomoc w przygotowywaniu ostatecznej wersji manuskryptu pracy naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
adiunkt
Paweł Krzyżek
dr n. med. Paweł Krzyżek

Prof. dr hab. Grażyna Gościniak
Katedra i Zakład Mikrobiologii, Uniwersytet Medyczny we Wrocławiu

Wrocław, dn. 6. 03. 2023 r.

OŚWIADCZENIE

Oświadczam, że w pracy

Czechowicz, P.; Nowicka, J.; Neubauer, D.; Chodaczek, G.; Krzyżek, P.; Gościniak, G. Activity of Novel Ultrashort Cyclic Lipopeptides against Biofilm of Candida albicans Isolated from VVC in the Ex Vivo Animal Vaginal Model and BioFlux Biofilm Model—A Pilot Study. Int. J. Mol. Sci. 2022, 23, 14453. https://doi.org/10.3390/ijms232214453

mój udział polegał na nadzorowaniu przebiegu badań oraz pomocy w przygotowywaniu ostatecznej wersji manuskryptu publikacji naukowej.

Podpis

Uniwersytet Medyczny we Wrocławiu
KATEDRA I ZAKŁAD MIKROBIOLOGII
kierownik
prof. dr hab. Grażyna Gościniak

12. KOMISJA BIOETYCZNA

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

OPINIA KOMISJI BIOETYCZNEJ Nr KB – 774/2018

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

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

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

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

„Badanie aktywności peptydów przeciwdrobnoustrojowych wobec szczepów *Candida spp.*"

zgłoszonym przez **mgr analityki medycznej Paulinę Czechowicz** uczestniczkę studiów doktoranckich w Katedrze i Zakładzie Mikrobiologii Wydziału Lekarskiego Uniwersytetu Medycznego we Wrocławiu oraz złożonymi wraz z wnioskiem dokumentami, w tajnym głosowaniu postanowiła wyrazić zgodę na przeprowadzenie badania w Katedrze i Zakładzie Mikrobiologii Wydziału Lekarskiego Uniwersytetu Medycznego we Wrocławiu pod nadzorem prof. dr hab. Grażyny Gościniak.

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

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

Opinia powyższa dotyczy: projektu badawczego będącego podstawą rozprawy doktorskiej

Wrocław, dnia 27 grudnia 2018 r.

Uniwersytet Medyczny we Wrocławiu
KOMISJA BIOETYCZNA
Przewodniczący
prof. dr hab. Jan Kornafel

BW