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Analiza bioróżnorodności izolatów bakterii *Listeria monocytogenes* pochodzących z przemysłu mięsnego w kontekście ich wrażliwości na chlorek benzalkoniowy

Analysis of the biodiversity of *Listeria monocytogenes* bacterial isolates originating from the meat industry in the context of their susceptibility to benzalkonium chloride

Rozprawa doktorska w dziedzinie nauk rolniczych
w dyscyplinie nauk o żywności i żywieniu

Doctoral dissertation in the field of agricultural sciences
in the discipline of food technology and nutrition

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Wykaz artykułów naukowych wchodzących w skład zbioru

W skład zbioru włączono cztery oryginalne publikacje badawcze (**P1, P2, P4** i **P5)** oraz jedną publikację prezentującą przegląd literatury (**P3**), zgodnie z poniższym wykazem. Głównymi kryteriami doboru czasopism było spełnienie warunku publikacji w trybie otwartego dostępu i jednocześnie niegenerowanie kosztów publikacyjnych.

Publikacja 1 (P1): Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2022, Genoserotyping of *Listeria monocytogenes* strains originating from meat products and meat processing environments, *Żywność. Nauka. Technologia. Jakość*, 29, 2 (131), 34 – 44, https://doi.org/10.15193/zntj/2022/131/414

Publikacja 2 (P2): Iwona Kawacka, Bernadeta Pietrzak, Marcin Schmidt, Agnieszka Olejnik-Schmidt, 2023, *Listeria monocytogenes* Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin, *Life*, 13, 821, https://doi.org/10.3390/life13030821

Publikacja 3 (P3): Iwona Kawacka, Agnieszka Olejnik-Schmidt, Marcin Schmidt 2022, Nonhemolytic *Listeria monocytogenes*—Prevalence Rate, Reasons Underlying Atypical Phenotype, and Methods for Accurate Hemolysis Assessment, *Microorganisms*, 10, 483, https://doi.org/10.3390/microorganisms10020483

Publikacja 4 (P4): Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2024, High Prevalence of Virulence-Associated Genes and Length Polymorphism in *actA* and *inlB* Genes Identified in *Listeria monocytogenes* Isolates from Meat Products and Meat-Processing Environments in Poland, *Pathogens*, 13, 444, https://doi.org/10.3390/pathogens13060444

Publikacja 5 (P5): Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2024, Gene *emrC* associated with resistance to quaternary ammonium compounds is common among *Listeria monocytogenes* from meat products and meat processing plants in Poland, *Antibiotics*, 13, 749, https://doi.org/10.3390/antibiotics13080749

Pokrycie opłaty za publikację **P1** w czasopiśmie *Żywność. Nauka. Technologia. Jakość* było nagrodą ufundowaną przez organizatorów Ogólnopolskiej Konferencji Studentów i Doktorantów „Żywność. Żywienie. Rynek. Innowacje w Nauce i Praktyce." (18.11.2021 r.), przyznaną za zajęcie pierwszego miejsca w konkursie na najlepszy wygłoszony referat. W związku z otrzymanymi zaproszeniami do darmowej publikacji, artykuły **P2, P3, P4** i **P5** także zostały wydane bez kosztów wydawniczych, które zostały w pełni zniesione przez zapraszające czasopisma.

Charakterystyka bibliometryczna wykazu artykułów

Charakterystyka bibliometryczna dorobku włączonego w zbiór artykułów oraz punkty zgodnie z listą MNiSW[1] zgodne z rokiem opublikowania pracy i współczynnik wpływu (IF) czasopisma z roku opublikowania pracy przedstawione są w Tabeli 1.

Tabela 1. Charakterystyka bibliometryczna dorobku

Publikacja (wydawnictwo)	**Punkty MNiSW**	**Współczynnik wpływu (IF)**	**Udział Doktorantki w powstaniu publikacji**
P1 (*Ż.N.T.J*)	20	--	75%
P2 (*Life*)	70	3,200	75%
P3 (*Microorganisms*)	40	4,926	80%
P4 (*Pathogens*)	100	3,300	75%
P5 (*Antibiotics*)	70	4,300	80%
Suma:	300	15,726	Średnia: 77%

[1] Ministerstwo Nauki i Szkolnictwa Wyższego

Spis treści

Wykaz skrótów stosowanych w autoreferacie

BHI	wyciąg mózgowo-sercowy (ang. *brain heart infusion*)
LIPI	Listeryjna wyspa patogenności (ang. *listeria pathogenicity island)*
PCR	reakcja łańcuchowa polimerazy (ang. *polymerase chain reaction*)
pz	pary zasad
RAPD	losowa amplifikacja polimorficznego DNA (ang. *randomly amplified polymorphic DNA*)
REP	polimorfizm sekwencji powtarzalnych (ang. *repetitive element sequence-based*)
RFLP	polimorfizm długości fragmentów restrykcyjnych (ang. *restriction fragment length polymorphism*)

Streszczenie

Bakterie *Listeria monocytogenes* są groźnym patogenem człowieka, a drogą zakażenia nimi jest spożycie zanieczyszczonej żywności. Gatunek ten często zasiedla środowiska produkcji żywności, w konsekwencji prowadząc do skażeń artykułów spożywczych. W celu eliminacji bakterii, w tym także *L. monocytogenes*, ze środowiska produkcyjnego, wykonywane są procedury dezynfekcji. Środkami wykorzystywanymi do tego celu są między innymi związki z grupy IV-rzędowych soli amoniowych, do których należy chlorek benzalkoniowy.

Podczas realizacji pracy zgromadzono 380 izolatów bakterii pochodzących z przemysłu mięsnego, wykazujących morfologię kolonii typową dla rodzaju *Listeria*. Na podstawie identyfikacji gatunkowej metodami genetycznymi (multiplex PCR i RFLP-PCR) oraz po analizie bioróżnorodności z wykorzystaniem techniki RAPD-PCR, wytypowano 153 izolaty *L. monocytogenes*, które stanowiły materiał badawczy do dalszych analiz. W czasie realizacji pracy wykonano analizy genoserotypu, antybiotykooporności, obecności wybranych genów warunkujących wirulencję, wrażliwości izolatów na chlorek benzalkoniowy i obecności wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe.

Izolaty w analizach genoserotypów zostały zakwalifikowane jako należące do czterech różnych serogrup (IIa, IIb, IIc i VIb), z których najbardziej powszechna była serogrupa IIa. Wszystkie izolaty były wrażliwe na zbadane antybiotyki, a w przypadku cyprofloksacyny 10 izolatów wykazało obniżoną wrażliwość, która jednak nie została zakwalifikowana jako oporność. Wszystkie izolaty posiadały aktywność beta-hemolityczną i 12 genów warunkujących wirulencję. W przypadku genów *actA* oraz *inlB* wykryto polimorfizm długości produktów reakcji PCR. Genem różnicującym zebrane izolaty w kontekście obecności genów warunkujących wirulencję był *ilsA*, wykryty u 18 z nich. U 79 izolatów (stanowiących 51,6%) zaobserwowano obniżoną wrażliwość na chlorek benzalkoniowy, a u 69 (stanowiących 45,1%) wykryto co najmniej jeden spośród badanych genów (*bcrABC*, *emrC* lub *qacH*) warunkujących oporność na IV-rzędowe sole amoniowe. Izolaty o obniżonej wrażliwości na chlorek benzalkoniowy częściej niż izolaty wrażliwe posiadały obniżoną wrażliwość na cyprofloksacynę. Nie znaleziono zależności między wrażliwością na chlorek benzalkoniowy a genoserotypem lub obecnością genu *ilsA*.

Badania wskazują na konieczność ciągłego śledzenia zmienności u *L. monocytogenes*.

Słowa kluczowe: bezpieczeństwo żywności, genotyp, wrażliwość

Summary

Listeria monocytogenes is a dangerous human pathogen, transmitted primarily through the consumption of contaminated food. This species frequently colonizes food production environments, leading to food contamination. To eliminate bacteria, including *L. monocytogenes*, from production environments, disinfection procedures are carried out. Quaternary ammonium compounds, such as benzalkonium chloride, are commonly used for this purpose.

During the study, 380 bacterial isolates from the meat industry were collected, exhibiting colony morphology typical of the genus *Listeria*. Based on genetic identification methods (multiplex PCR and RFLP-PCR) and after analyzing biodiversity using the RAPD-PCR technique, 153 *L. monocytogenes* isolates were selected and used as the research material for further analyses. During the study, analyses of the genoserotype, antibiotic resistance, the presence of selected virulence-associated genes, sensitivity of isolates to benzalkonium chloride, and the presence of selected genes conferring resistance to quaternary ammonium salts were performed.

The isolates were classified in the genoserotyping analyses as belonging to four different serogroups (IIa, IIb, IIc, and VIb), with serogroup IIa being the most common. All isolates were susceptible to the tested antibiotics, and in the case of ciprofloxacin 10 isolates exhibited reduced susceptibility, which, however, was not classified as resistance. All isolates possessed beta-hemolytic activity and 12 virulence genes. In the case of the *actA* and *inlB* genes, polymorphism of the length of PCR product was detected. The *ilsA* gene, was a differentiating gene for the collected isolates in the context of the presence of virulence genes and was detected in 18 of them. In 79 isolates (51.6%), reduced susceptibility to benzalkonium chloride was observed, and in 69 (45.1%) at least one of the tested genes (*bcrABC*, *emrC*, or *qacH*) conferring resistance to quaternary ammonium salts was detected. Isolates with reduced susceptibility to benzalkonium chloride had reduced susceptibility to ciprofloxacin more often than susceptible isolates. No relationship was found between susceptibility to benzalkonium chloride and serogroup or the presence of the *ilsA* gene.

The results indicate the need for continuous monitoring of the diversity and variability of *L. monocytogenes*.

Key words: food safety, genotypes, sensitivity

1. Wstęp

1.1. Uzasadnienie i charakterystyka problemu badawczego

1.1.1. *Listeria monocytogenes* jako problem społeczny i ekonomiczny

Głównym wyzwaniem związanym z dostarczaniem bezpiecznej żywności, która uważana jest za podstawowe prawo człowieka, jest jej bezpieczeństwo mikrobiologiczne, szczególnie pod kątem bakteryjnym. W porównaniu do innych grup drobnoustrojów (np. wirusów lub pierwotniaków) to właśnie bakterie powodują najwięcej incydentów zdrowotnych związanych ze spożyciem zakażonej żywności (Fung i in., 2018).

Najistotniejszymi rodzajami bakterii mającymi negatywny wpływ na bezpieczeństwo produktów spożywczych są przedstawiciele: *Salmonella*, *Vibrio*, *Escherichia coli*, *Shigella*, *Brucella*, *Campylobacter* i *Listeria*. Spośród tych grup mikroorganizmów gatunek *Listeria monocytogenes* powoduje najwyższy odsetek zgonów (Erickson i Doyle, 2017; Fung i in., 2018; Huang i in., 2016). Objawy zakażenia *L. monocytogenes* w przypadku zdrowych osób dorosłych zazwyczaj ograniczają się do silnego zatrucia pokarmowego przebiegającego z gorączką. Jednak gdy dochodzi rozwinięcia się inwazyjnej listeriozy, objawy obejmują między innymi sepsę, zapalenie opon mózgowych lub zapalenie mózgu oraz poronienia w przypadku kobiet ciężarnych. Na taki przebieg infekcji szczególnie narażone są osoby starsze, dzieci, osoby z osłabioną odpornością i kobiety w ciąży (Osek i Wieczorek, 2022; Rogalla i Bomar, 2020). W wyniku wystąpienia ciężkiej inwazyjnej listeriozy odsetek zgonów osiąga około 20-30% (Kaptchouang Tchatchouang i in., 2020; Osek i in., 2022a; Zhu i in., 2017).

L. monocytogenes odpowiada za dużą część zgonów spowodowanych spożyciem skażonych produktów spożywczych, która na przykład w USA szacowana jest na około 19 % (Scallan i in., 2011). Ze względu na wysoką śmiertelność, jak również wysoki wskaźnik hospitalizacji u zarażonych pacjentów, bakterie te powodują także ogromne roczne straty ekonomiczne. Przykładowo w USA szacowane są one na 18% całości obciążenia ekonomicznego spowodowanego spożyciem żywności skażonej mikroorganizmami patogennymi (Erickson i Doyle, 2017; Huang i in., 2016).

1.1.2. *L. monocytogenes* jako problem przemysłu spożywczego

Bakterie *L. monocytogenes* są izolowane z różnego typu produktów spożywczych, takich jak: mięso, ryby, mleko, owoce i warzywa, a także z przetworów surowców tych grup (Kaptchouang Tchatchouang i in., 2020; Quereda i in., 2021). Obróbka cieplna jest czynnikiem

skutecznie inaktywującym bakterie tego gatunku, jednak nawet produkty utrwalane termicznie mogą zostać zanieczyszczone w trakcie procesów technologicznych. W związku z tym *L. monocytogenes* można znaleźć nie tylko w żywności surowej, ale także przetworzonej (Bintsis, 2017).

Gatunek ten charakteryzuje się łatwą adaptacją do warunków środowiskowych, w tym zdolnością do wzrostu w szerokim zakresie temperatur (0°C–45°C), pH (4,3–9,6), tolerancją wysokich stężeń soli (do 10,0% NaCl) i niskiej aktywności wody (A_w do 0,90) (Wiktorczyk-Kapischke i in., 2023). Cechy te ułatwiają przetrwanie i namnażanie w zakładach przetwórstwa żywności (Osek i in., 2022a). Prowadzi to do rozprzestrzenienia się *L. monocytogenes*, a czasem także do zasiedlania środowiska produkcyjnego (Osek i in., 2022b).

W związku z możliwością bytowania bakterii tego gatunku w zakładach przetwórstwa spożywczego, w pracy niniejszej doktorskiej postawiono hipotezę badawczą, mówiącą, że „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego są różnorodne genetycznie". Hipoteza ta częściowo odnosi się pochodzenia izolatów, ponieważ gdy żywność zostaje zanieczyszczona w czasie procesów technologicznych, to poszczególne izolaty mogą być bardzo zbliżone genetycznie. W przeciwnym wypadku, gdy bakterie znajdujące się gotowym wyrobie są pochodzenia surowcowego, spodziewana jest ich różnorodność genetyczna.

Ze względu na powodowane niebezpieczeństwo dla zdrowia, obecność *L. monocytogenes* w produktach spożywczych jest ściśle regulowana w większości krajów rozwiniętych, w tym w krajach Unii Europejskiej (Jordan i McAuliffe, 2018). Zatem w celu redukcji ryzyka zanieczyszczenia wyrobów gotowych bakteriami zasiedlającymi środowisko produkcyjne, a tym samym także w celu zapewnienia zgodności produktów spożywczych z wymaganiami prawnymi, konieczne jest przeprowadzanie skutecznej i wydajnej dezynfekcji, skutkującej eliminacją *L. monocytogenes* ze środowiska produkcji żywności.

1.1.3. Oporność *L. monocytogenes* na IV-rzędowe sole amoniowe

Do procesów dezynfekcji w zakładach przemysłu spożywczego wykorzystuje się między innymi związki z grupy IV-rzędowych soli amoniowych, do których należy chlorek benzalkoniowy. Powszechność tej grupy związków wynika między innymi z ich skuteczności w zwalczaniu różnych bakterii, grzybów i wirusów, biodegradowalności oraz braku właściwości korozyjnych (Elhanafi i in., 2010; Kovacevic i in., 2016; Palaiodimou i in., 2021; Tezel i Pavlostathis, 2015).

Środki dezynfekujące mogą jednak wywierać na mikroorganizmach presję selekcyjną, powodując utrzymywanie lub nabywanie genów związanych z opornością na te związki.

Zjawisko to jest obserwowane między innymi w przypadku oporności na IV-rzędowe sole amoniowe u *L. monocytogenes* izolowanych ze środowiska przetwórstwa żywności (Osek i in., 2022a).

Monitorowanie wrażliwości izolatów *L. monocytogenes* na IV-rzędowe sole amoniowe jest zatem istotnym zagadnieniem z punktu widzenia bezpieczeństwa żywności, ponieważ dane o wrażliwości izolatów pozwalają na określenie potencjalnej skuteczności procesów dezynfekcji w zakładach przemysłu spożywczego. Umożliwia to opracowanie skutecznych procedur dezynfekcji, tym samym zwiększając bezpieczeństwo konsumentów.

1.1.4. Geny warunkujące wirulencję *L. monocytogenes*

Poszczególne szczepy gatunku *L. monocytogenes* mogą różnić się między sobą nie tylko wrażliwością na środki dezynfekcyjne. Zdarza się na przykład, że niektóre izolaty nie wykazują aktywności beta-hemolitycznej, czyli zdolności do rozkładu krwinek czerwonych, choć jest ona uznawana za specyficzną cechę gatunkową *L. monocytogenes* (Maury i in., 2017). Cecha ta jest często wykorzystywana do potwierdzania przynależności izolatów rodzaju *Listeria* do gatunku *L. monocytogenes* (Fox i in., 2021; Hitchins i in., 2017; Rogalla i Bomar, 2020). Historycznie fenotyp hemolityczny uważano za marker wirulencji *L. monocytogenes* (Fernandez-Garayzabal i in., 1992), a obecnie wiadomo, że brak tej cechy jest najczęściej efektem mutacji istotnych genów warunkujących wirulencję tych bakterii (Maury i in., 2017).

Najważniejsze geny związane z wirulencją *L. monocytogenes* są zlokalizowane na pierwszej listeryjnej wyspie patogenności (LIPI-1), zawierającej geny: *hly*, *actA*, *plcA*, *plcB* i *mpl* (Quereda i in., 2021; Sibanda i Buys, 2022; Wiktorczyk-Kapischke i in., 2023). Wszystkie te geny są regulowane przez gen *prfA*, kodujący białko PrfA, czyli główny pozytywny czynnik regulacyjny genów wirulencji u gatunku *L. monocytogenes* (Guariglia-Oropeza i in., 2014; Ollinger i in., 2009; Quereda i in., 2021). Listeryjna wyspa patogenności LIPI-3 zawiera gen kodujący listeriolizynę S, ulegający ekspresji podczas zakażeń układu pokarmowego. Z kolei geny znajdujące się w obrębie LIPI-4 odgrywają rolę podczas zakażenia ośrodkowego układu nerwowego i łożyska. Zarówno LIPI-3, jak i LIPI-4 występują w genomach bakterii *L. monocytogenes* znacznie rzadziej niż LIPI-1 (Tavares i in., 2020; Wiktorczyk-Kapischke i in., 2023). Jednymi z istotniejszych genów w kontekście wirulencji *L. monocytogenes* są także geny kodujące internaliny, głównie *inlA* i *inlB* (Lopes-Luz i in., 2021; Mir, 2021; Osek i Wieczorek, 2022; Quereda i in., 2021).

Istnieje także szereg innych genów warunkujących wirulencję *L. monocytogenes*, jednak wymienione wyżej należą do najistotniejszych (Guariglia-Oropeza i in., 2014; Lopes-

Luz i in., 2021; Mir, 2021; Osek i Wieczorek, 2022; Quereda i in., 2021; Sibanda i Buys, 2022; Wiktorczyk-Kapischke i in., 2023).

W nawiązaniu do częstotliwości występowania genów wirulencji w genomach bakterii tego gatunku, w niniejszej pracy doktorskiej postawiono hipotezę badawczą, mówiącą, że „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się częstotliwością występowania genów warunkujących wirulencję".

1.1.5. Serotypy *L. monocytogenes* a bezpieczeństwo żywności

Poszczególne izolaty *L. monocytogenes* mogą różnić się między sobą nie tylko obecnością w ich genomach genów kluczowych w przebiegu infekcji, ale także innymi cechami. Jedną z nich jest na przykład serotyp, będący kombinacją antygenów somatycznych (O) i wiciowych (H), znajdujących się na powierzchni komórek (Borucki i Call, 2003; Gorski, 2021; Seeliger i Höhne, 1979).

Obecnie znanych jest 14 serotypów *L. monocytogenes* (oznaczonych symbolami: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab 4b, 4c, 4d, 4e, 7, 4h) (Doumith i in., 2004; Yin i in., 2019), ale tylko cztery z nich (4b, 1/2a, 1/2b, 1/2c) powodują ponad 95% przypadków listeriozy i stanowią ponad 95% izolatów z produktów spożywczych (Doumith i in., 2004). Izolat 1/2a jest najczęściej izolowany z żywności, natomiast to serotyp 4b odpowiada za większość wywołanych infekcji (Borucki i Call, 2003; Simonavičienė i in., 2021). Serotyp 4h, odkryty w 2019 roku, również jest uważany za stwarzający duże potencjalne zagrożenie dla zdrowia konsumentów i jest opisywany jako hiperwirulentny (Yin i in., 2019).

Rozróżnianie izolatów *L. monocytogenes* na podstawie ich serotypu można wykonywać metodami aglutynacji z przeciwciałami, natomiast technika ta nie zawsze daje zadowalającą powtarzalność wyników, jak również jest stosunkowo kosztochłonna (Doumith i in., 2004). Genoserotypowanie natomiast opiera się na wykrywaniu określonych genów, co pozwala na klasyfikację izolatów do określonych grup serotypów, nazwanych serogrupami. Technika ta w stosunku do klasycznego serotypowania zwiększa powtarzalność wyników i ułatwia wykonywanie rutynowych badań (Borucki i Call, 2003). Powszechnie uznana metodyka genoserotypowania, oparta na technice multiplex-PCR, klasyfikuje cztery główne serotypy *L. monocytogenes* (4b, 1/2a, 1/2b, 1/2c) do czterech różnych serogrup (Doumith i in., 2004), jednak hiperwirulentny serotyp 4h jest w tej analizie nieodróżnialny od pozostałych serotypów. Z tego względu Feng i in. (2020) opracowali metodykę, w której serotyp 4h jest wyróżniony do osobnej serogrupy, co umożliwia jego identyfikację.

Analiza genoserotypów jest jedną z metod różnicowania izolatów *L. monocytogenes*, a analiza różnic między izolatami jest istotna z punktu widzenia ekogenetyki. Umożliwia ona także śledzenie źródeł zanieczyszczeń pojawiających się w zakładach przetwórstwa spożywczego. Jest to zagadnienie o istotnym znaczeniu w kontekście bezpieczeństwa żywności, ponieważ zdolność przedstawicieli tego gatunku do przetrwania w środowisku zakładów przetwórstwa spożywczego może być powiązana z serotypami (Alía i in., 2020).

1.1.6. Istotność problemu antybiotykooporności u *L. monocytogenes*

Poszczególne izolaty bakterii mogą różnić się między sobą także wrażliwością na antybiotyki, a antybiotykooporność jest zjawiskiem naturalnym, występującym gdy bakterie są narażone na działanie tych związków. Powoduje to presję selekcyjną i skutkuje śmiercią lub hamowaniem wzrostu izolatów wrażliwych, podczas gdy bakterie oporne mają większe szanse na przeżycie i namnażanie się (Prestinaci i in., 2015), analogicznie jak w przypadku zjawiska oporności na środki dezynfekcyjne.

Do czynników odpowiedzialnych za szybkie rozprzestrzenianie się zjawiska antybiotykooporności zalicza się między innymi powszechne stosowanie antybiotyków w hodowlach zwierząt oraz nieuregulowaną sprzedaż tanich antybiotyków, szczególnie w krajach, w których dostęp do powszechnej służby zdrowia jest ograniczony (Laxminarayan i Chaudhury, 2016). Do zjawiska krzyżowej oporności izolatów *L. monocytogenes* na antybiotyki może prowadzić także adaptacja na środki dezynfekcyjne, takie jak IV-rzędowe sole amoniowe (Bland i in., 2021; Kode i in., 2021a; Kode i in., 2021b). Problem antybiotyko-oporności bakterii jest istotny i dotyka wszystkich regionów świata (Murray i in., 2022).

Ponieważ podczas chowu zwierząt powszechnie wykorzystywane są antybiotyki, to środowisko produkcji żywca jest istotnym źródłem pochodzenia antybiotykoopornych mikroorganizmów (Spellberg i in., 2016). Bakterie pochodzące ze środowiska chowu zwierząt stanowią natomiast źródło zanieczyszczenia produktów spożywczych pochodzenia zwierzęcego. W związku z tym w niniejszej pracy postawiono hipotezę badawczą, mówiącą, że „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się pod kątem ich wrażliwości na substancje o charakterze przeciwdrobnoustrojowym". Hipoteza ta odnosi się między innymi do antybiotykooporności izolatów, ale także do ich wrażliwości na chlorek benzalkoniowy.

W przypadku bakterii patogennych, w tym także *L. monocytogenes,* ciągłe prowadzenie badań nad ich opornością na antybiotyki jest konieczne i niezbędne do oceny potencjalnej skuteczności leczenia infekcji tymi bakteriami.

1.1.7. Podsumowanie

Bakterie z rodzaju *L. monocytogenes* są patogenami człowieka, a infekcje przez nie wywoływane charakteryzują się dużym odsetkiem hospitalizacji i zgonów, co stanowi istotny problem społeczny i gospodarczy. Gatunek ten cechuje się zdolnością do adaptacji do warunków środowiskowych, co sprawia, że potrafi on zasiedlać zakłady przetwórstwa spożywczego, prowadząc do wtórnych skażeń produktów spożywczych. Wieloaspektowe monitorowanie *L. monocytogenes* jest ważnym zagadnieniem z punktu widzenia bezpieczeństwa mikrobiologicznego żywności i zdrowia społecznego. Posiadanie aktualnej wiedzy o izolatach pozwala między innymi na dostosowanie procedur dezynfekcji, w rezultacie umożliwiając produkcję bezpiecznych artykułów spożywczych.

1.2. Cel i zakresy badań

Celem pracy była analiza bioróżnorodności izolatów bakterii *L. monocytogenes* pochodzących z surowców, produktów i środowiska przemysłu mięsnego w kontekście ich wrażliwości na chlorek benzalkoniowy. Główny cel zrealizowano w oparciu o liczne cele szczegółowe (oznaczone **C1-C8**), wymienione poniżej. W ramach opisu każdego z celów przedstawiono zakres jego realizacji.

C1. Stworzenie kolekcji izolatów bakterii *L. monocytogenes* pochodzących z przemysłu mięsnego.

Zabezpieczano kolonie bakterii pochodzących z przemysłu mięsnego, które wykazywały typową dla rodzaju *Listeria* morfologię na podłożach selektywnych. Identyfikacja gatunkowa zebranych izolatów została wykonana z wykorzystaniem dwóch technik genetycznych opisanych w literaturze, tj. RFLP-PCR wg. Paillard i in. (2003) oraz multiplex PCR wg. Li i in. (2021). Na wybranych izolatach wykonano analizy służące ocenie różnorodności genetycznej izolatów, tj. RAPD-PCR wg. Wernars i in. (1996) i REP-PCR wg. Jeršek i in. (1999).

C2. Analiza różnorodności genoserotypów izolatów *L. monocytogenes*.

Analiza została przeprowadzona w zakresie dwóch dostępnych w literaturze protokołów służących do genoserotypowania tj. Doumith i in. (2004) i Feng i in. (2020), które dostarczają uzupełniających się informacji.

C3. Analiza oporności izolatów *L. monocytogenes* na antybiotyki.

Analizę przeprowadzono z wykorzystaniem metody dyfuzyjno-krążkowej w zakresie 10 antybiotyków, w tym powszechnie stosowanych do leczenia infekcji wywołanych bakteriami *L. monocytogenes*, tj. ampicyliny, chloramfenikolu, cyprofloksacyny, erytromycyny,

gentamycyny, penicyliny, streptomycyny, sulfametoksazolu/trimetoprimu, tetracykliny i wankomycyny.

C4. Analiza aktywności beta-hemolitycznej izolatów *L. monocytogenes*.

Analizę przeprowadzono na podstawie hodowli izolatów na podłożach stałych zawierających krew baranią i oceny powstania strefy beta-hemolizy w obszarze wyrosłych po inkubacji kolonii.

C5. Analiza występowania w genomach izolatów *L. monocytogenes* wybranych genów warunkujących wirulencję.

Analiza została wykonana w zakresie detekcji 14 genów warunkujących wirulencję, tj. *prfA*, *sigB*, *plcA*, *plcB*, *hlyA*, *mpl*, *actA*, *inlA*, *inlB*, *inlC*, *inlJ, iap, flaA* i *ilsA*. Badania przeprowadzono z wykorzystaniem klasycznej techniki PCR.

C6. Analiza różnic we wrażliwości izolatów na chlorek benzalkoniowy.

Analiza została przeprowadzona w zakresie porównania wrażliwości izolatów na chlorek benzalkoniowy na podstawie różnic między średnicami stref zahamowania wzrostu otrzymanymi w teście dyfuzyjnym.

C7. Analiza obecności wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe w genomach izolatów.

Analiza została wykonana w zakresie detekcji 3 genów warunkujących oporność na IV-rzędowe sole amoniowe, tj. *bcrABC*, *emrC* i *qacH*. Badania wykonano z wykorzystaniem klasycznej techniki PCR.

C8. Analizy zależności między badanymi cechami.

Analizy zależności między badanymi cechami przeprowadzono w oparciu o wyniki badań otrzymane w ramach realizacji pozostałych celów. Badaniu poddano występowanie zależności pomiędzy:

1. występowaniem genów warunkujących oporność na IV-rzędowe sole amoniowe (*bcrABC* lub *emrC* lub *qacH*) a wrażliwością na chlorek benzalkoniowy,
2. wrażliwością na chlorek benzalkoniowy a wrażliwością na antybiotyki,
3. występowaniem genów warunkujących oporność na IV-rzędowe sole amoniowe (*bcrABC* lub *emrC* lub *qacH*) a obecnością genu *ilsA*,
4. wrażliwością na chlorek benzalkoniowy a obecnością genu *ilsA*,
5. wrażliwością na chlorek benzalkoniowy a genoserotypem,
6. wrażliwością na antybiotyki i obecnością genu *ilsA*.

Analizy zależności wykonano z wykorzystaniem statystycznego testu chi-kwadrat, badającego niezależność analizowanych cech. Przyjęty poziom istotności wynosił 0,05.

1.3. Hipotezy badawcze

W oparciu o analizę literatury postawiono trzy hipotezy badawcze, oznaczone **H1**-**H3**, wymienione poniżej.

H1. Izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego są różnorodne genetycznie.

H2. Izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się pod kątem ich wrażliwości na substancje o charakterze przeciwdrobnoustrojowym.

H3. Izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się częstotliwością występowania genów warunkujących wirulencję.

Przedstawione hipotezy zweryfikowano na podstawie oryginalnych badań, w oparciu wyniki otrzymane w ramach realizacji wyznaczonych celów.

2. Wyniki

2.1. Stworzenie kolekcji izolatów

Realizację pracy doktorskiej rozpoczęto od stworzenia kolekcji izolatów *L. monocytogenes*. Mikroorganizmy pochodzące z produktów mięsnych (zarówno przetworzonych, jak i surowych) oraz ze środowiska produkcji zebrano w ramach współpracy z laboratorium badającym rutynowo żywność i stan higieny zakładów spożywczych. Materiał biologiczny był otrzymywany w postaci posiewów na podłożach selektywnych ALOA lub OXFORD. Kolonie o morfologii typowej dla rodzaju *Listeria* (niebiesko-zielone na podłożach ALOA lub ciemnoszare z zielonkawym odcieniem na podłożach OXFORD) przesiewano na podłoże mózgowo-sercowe (BHI) z agarem, celem ich zabezpieczenia. W przypadku zaobserwowania różnic morfologii kolonii na podłożach selektywnych, pobierano z jednej płytki różniące się kolonie, a powstałe w ten sposób izolaty otrzymywały różne oznaczenia.

Kolekcję tworzono na przestrzeni 13 miesięcy w okresie od października 2020 r. do listopada 2021 r., łącznie gromadząc 380 izolatów, zabezpieczonych poprzez zamrożenie w podłożu BHI z dodatkiem 50% glicerolu w -80°C. Ze wszystkich izolatów wyizolowano DNA, które również zabezpieczono poprzez zamrożenie w -20°C. Do bieżących analiz genetycznych wykorzystywano DNA w stężeniu roboczym, wynoszącym 10 ng/µL.

Do identyfikacji izolatów należących do gatunku *L. monocytogenes* wykorzystano dwie niezależne techniki: RFLP-PCR oraz multiplex PCR. Technikę PCR-RFLP wykonywano w oparciu o protokół wg. Paillard i in. (2003) w zakresie amplifikacji fragmentu genu 23S rRNA (oznaczonego S2), a następnie przeprowadzenia reakcji hydrolizy powstałego w reakcji PCR produktu. Hydrolizę wykonywano enzymami restrykcyjnymi *Hha*I oraz osobno *Xmn*I, co pozwalało na zidentyfikowanie bakterii gatunku *L. monocytogenes*. Wynik potwierdzano z wykorzystaniem techniki multiplex PCR wg. Li i in. (2021). W przypadku 218 izolatów z wykorzystaniem obydwu stosowanych technik jednoznacznie potwierdzono ich przynależność do gatunku *L. monocytogenes*.

Na puli 218 izolatów *L. monocytogenes* przeprowadzono analizę różnorodności genetycznej z wykorzystaniem techniki RAPD-PCR wg. Wernars i in. (1996). Gdy izolaty pochodzące z tego samego źródła wykazały taki sam profil prążków w tym oznaczeniu, to do dalszych badań wybierano tylko jeden izolat. Na podstawie tej analizy zawężono badaną pulę izolatów do 153. Tym samym zrealizowano pierwszy cel (**C1**), którym było stworzenie kolekcji różnorodnych genetycznie izolatów *L. monocytogenes*. Na tej puli izolatów (*n*=153)

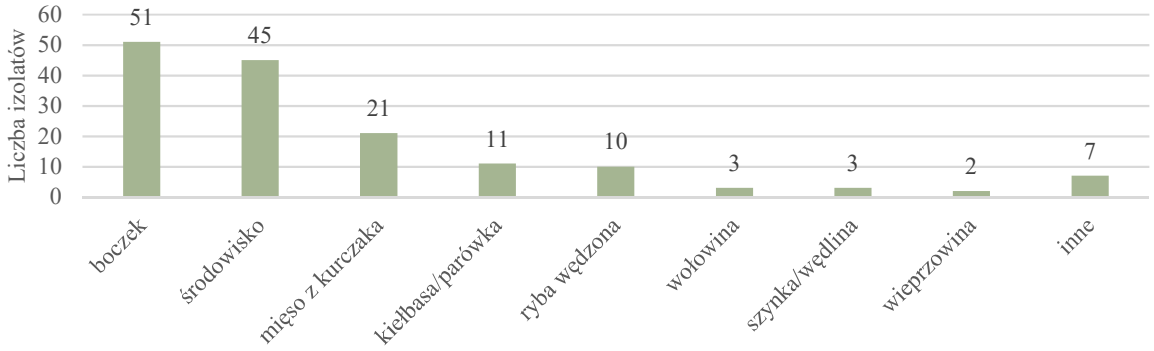
wykonano badania opisane w publikacjach włączonych do rozprawy doktorskiej**,** oznaczonych **P1, P2, P4** i **P5**.

Wyniki analizy RAPD-PCR zostały przedstawione na konferencji w wystąpieniu posterowym pt.: „RAPD-PCR diversity analysis of *Listeria monocytogenes* isolates originating from meat products and meat processing plants in Poland" zaprezentowanym na XXVI Sesji Naukowej Sekcji Młodej Kadry Naukowej "Żywność dzisiaj lokalna czy globalna? tradycyjna czy innowacyjna?" w Poznaniu (19-20 maja 2022).

W celu pogłębienia charakterystyki kolekcji na wybranych izolatach (*n*=144) wykonano także analizę REP-PCR wg. Jeršek i in. (1999), której wyniki również przedstawiono w postaci wystąpienia posterowego pt. „Analiza różnorodności genetycznej izolatów *Listeria monocytogenes* z wykorzystaniem techniki REP-PCR" na XXIX Ogólnopolskim Zjeździe Polskiego Towarzystwa Mikrobiologów w Warszawie (15-17 września 2022). Wyniki analiz RAPD-PCR oraz REP-PCR wykorzystano częściowo w publikacji **P2**.

Stworzona kolekcja 380 zabezpieczonych w kriobanku izolatów bakterii rodzaju *Listeria* oraz zawężona pula 153 izolatów zawierająca scharakteryzowane genetycznie izolaty gatunku *L. monocytogenes*, a także wyizolowane i zabezpieczone DNA tych izolatów stanowi cenny materiał badawczy. Został on wykorzystany nie tylko w publikacjach naukowych wchodzących w skład niniejszej rozprawy, ale także służył realizacji prac magisterskich wykonywanych w Katedrze Biotechnologii i Mikrobiologii Żywności Uniwersytetu Przyrodniczego w Poznaniu. Co więcej, stworzona kolekcja może zostać wykorzystana w przyszłości, służąc jako obiekt kolejnych badań naukowych.

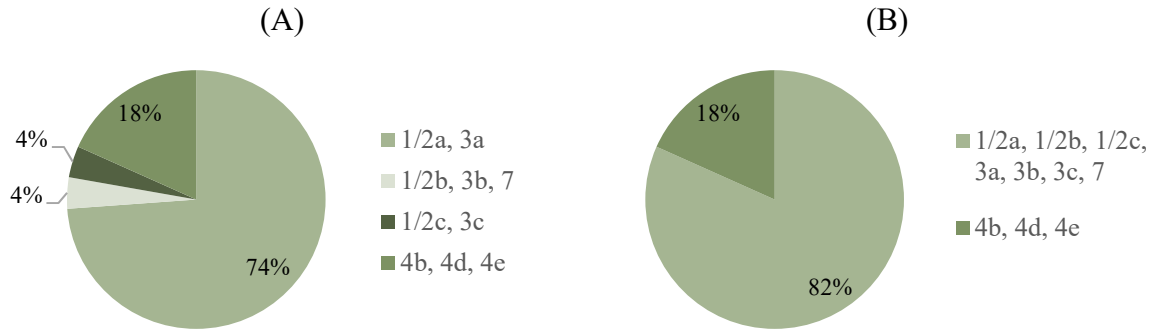
Na Rycinie 1 przedstawiono źródła izolacji 153 izolatów *L. monocytogenes* pochodzących z przemysłu mięsnego, stanowiących materiał badawczy wykorzystany w publikacjach **P1, P2, P4** i **P5**.



Rycina 1. Wykres słupkowy prezentujący źródła pochodzenia 153 izolatów *L. monocytogenes* znajdujących się w badanej puli (źródło: **P1**)

2.2. Analiza genoserotypów

W ramach realizacji pracy doktorskiej wykonano analizę różnorodności genoserotypów skolekcjonowanych izolatów *L. monocytogenes*. Przeprowadzono analizy genoserotypu powszechnie uznaną metodą rozdzielającą cztery główne serotypy *L. monocytogenes* (4b, 1/2a, 1/2b, 1/2c) do czterech różnych serogrup (Doumith i in., 2004), jak również z wykorzystaniem protokołu pozwalającego na rozróżnienie hiperwirulentnego serotypu 4h od pozostałych serotypów (Feng i in., 2020). Badania te opisano w publikacji **P1**, a wyniki genoserotypowania przedstawiono na wykresach na Rycinie 2.



Rycina 2. Wykresy kołowe przedstawiające wyniki genoserotypowania 153 izolatów z badanej puli otrzymane według metodyk: (A) Doumith i in. (2004); (B) Feng i in. (2020) (źródło: **P1**)

Wyniki uzyskane obiema metodami były spójne, co oznacza, że wszystkie izolaty zostały przypisanie do odpowiadających sobie w ramach obu metodyk serogrup. W protokole według Feng i in. (2020) żaden z izolatów nie został zidentyfikowany jako serotyp 4h. W związku z tym metodologia Doumith i in. (2004) pozwoliła większe zróżnicowanie izolatów miedzy sobą, ponieważ jedna serogrupa (obejmująca serotypy 1/2a, 1/2b, 1/2c, 3a, 3b, 3c i 7) z protokołu Feng i in. (2020) została dodatkowo rozdzielona na trzy odrębne grupy w protokole Doumith i in. (2004).

Wykorzystanie do genoserotypowania tylko jednego protokołu, nawet pozwalającego na zróżnicowanie większej liczby izolatów miedzy sobą (Doumith i in., 2004), może doprowadzić do przeoczenia hiperwirulentnego serotypu 4h w badanej próbie, a z kolei metoda pozwalająca na detekcję serotypu 4h (Feng i in., 2020) dostarcza mniej informacji o badanych izolatach. Choć serotyp 4h występuje rzadko, to stwarza zagrożenie dla bezpieczeństwa konsumentów, dlatego najlepiej do technik genoserotypowania wykorzystywać jednocześnie obie metody, które dostarczają uzupełniających się informacji o badanych izolatach.

W związku z niewystępowaniem izolatów serotypu 4h w badanej puli izolatów, w Tabeli 2 przedstawiono wyniki uzyskane na podstawie metodyki wg. Doumith i in. (2004).

Wyniki zestawiono dla izolatów ogółem oraz z podziałem na izolaty pochodzące ze środowiska produkcyjnego i z produktów spożywczych.

Tabela 2. Wyniki genoserotypowania izolatów (źródło: **P1**)

Serogrupa		**Wszystkie izolaty**		**Izolaty z produktów spożywczych**		**Izolaty ze środowiska produkcyjnego**	
Symbol	Serotypy	Liczba	%	Liczba	%	Liczba	%
IIa	1/2a, 3a	113	73,9%	82	75,9%	31	68,9%
IIb	1/2b, 3b, 7	6	3,9%	4	3,7%	2	4,4%
IIc	1/2c, 3c	6	3,9%	6	5,6%	0	0,0%
IVb	4b, 4d, 4e	28	18,3%	16	14,8%	12	26,7%
Suma:		153	100%	108	100%	45	100%

Większość izolatów z produktów spożywczych i środowiska przetwórstwa żywności, tj. 113 (stanowiące 73,9%) należała do serogrupy IIa. Serogrupa IVb była drugą najbardziej liczną, do której należało 28 izolatów (stanowiących 18,3%). W analizowanej puli było także 6 izolatów (stanowiących 3,9%) należących do serogrupy IIb, z czego 4 wykryto w żywności (były to próbki z salcesonu, mięsa wieprzowego, parówki i mięsa z kurczaka), a 2 izolaty pochodziły ze środowiska produkcyjnego (z powierzchni noża i podeszwy buta). Z kolei 6 izolatów zaklasyfikowanych do serogrupy IIc (stanowiących 3,9%) zostało wyizolowane wyłącznie z produktów spożywczych: parówek (3 izolaty), mięsa z kurczaka (2 izolaty) i białej kiełbasy parzonej (1 izolat). Żaden z izolatów z kolekcji nie należał do serogrupy L, reprezentującej serotypy 4a, 4c, 4ab i 4h.

Spośród 51 izolatów pochodzących z boczku, 47 (92,2%) należało do serogrupy IIa, a 4 izolaty do serogrupy IVb. Natomiast izolaty z mięsa z kurczaka (n=21) reprezentowały trzy różne serotypy: 18 izolatów zostało scharakteryzowanych jako serogrupa IIa, 2 izolaty należały do serogrupy IIc, a 1 izolat należał do serogrupy IIb. Izolaty pochodzące z kiełbas i parówek (11 izolatów) również zostały zakwalifikowane do różnych grup: 6 izolatów (w tym 3 z kabanosów, 2 z parówek i 1 z kiełbasy śląskiej) zostało scharakteryzowanych jako serogrupa IIa, 4 izolaty (3 z parówek i 1 z białej kiełbasy parzonej) należały do serogrupy IIc, a 1 izolat (z parówki) należał do serogrupy IIb. Duża różnorodność serogrup sugeruje, że zanieczyszczenie *L. monocytogenes* w tych produktach jest prawdopodobnie pochodzenia surowcowego. Z kolei w przypadku prób wędzonej ryby, wszystkie 10 izolatów należało do serogrupy IVb, sugerując możliwe wtórne skażenie izolatami ze środowiska produkcyjnego.

Badania genoserotypów służyły realizacji celu **C2**. Co więcej, pozwoliły one na wytypowanie izolatów należących do serogrupy IVb (n=28), wśród których znajduje się serotyp 4b, najczęściej wywołujący infekcje u ludzi.

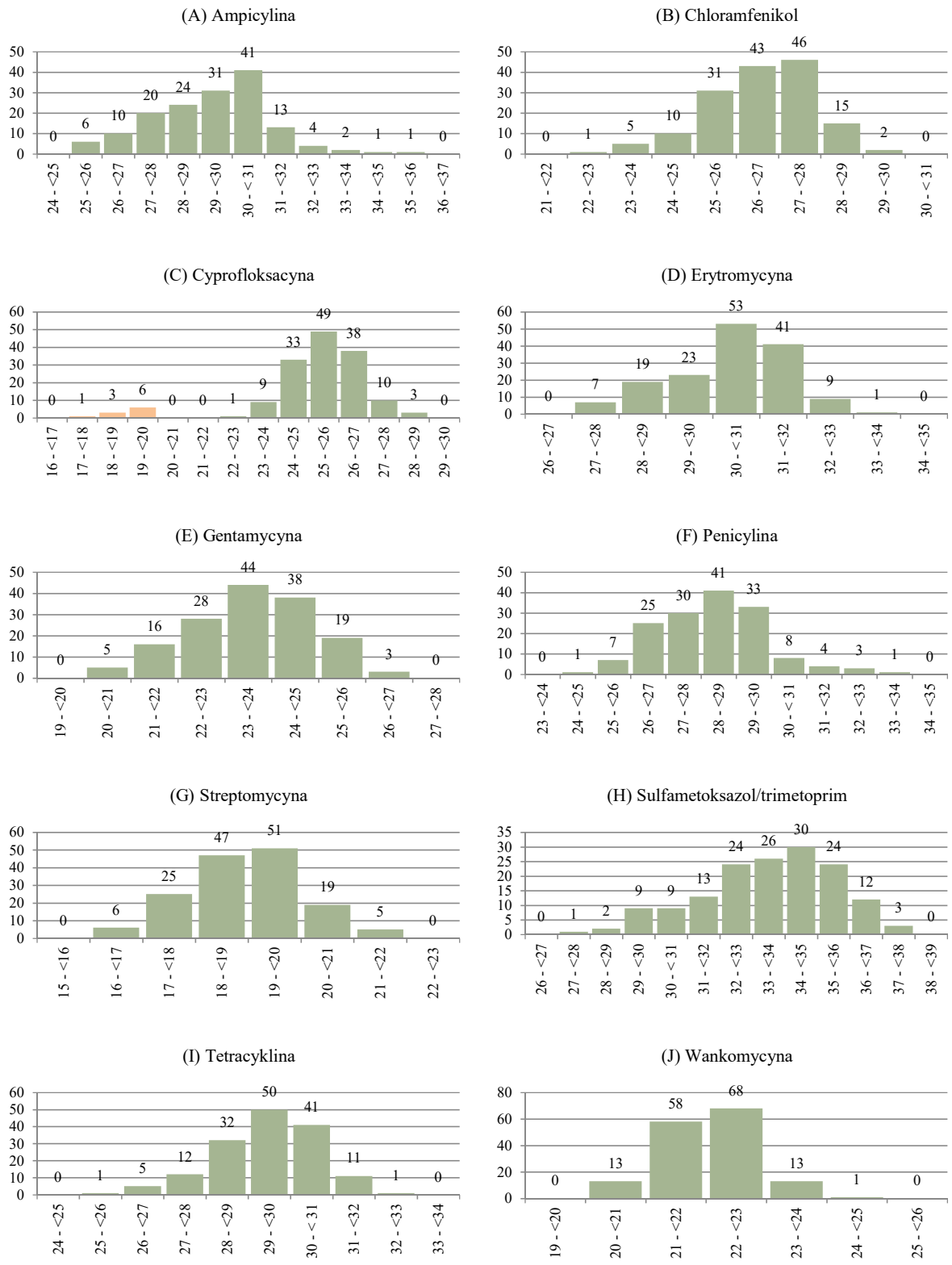
2.3. Analiza antybiotykooporności

Następnym etapem pracy doktorskiej była ocena wrażliwości izolatów na antybiotyki, w tym szczególnie na powszechnie wykorzystywane w leczeniu infekcji spowodowanych *L. monocytogenes*. Badania antybiotykooporności izolatów wykonano z wykorzystaniem metody dyfuzyjno-krążkowej, sprawdzając wrażliwość na 10 antybiotyków (ampicylinę, chloramfenikol, cyprofloksacynę, erytromycynę, gentamycynę, penicylinę, streptomycynę, sulfameto-ksazol/trimetoprim, tetracyklinę i wankomycynę), tym samym realizując cel **C3**. Wyniki tych analiz opublikowano w artykule **P2**.

Otrzymane w badaniach strefy zahamowania wzrostu analizowano w oparciu o przyjęte kryteria, zgodnie z wytycznymi CLSI (Clinical and Laboratory Standards Institute, 2020). W przypadku dziewięciu uwzględnionych antybiotyków (wszystkich z wyjątkiem cyprofloksacyny), wszystkie izolaty charakteryzowały się wrażliwością na te związki. Jedynie w przypadku cyprofloksacyny 10 izolatów (stanowiących 6,5%) nie zostało scharakteryzowanych jako wrażliwe, chociaż według zastosowanych kryteriów izolaty nie były one oporne, a prezentowały pośrednią wrażliwość.

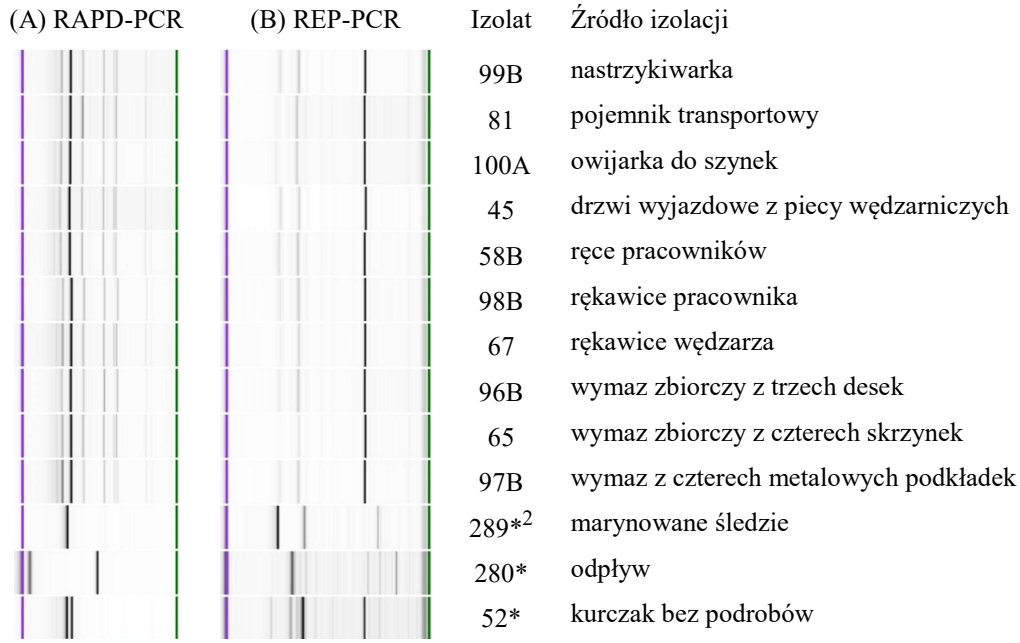
Na Rycinie 3 przedstawiono wyniki rozkładu średnic stref zahamowania wzrostu izolatów *L. monocytogenes* uzyskanych w analizie antybiotykooporności. Otrzymany rozkład stref zahamowania wzrostu był jednomodalny w przypadku wszystkich antybiotyków z wyjątkiem cyprofloksacyny. W jej przypadku rozkład stref ma charakter dwumodalny, z widoczną subpopulacją izolatów (zaznaczoną wykresie kolorem pomarańczowym) o obniżonej wrażliwości na ten antybiotyk. Wyniki analizy histogramów są spójne z interpretacją otrzymanych wielkości stref przejaśnienia zgodnie z przyjętymi kryteriami.

Wyniki tej analizy wzbudziły ciekawość dotyczącą możliwego powodu obniżonej wrażliwości izolatów na cyprofloksacynę, a wykonane badania literaturowe wskazały na możliwość oporności krzyżowej między tym antybiotykiem, a związkami z grupy IV-rzędowych soli amoniowych. Właśnie z tego względu wykonano analizę różnic wrażliwości izolatów na chlorek benzalkoniowy, należący do tej grupy związków.



Rycina 3. Histogramy przedstawiające rozkłady średnic stref zahamowania wzrostu (wyrażonych w mm) izolatów *L. monocytogenes* dla następujących antybiotyków: (A) ampicylina (10 µg), (B) chloramfenikol (30 µg), (C) cyprofloksacyna (5 µg), (D) erytromycyna (15 µg), (E) gentamycyna (10 µg), (F) penicylina (10 IU), (G) streptomycyna (10 µg), (H) sulfametoksazol/trimetoprim (1,25/23,75 µg), (I) tetracyklina (30 µg), (J) wankomycyna (30 µg). Na wykresach kolorem zielonym zaznaczono izolaty wrażliwe, a kolorem pomarańczowym wskazano izolaty o obniżonej wrażliwości na dany antybiotyk (źródło: **P2**)

Wszystkie 10 izolatów o obniżonej wrażliwości na cyprofloksacynę pochodziło z wymazów środowiskowych i wszystkie posiadały genoserotyp VIb (reprezentujący serotypy 4b, 4d i 4e). W celu pogłębienia analizy różnorodności tych izolatów przeanalizowano ich (a także dla porównania trzech wrażliwych izolatów) profile prążków uzyskane w badaniach RAPD-PCR i REP-PCR. Wyniki analizy przedstawiono na Rycinie 4.



Rycina 4. Wyniki rozdziałów produktów reakcji (A) RAPD-PRC i (B) REP-PCR zestawione dla 10 izolatów o obniżonej wrażliwości na cyprofloksacynę oraz dla izolatów 289, 280, 52 wrażliwych na ten antybiotyk (źródło: **P2**)

Uzyskane profile prążków dla 10 izolatów o obniżonej wrażliwości na cyprofloksacynę są zbliżone w przypadku obu zastosowanych metodyk. W analizach uwzględniono również trzy izolaty wrażliwe na cyprofloksacynę (289, 280, 52), których profile są różne pomiędzy sobą i odmienne od izolatów o obniżonej wrażliwości. Podobieństwo między 10 izolatami o obniżonej wrażliwości na cyprofloksacynę zostało wykazane także w analizie filogenetycznej przedstawionej na wykresie (*Figure 3*) w publikacji **P2**. Wyznaczony współczynnik różnic (ang. *distance coefficient*) między izolatami o obniżonej wrażliwości wynosił maksymalnie 0,245. W związku z tym można przypuszczać, że izolaty z tej grupy prawdopodobnie stanowią rozprzestrzenione w środowisku przetwórstwa żywności zanieczyszczenie, a nie mikroflorę pochodzenia surowcowego.

Według otrzymanych wyników stopień oporności izolatów *L. monocytogenes* z badanej puli jest niski, a powszechna wrażliwość izolatów na działanie antybiotyków jest zjawiskiem pozytywnym.

[2] * oznacza izolaty wrażliwe na cyprofloksacynę

2.4. Analiza cech związanych z wirulencją

2.4.1. Ocena aktywności beta-hemolitycznej

Kolejnym krokiem była ocena aktywności beta-hemolitycznej izolatów. Zdecydowano się wykonać tę analizę, mimo że aktywność beta-hemolityczna jest uznawana za cechę gatunkową *L. monocytogenes*, ponieważ istnieją doniesienia literaturowe o występowaniu niehemolitycznych izolatów (Burall i in., 2014; Maury i in., 2017; Szymczak, 2023). Doniesienia te dotyczą najczęściej izolatów pochodzących z produktów spożywczych i środowiska produkcji żywności (źródło: **P3**), zatem badanie było szczególnie uzasadnione. Dodatkowo, najczęstszą przyczyną braku hemolitycznego fenotypu u *L. monocytogenes* są mutacje w obrębie genu *prfA*, kodującego białko będące regulatorem ekspresji genów wirulencji, między innymi *hly*, kodującego hemolizynę. Innym powodem niehemolitycznego fenotypu bywają także mutacje samego genu *hly* (źródło: **P3**). Ponieważ geny *prfA* oraz *hly* są bardzo istotnymi czynnikami wirulencji, to wykrycie izolatów o niehemolitycznym fenotypie dostarczyłoby informacji o prawdopodobnej zredukowanej wirulentności niektórych izolatów z badanej puli.

Analizę wykonano na komercyjnie dostępnym podłożu Columbia, zawierającym dodatek 5% krwi baraniej. Odczytu dokonywano po inkubacji w 37°C zarówno po 24, jak i po 48 godzinach. Jako kontrole negatywne wykorzystano 3 izolaty *L. innocua* pochodzące z kolekcji stworzonej w ramach realizacji niniejszej pracy, a jako kontrolę pozytywną wybrano izolat *L. ivanovii* ATCC 19119 o znanych właściwościach beta-hemolitycznych.

W przypadku kontroli pozytywnej zaobserwowano silną hemolizę, zauważalną jako przejaśnienie wokół wyrosłych kolonii, sięgające od około 5 do około 10 mm za ich brzegi, natomiast w przypadku izolatów *L. monocytogenes* hemoliza (zarówno po 24, jak i po 48 godzinach) była obserwowana wyłącznie pod biomasą wyrosłych kolonii. Usunięcie biomasy za pomocą ezy było czynnikiem ułatwiającym odczyt.

W związku z otrzymanymi wynikami, w celu pogłębienia wiedzy dotyczącej aktywności beta-hemolitycznej bakterii *L. monocytogenes*, a także w celu weryfikacji możliwości ulepszenia techniki badawczej, wykonano obszerne badania literaturowe dotyczące metod służących do oceny zdolności hemolitycznych u izolatów z tego gatunku. Co więcej, podsumowano także doniesienia dotyczące nietypowych, niehemolitycznych izolatów *L. monocytogenes*, a całość badań literaturowych poskutkowała wydaniem publikacji przeglądowej **P3**.

Wykonane analizy aktywności beta-hemolitycznej, służące realizacji celu **C4**, wykazały, że białko Hly (listeriolizyna O) kodowane przez gen *hly* jest funkcjonalne u wszystkich 153 badanych izolatów, co pozwala wysnuć wniosek, że główny pozytywny regulator genów wirulencji u *L. monocytogenes* kodowany przez gen *prfA* również jest aktywny.

Wyniki analizy aktywności beta-hemolitycznej w badanej puli izolatów opublikowano w artykule **P4** po wykonaniu wspomnianych wcześniej badań literaturowych, zawartych w publikacji przeglądowej **P3**.

2.4.2. Detekcja wybranych genów warunkujących wirulencję

Zgodnie z celem **C5** u badanych izolatów przeprowadzono analizę obecności 14 kluczowych genów warunkujących wirulencję: *prfA*, *sigB*, *plcA*, *plcB*, *hlyA*, *mpl*, *actA*, *inlA*, *inlB*, *inlC*, *inlJ, iap, flaA* i *ilsA*. Badania wykonano wykorzystując technikę PCR. Wyniki tych analiz przedstawiono w publikacji **P4** w zakresie trzynastu genów, a w zakresie genu *ilsA* także w publikacji **P5**.

Dziesięć zbadanych genów: *prfA*, *sigB*, *plcA*, *plcB*, *hlyA*, *mpl*, *inlA*, *inlC*, *inlJ* i *iap* zostało zidentyfikowanych u wszystkich 153 izolatów, a produkty reakcji PCR były oczekiwanej wielkości, co wskazuje na ich pełną sekwencję w obrębie amplifikowanych fragmentów.

Gen *actA* wykazał polimorfizm w analizowanej grupie izolatów, a jego detekcja z użyciem dwóch par starterów (oznaczonych odpowiednio jako para actA1 i para actA2) pozwoliła na wyróżnienie czterech grup izolatów. Grupy zostały wyróżnione w związku z istnieniem dwóch polimorficznych form długości genów, jak również w związku z polimorfizmem sekwencji występującym w obrębie rejonów hybrydyzacji pary starterów actA1. Podział izolatów na cztery genotypy na podstawie analizy genu *actA* przedstawiono w Tabeli 3.

Tabela 3. Liczebność grup izolatów wyróżnionych na podstawie analizy genu *actA* (źródło: **P4**)

		Produkt reakcji z parą starterów actA1		
		Wariant krótszy (839 pz)	Wariant dłuższy (950 pz)	Brak produktu
Produkt reakcji z parą starterów actA2	Wariant krótszy (268 pz)	15	0	13
	Wariant dłuższy (385 pz)	0	120	5

W oparciu o wyniki badań i analizy dostępnej literatury nie można wyciągnąć jednoznacznych wniosków o potencjalnie większej lub mniejszej wirulencji izolatów posiadających wydłużony lub skrócony wariant genu *actA* (Bania i in., 2009; Conter i in., 2010; Moriishi i in., 1998; Zhou i in., 2005). Analiza genotypów *actA* dostarczyła

jednak dodatkowych informacji o różnorodności badanej puli izolatów, ponieważ warianty *actA* są niezależne od genoserotypów.

Gen *inlB* został wykryty w przypadku wszystkich badanych izolatów, jednak u dwóch izolatów w genie wykryto delecję długości 141 par zasad. Oba izolaty z delecją, pochodzące z salcesonu i parówki, należały do serogrupy IIb. W przypadku izolatów posiadających delecję w obrębie genu *inlB* ich stopień inwazyjności może być obniżony, natomiast istnieją także doniesienia o zachowaniu wirulencji izolatów, mimo nieposiadania aktywnego genu *inlB* (Roche i in., 2009).

Gen *flaA*, został wykryty u wszystkich 113 izolatów (stanowiących 74% kolekcji) charakteryzujących się genoserotypem IIa (reprezentującym serotypy 1/2a, 3a), natomiast izolaty innych serotypów nie posiadały tego genu. Otrzymany wynik jest zgodny z oczekiwanym, ponieważ gen *flaA* jest genem typowym dla izolatów serogrupy IIa.

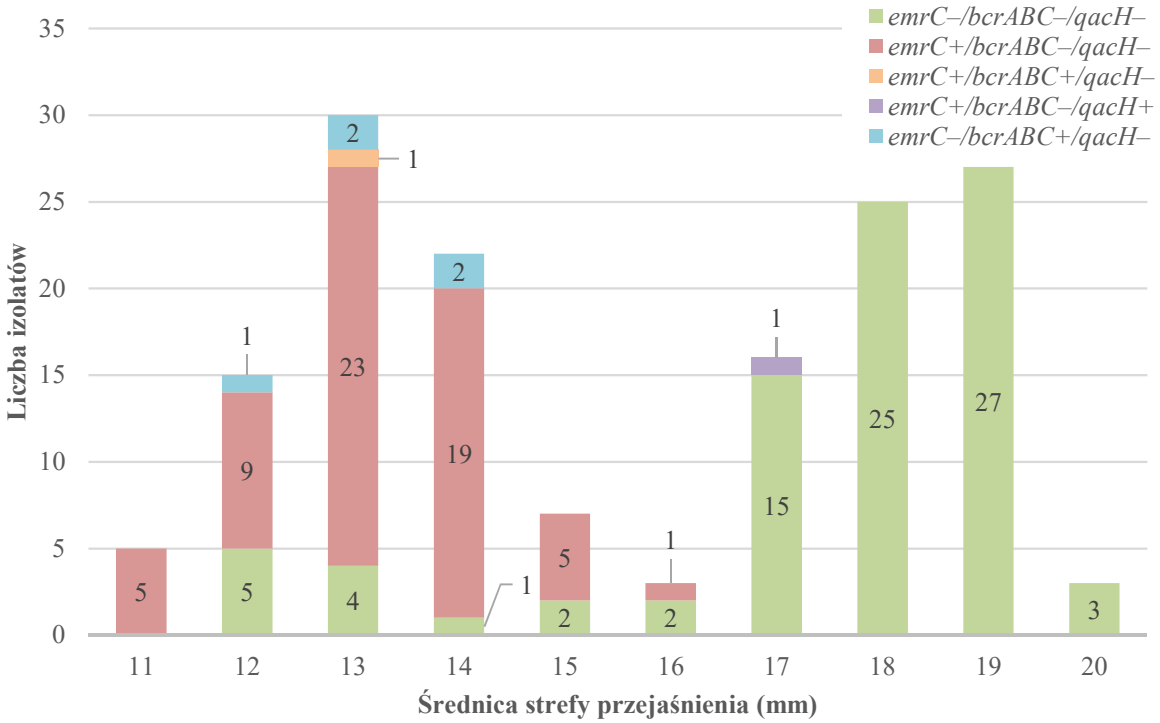
Genem różnicującym zebrane izolaty w kontekście obecności genów warunkujących wirulencję był *ilsA*. Został on wykryty u 18 izolatów (stanowiących 12%), w tym u 16 o genoserotypie IVb a także u 2 o genoserotypie IIb.

2.5. Analiza oporności na IV-rzędowe sole amoniowe

2.5.1. Analiza różnic we wrażliwości izolatów na chlorek benzalkoniowy

Jak wspomniano wcześniej, wykryta u 10 izolatów obniżona wrażliwość na cyprofloksacynę mogła być, według doniesień literaturowych, spowodowana procesem selekcji następującym w wyniku długotrwałej ekspozycji izolatów na związki z grupy IV-rzędowych soli amoniowych. Z tego właśnie względu wykonano analizę różnic we wrażliwości izolatów na chlorek benzalkoniowy. Tym samym zrealizowano cel **C6**, a wyniki analizy opisano w ostatniej publikacji cyklu, oznaczonej **P5**.

Rozkład stref zahamowania wzrostu izolatów (wyrażonych w mm) uzyskanych w testach dyfuzyjnych z chlorkiem benzalkoniowym (o stężeniu 6 mg/mL) przedstawiono na Rycinie 5.



Rycina 5. Rozkład stref przejaśnienia (wyrażonych w mm) uzyskanych w testach dyfuzyjnych z chlorkiem benzalkoniowym zawierający informacje o profilu genów związanych z opornością na IV-rzędowe sole amoniowe (źródło: **P5**)

Na histogramie widoczny jest wyraźny rozkład dwumodalny, wskazujący na istnienie dwóch subpopulacji izolatów pod względem ich wrażliwości na chlorek benzalkoniowy. Na podstawie analizy histogramu, średnica strefy zahamowania wzrostu równa lub mniejsza niż 15 mm została wybrana jako kryterium stosowane do scharakteryzowania izolatów jako wykazujące obniżoną wrażliwość na chlorek benzalkoniowy (w stosunku do izolatów bardziej wrażliwych). Izolaty o średnicy strefy równej lub większej niż 17 mm uznano za wrażliwe, natomiast izolaty posiadające strefę zahamowania równą 16 mm uznano za wykazujące wrażliwość pośrednią.

Po zastosowaniu wybranych kryteriów ponad połowa, tj. 79 izolatów (51,6%) została sklasyfikowana jako wykazująca obniżoną wrażliwość na chlorek benzalkoniowy, co wskazuje na powszechne występowanie obniżonej wrażliwości na IV-rzędowe sole amoniowe wśród izolatów *L. monocytogenes* pochodzących z przemysłu mięsnego. Podsumowanie wyników analizy fenotypowej przedstawiono w Tabeli 4.

Tabela 4. Podsumowanie analizy wrażliwości izolatów na chlorek benzalkoniowy (źródło: **P5**)

Wrażliwość na chlorek benzalkoniowy	**Liczba izolatów (%)**
wrażliwość	71 (46,4%)
wrażliwość pośrednia	3 (2,0%)
obniżona wrażliwość	79 (51,6%)

Wszystkie 10 izolatów o obniżonej wrażliwości na cyprofloksacynę wykazało obniżoną wrażliwość na chlorek benzalkoniowy w tym oznaczeniu, co mogłoby potwierdzać podejrzenie o występującej oporności krzyżowej. Jednakże, w badanej puli zidentyfikowano także 69 innych izolatów o obniżonej wrażliwości na chlorek benzalkoniowy, które nie wykazywały obniżonej wrażliwości na cyprofloksacynę.

Powszechność izolatów o obniżonej wrażliwości na chlorek benzalkoniowy w badanej puli izolatów wzbudziła ciekawość dotyczącą jej przyczyn, prowadząc do wykonania analizy detekcji wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe.

2.5.2. Detekcja genów warunkujących oporność na IV-rzędowe sole amoniowe

Do analizy obecności wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe w genomach izolatów, stanowiącej cel **C7**, wybrano geny: *bcrABC*, *emrC* i *qacH*, powszechnie występujące w genomach *L. monocytogenes* według doniesień literaturowych. Spośród zbadanych genów, gen *emrC* był najczęściej obecny w genomach izolatów z badanej puli i został wykryty u 64 izolatów (41,8%). Gen *bcrABC* był obecny w genomach sześciu izolatów (3,9%), a *qacH* został wykryty w jednym przypadku (0,7%). Na podstawie analizy tych trzech genów, wśród badanych izolatów wyróżniono 5 genotypów, przedstawionych w Tabeli 5. Wyniki tych badań opisano w publikacji **P5**.

Tabela 5. Wyniki analizy genów warunkujących oporność na IV-rzędowe sole amoniowe (źródło: **P5**)

Geny			**Liczba izolatów (%)**
emrC	*bcrABC*	*qacH*	
–[3]	–	–	84 (54,9%)
+[4]	–	–	62 (40,5%)
–	+	–	5 (3,3%)
+	–	+	1 (0,7)%
+	+	–	1 (0,7)%

U 84 izolatów (stanowiących 54,9% kolekcji) nie wykryto żadnego spośród badanych genów warunkujących oporność na IV-rzędowe sole amoniowe. W przypadku 67 izolatów wykryto jeden gen (u 62 izolatów był to gen *emrC*, natomiast u 5 izolatów *bcrABC*). Dwa izolaty miały jednocześnie po dwa geny warunkujące oporność, mianowicie *emrC* i *bcrABC* (w przypadku izolatu pochodzącego z wymazu z kratki ściekowej) lub *emrC* i *qacH* (u izolatu pochodzącego z wymazu z taśmy transportującej mięso).

[3] – oznacza niewykrycie obecności genu
[4] + oznacza wykrycie obecności genu

2.5.3. Zależność fenotypu od genotypu we wrażliwości na IV-rzędowe sole amoniowe

Kolejnym wykonanym krokiem była analiza zależności między występowaniem genów warunkujących oporność na IV-rzędowe sole amoniowe a wrażliwością na chlorek benzalkoniowy w badaniach fenotypowych. Wyniki tej analizy przedstawiono w publikacji **P5**.

Zależność między obecnością badanych genów a fenotypem jest przedstawiona na Rycinie 5 na histogramie rozkładu stref przejaśnienia uzyskanych w teście dyfuzyjnym. Na wykresie kolorami, zgodnie z legendą, zaznaczono profile genów uzyskane w testach detekcji genów związanych z opornością na IV-rzędowe sole amoniowe.

Spośród 79 izolatów scharakteryzowanych jako mające obniżoną wrażliwość na chlorek benzalkoniowy, 67 (84,8%) miało co najmniej jeden gen związany z opornością na IV-rzędowe sole amoniowe. Spośród 71 izolatów uznanych za wrażliwe, 70 izolatów (98,6%) nie miało żadnych analizowanych genów związanych z opornością na IV-rzędowe sole amoniowe. Pozostały wrażliwy izolat (stanowiący 1,4%) posiadał dwa geny i charakteryzował się profilem *emrC+/bcrABC-/qacH+*.

W kolekcji zidentyfikowano łącznie 12 izolatów (15,2%) o obniżonej wrażliwości na chlorek benzalkoniowy, których fenotyp nie wynikał z obecności żadnego ze zbadanych genów (*emrC*, *bcrABC* lub *qacH*).

Mimo że w pojedynczych przypadkach zaobserwowano rozbieżność fenotypu i genotypu, to z użyciem testu chi-kwadrat, którego wartość p wynosiła $< 0{,}0001$, potwierdzono występowanie zależności między występowaniem w genomach co najmniej jednego z badanych genów warunkujących oporność na IV-rzędowe sole amoniowe (*emrC* lub *bcrABC* lub *qacH*) a wrażliwością na chlorek benzalkoniowy (określoną jako wrażliwość, wrażliwość pośrednia lub obniżona wrażliwość).

2.6. Analiza zależności między zbadanymi cechami

2.6.1. Wrażliwość na chlorek benzalkoniowy a antybiotykooporność

Przeprowadzono analizy zależności między cechami fenotypowymi izolatów, tj. między opornością na antybiotyki a wrażliwością na chlorek benzalkoniowy. Ponieważ zbadane izolaty nie różniły się wrażliwością na antybiotyki inne niż cyprofloksacyna, dlatego w tej analizie zależności uwzględniono tylko ten antybiotyk. W Tabeli 6 przedstawiono rozkład liczebności izolatów w kontekście ich wrażliwości na cyprofloksacynę w odniesieniu do ich wrażliwości na chlorek benzalkoniowy.

Tabela 6. Rozkład liczebności izolatów w kontekście ich wrażliwości na cyprofloksacynę w odniesieniu do wrażliwości na chlorek benzalkoniowy

		Wrażliwość na chlorek benzalkoniowy		
		Wrażliwość	Wrażliwość pośrednia	Obniżona wrażliwość
Wrażliwość na cyprofloksacynę	Wrażliwość	71	3	69
	Wrażliwość pośrednia	0	0	10

Wartość p testu chi-kwadrat badającego niezależność wrażliwości izolatów na cyprofloksacynę od ich wrażliwości na chlorek benzalkoniowy wynosiła < 0,0001, co oznacza, że można przyjąć hipotezę alternatywną, mówiącą o zależności między badanymi cechami. Zatem w badanej puli izolaty o obniżonej wrażliwości na chlorek benzalkoniowy wykazywały obniżoną wrażliwość na cyprofloksacynę (określoną jako wrażliwość pośrednia) statystycznie istotnie częściej niż izolaty o standardowej wrażliwości.

W kolekcji zidentyfikowano 12 izolatów nie posiadających żadnego z badanych genów warunkujących oporność na IV-rzędowe sole amoniowe (*emrC*, *bcrABC* lub *qacH*), a jednocześnie wykazujących obniżoną wrażliwość na chlorek benzalkoniowy. W grupie tych 12 izolatów, znalazły się wszystkie izolaty (n=10) o zmniejszonej wrażliwości na cyprofloksacynę.

Wyniki potwierdzają pierwotne podejrzenie, że przyczyną obniżonej wrażliwości na cyprofloksacynę może być wcześniejsza adaptacja izolatów do IV-rzędowych soli amoniowych. Prawdopodobnym powodem występującej u *L. monocytogenes* oporności krzyżowej między tymi związkami mogą być mutacje w obrębie genu represorowego *fepR*, które są obserwowane u izolatów wykazujących obniżoną wrażliwość na cyprofloksacynę po adaptacji do IV-rzędowych soli amoniowych (Douarre i in., 2022). Innym możliwym powodem może być obecność mniej powszechnych genów warunkujących oporność na IV-rzędowe sole amoniowe w genomach izolatów.

2.6.2. Obecność genów *bcrABC* lub *emrC* lub *qacH* a obecność genu *ilsA*

Wykonano analizę zależności między obecnością genu *ilsA* a obecnością wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe (*bcrABC* lub *emrC* lub *qacH*). Wyniki przedstawiono w publikacji **P5**.

W Tabeli 7 przedstawiono rozkład liczebności izolatów w obrębie grup w zależności od ich genotypu (tutaj określonego jako posiadanie co najmniej jednego z badanych genów warunkujących oporność na IV-rzędowe sole amoniowe lub nieposiadanie żadnego z tych genów) i obecności lub nieobecności genu *ilsA*.

Tabela 7. Rozkład liczebności izolatów w kontekście ich obecności genu *ilsA* w odniesieniu do obecności lub braku wybranych genów warunkujących oporność na IV-rzędowe sole amoniowe

		Obecność genów *bcrABC* lub *emrC* lub *qacH*	
		bcrABC+ lub *emrC*+ lub *qacH*+	*bcrABC*- i *emrC*- i *qacH*-
Obecność genu *ilsA*	*ilsA*+	66	69
	ilsA-	15	3

Na podstawie powyższego rozkładu wykonano test chi-kwadrat weryfikujący niezależność między badanymi cechami. Wartość p testu chi-kwadrat wynosiła 0,006, co wskazuje, że izolaty posiadające gen *ilsA* statystycznie rzadziej posiadają geny warunkujące oporność na IV-rzędowe sole amoniowe, niż izolaty nieposiadające *ilsA*.

2.6.3. Wrażliwość na chlorek benzalkoniowy a obecność genu *ilsA*

Wykonano także analizę zależności między obecnością lub nieobecnością genu *ilsA*, a wrażliwością izolatów na chlorek benzalkoniowy otrzymaną w teście fenotypowym. Wyniki przedstawiono w publikacji **P5**. Rozkład liczebności izolatów z podziałem na te grupy przedstawiono w Tabeli 8.

Tabela 8 Rozkład liczebności izolatów w kontekście obecności lub nieobecności genu *ilsA* w odniesieniu do wrażliwości izolatów na chlorek benzalkoniowy w teście fenotypowym

		Wrażliwość na chlorek benzalkoniowy		
		Wrażliwość	Wrażliwość pośrednia	Obniżona wrażliwość
Obecność genu *ilsA*	*ilsA*+	6	1	11
	ilsA-	65	2	68

Analiza zależności pomiędzy obecnością genu *ilsA* a wrażliwością na chlorek benzalkoniowy w teście fenotypowym wykonana na podstawie powyższego rozkładu pozwoliła na przyjęcie hipotezy, mówiącej o niezależności tych dwóch zmiennych. Wartość p testu chi-kwadrat wyniosła 0,294. Oznacza to, że nie zaobserwowano statystycznej zależności między występowaniem genu *ilsA* a wrażliwością na chlorek benzalkoniowy.

2.6.4. Wrażliwość na chlorek benzalkoniowy a genoserotyp

Sprawdzono także zależność między genoserotypem badanych izolatów a ich wrażliwością na chlorek benzalkoniowy. Zarówno w grupie izolatów wrażliwych, jak i w grupie izolatów o obniżonej wrażliwości na chlorek benzalkoniowy znajdowały się izolaty przyporządkowane do wszystkich czterech genoserotypów. Rozkład liczebności izolatów w obrębie serogrup w kontekście ich wrażliwości na chlorek benzalkoniowy przedstawiono w Tabeli 9.

Tabela 9. Rozkład liczebności izolatów w obrębie serogrup w kontekście ich wrażliwości na chlorek benzalkoniowy

Serogrupa		**Wrażliwość na chlorek benzalkoniowy**		
Symbol	Serotypy	Wrażliwość	Wrażliwość pośrednia	Obniżona wrażliwość
IIa	1/2a, 3a	50	2	61
IIb	1/2b, 3b, 7	4	1	1
IIc	1/2c, 3c	3	0	3
IVb	4b, 4d, 4e	14	0	14

Wartość p testu chi-kwadrat badającego niezależność genoserotypu od wrażliwości na chlorek benzalkoniowy wynosiła 0,142, co oznacza, że można przyjąć hipotezę, mówiącą o braku zależności między badanymi cechami. Wynika z tego, że w badanej próbie wrażliwość na chlorek benzalkoniowy nie była zależna od genoserotypu izolatu, a wrażliwości izolatów nie da się przewidzieć na podstawie analizy genoserotypu.

2.6.5. Oporność na antybiotyki a obecność genu *ilsA*

Ostatnim badaniem zależności między cechami izolatów była analiza zależności między opornością na antybiotyki a obecnością genu *ilsA*. Jak wcześniej wspominano, w trakcie badań zidentyfikowano 10 izolatów o obniżonej wrażliwości na cyprofloksacynę, a gen *ilsA* został wykryty u 18 izolatów. W Tabeli 10 przedstawiono liczebności grup izolatów w kontekście tych dwóch cech.

Tabela 10. Rozkład liczebności izolatów w kontekście obecności lub nieobecności genu *ilsA* w odniesieniu do wrażliwości izolatów na cyprofloksacynę

		wrażliwość na cyprofloksacynę	
		Wrażliwość	Wrażliwość pośrednia
Obecność genu *ilsA*	*ilsA*+	10	8
	ilsA-	133	2

U 8 spośród 10 izolatów o obniżonej wrażliwości na cyprofloksacynę (określonej jako wrażliwość pośrednia) wykryto gen *ilsA*. W tym przypadku wartość p testu chi-kwadrat badającego niezależność między obecnością genu *ilsA* a obniżoną wrażliwością na cyprofloksacynę wynosiła < 0,0001, co oznacza, że w badanej próbie izolaty posiadające gen *ilsA* wykazały obniżoną wrażliwość na cyprofloksacynę statystycznie istotnie częściej, niż izolaty nieposiadające tego genu.

3. Podsumowanie

3.1. Odniesienie do celu głównego

Głównym celem pracy była analiza różnorodności tych izolatów w kontekście ich wrażliwości na chlorek benzalkoniowy, a cel ten zrealizowano w oparciu o wyznaczone cele szczegółowe, oznaczone **C1-C8**.

Zgromadzono i zabezpieczono 380 izolatów bakterii o morfologiach kolonii typowych dla bakterii z rodzaju *Listeria* na podłożach selektywnych. Ze stworzonej kolekcji, na podstawie analiz multiplex PCR, RFLP-PCR oraz RAPD-PCR wytypowano 153 izolaty *L. monocytogenes*, które poddano szczegółowej analizie w ramach niniejszej pracy doktorskiej. Stworzona kolekcja stanowi cenny materiał badawczy, który pozwoli na kontynuację badań w przyszłości.

Ogółem, na podstawie wszystkich przeprowadzonych analiz ustalono, że cechami wspólnymi wszystkich 153 zbadanych izolatów *L. monocytogenes* były: wrażliwość na 9 antybiotyków (ampicylinę, chloramfenikol, erytromycynę, gentamycynę, penicylinę, streptomycynę, sulfametoksazol/trimetoprim, tetracyklinę i wankomycynę), aktywność beta-hemolityczna i obecność 10 genów warunkujących wirulencję (*iap*, *sigB*, *prfA*, *hlyA*, *inlC*, *inlA*, *inlJ*, *plcA*, *plcB* i *mpl*). Izolaty różniły się natomiast: genoserotypem (a tym samym także obecnością lub brakiem genu *flaA*), stopniem wrażliwości na cyprofloksacynę, wrażliwością na chlorek benzalkoniowy, polimorfizmem długości genów *inlB* i *actA*, jak również obecnością lub brakiem genów: *ilsA*, *bcrABC*, *emrC* i *qacH*.

Na podstawie wszystkich analiz ostatecznie otrzymano 25 różnych kombinacji profili geno- i fenotypów wśród skolekcjonowanych izolatów. Unikatowe kombinacje tych profili pozwoliły na wyróżnienie 25 grup izolatów, które oznaczono alfabetycznie, literami od A do Z. Wyniki podsumowano w Tabeli 11, w której dla zwiększenia czytelności nie ujęto opisanych wyżej cech wspólnych dla wszystkich badanych izolatów.

Tabela 11. Podsumowanie wyników analiz uwzględnionych w pracy doktorskiej, z wyłączeniem cech wspólnych dla wszystkich badanych izolatów, umożliwiające wyróżnienie 25 grup

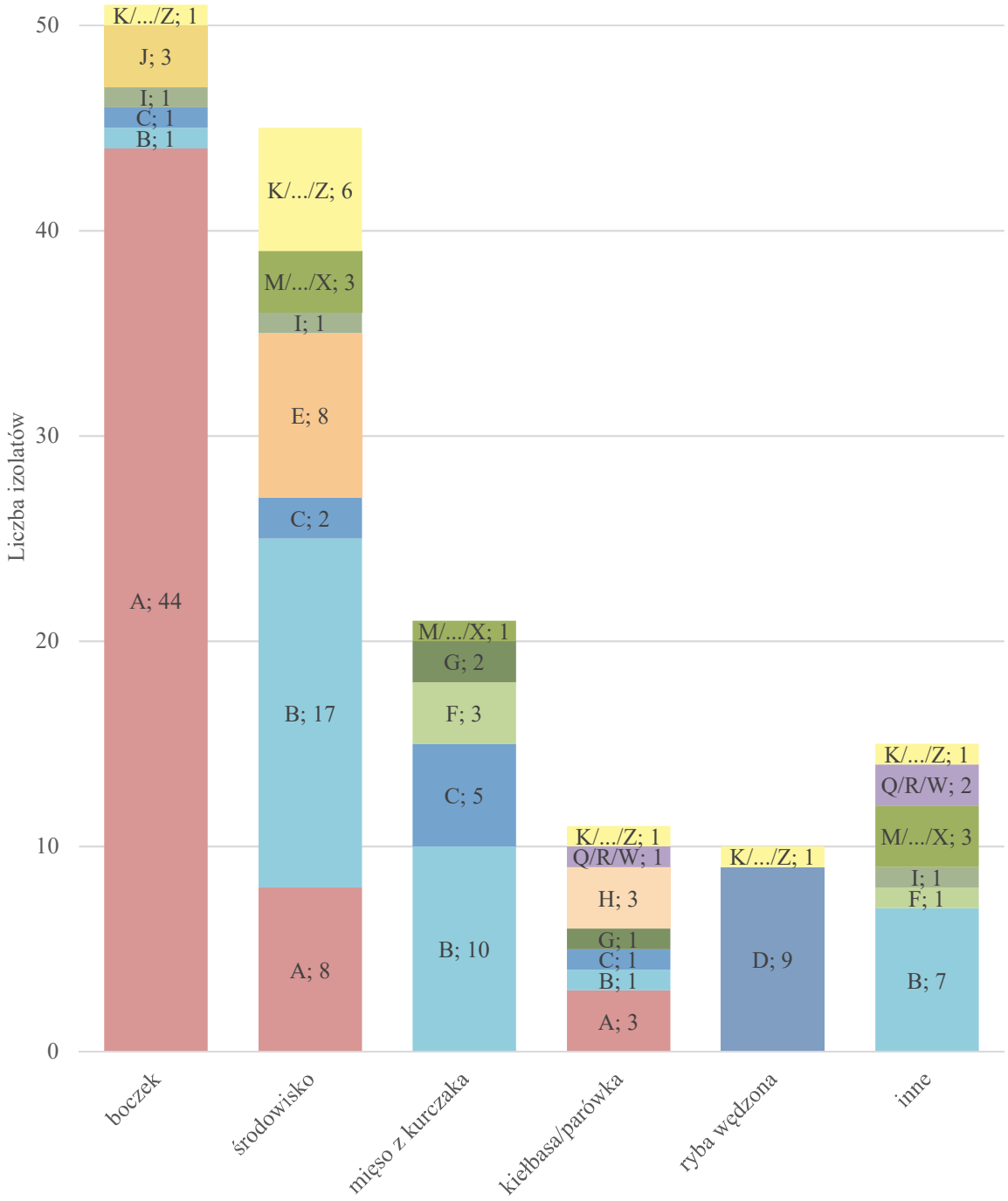
Genoserotyp	***flaA***	***emrC***	***bcrABC***	***qacH***	***ilsA***	***actA2***	***actA1***	***inlB***	**Wrażliwość na chlorek benzalkoniowy**	**Wrażliwość na cyprofloksacynę**	**Liczba izolatów**	**Oznaczenie grupy izolatów**
1/2a, 3a	+[5]	+	–[6]	–	–	D[7]	D	+	R[8]	S[9]	55	**A**
1/2a, 3a	+	–	–	–	–	D	D	+	S	S	36	**B**
1/2a, 3a	+	–	–	–	–	K[10]	–	+	S	S	9	**C**
4b, 4d, 4e	–	–	–	–	–	D	D	+	S	S	9	**D**
4b, 4d, 4e	–	–	–	–	+	K	K	+	R	I[11]	8	**E**
1/2a, 3a	+	–	–	–	–	D	–	+	S	S	4	**F**
1/2c, 3c	–	–	–	–	–	D	D	+	S	S	3	**G**
1/2c, 3c	–	–	+	–	–	D	D	+	R	S	3	**H**
4b, 4d, 4e	–	–	–	–	+	D	D	+	S	S	3	**I**
4b, 4d, 4e	–	+	–	–	+	D	D	+	R	S	3	**J**
1/2a, 3a	+	–	+	–	–	D	D	+	R	S	2	**K**
1/2a, 3a	+	+	–	–	–	K	–	+	R	S	2	**L**
1/2b, 3b, 7	–	–	–	–	–	D	D	+	S	S	2	**M**
4b, 4d, 4e	–	–	–	–	+	K	K	+	S	S	2	**N**
4b, 4d, 4e	–	–	–	–	–	K	K	+	R	I	2	**O**
1/2a, 3a	+	–	–	–	–	D	–	+	R	S	1	**P**
1/2a, 3a	+	–	–	–	–	K	–	+	I	S	1	**Q**
1/2a, 3a	+	+	–	–	–	K	–	+	I	S	1	**R**
1/2a, 3a	+	+	–	+	–	D	D	+	S	S	1	**S**
1/2a, 3a	+	+	+	–	–	D	D	+	R	S	1	**T**
1/2b, 3b, 7	–	–	–	–	+	D	D	+	S	S	1	**U**
1/2b, 3b, 7	–	–	–	–	+	K	K	+	I	S	1	**W**
1/2b, 3b, 7	–	–	–	–	–	K	K	K	S	S	1	**X**
1/2b, 3b, 7	–	+	–	–	–	K	K	K	R	S	1	**Y**
4b, 4d, 4e	–	–	–	–	–	D	D	+	R	S	1	**Z**

[5] + oznacza wykrycie danego genu
[6] – oznacza niewykrycie danego genu
[7] D oznacza dłuższą formę polimorficzną genu
[8] R oznacza obniżoną wrażliwość na związek (ang. *reduced sensitivity*)
[9] S oznacza wrażliwość na związek (ang. *sensitivity*)
[10] K oznacza krótszą formę polimorficzną genu
[11] I oznacza pośrednią wrażliwość na związek (ang. *intermediate response*)

W przypadku 10 grup, oznaczonych P-Z uzyskane profile geno- i fenotypowe były unikatowe i charakteryzował się nimi każdorazowo tylko 1 izolat. Grupy oznaczone F-O charakteryzowały każdorazowo od 2 do 4 izolatów. Do bardziej powszechnych grup (oznaczonych A-E) przypisano od 8 do 55 izolatów, z czego dwie najbardziej liczne grupy (oznaczone A i B) charakteryzowały się: genoserotypem 1/2a, 3a (zatem także obecnością genu *flaA*), dłuższym wariantem genu *actA* wykrytym oboma parami starterów, standardową długością genu *inlB*, nieobecnościami genów *ilsA, bcrABC* i *qacH* oraz wrażliwością na cyprofloksacynę. Te dwie najbardziej liczne grupy różniły się między sobą jednocześnie obecnością genu *emrC* i obniżoną wrażliwością na chlorek benzalkoniowy (w przypadku liczniejszej grupy, oznaczonej A, zawierającej 55 izolatów) lub brakiem obecności genu *emrC* oraz standardową wrażliwością na chlorek benzalkoniowy (w przypadku mniej licznej grupy, oznaczoną B, zawierającą 36 izolatów). Rozkład zależności pomiędzy pochodzeniem izolatów a ich grupami geno- i fenotypowymi została przedstawiona na Rycinie 6.

Wśród analizowanych mikroorganizmów znajdowało się 51 izolatów pochodzących z boczku (skolekcjonowanych na przestrzeni 13 miesięcy), a 44 spośród nich należało do profilu A, zatem według badań nie różniły się one między sobą, a charakteryzowało je między innymi posiadanie genu *emrC* i obniżona wrażliwość na chlorek benzalkoniowy. Oporność tych izolatów na IV-rzędowe sole amoniowe może być potencjalną przyczyną ich uporczywej obecności w próbach boczku, ponieważ izolaty o obniżonej wrażliwości na chlorek benzalkoniowy były prawdopodobnie mniej wrażliwe na procesy dezynfekcji. Pozostałe izolaty profilu A były wyizolowane ze środowiska produkcyjnego (8 izolatów, spośród których 6 pochodziło z wymazów z kratek ściekowych) oraz z kiełbas (3 izolaty pochodzące z kabanosów). Izolaty z drugiego pod kątem częstości pojawiania się profilu, oznaczonego B, zostały wyizolowane głównie ze środowiska produkcyjnego (17 izolatów), prób mięsa kurczaka (10 izolatów) i innych źródeł (w których znalazły się między innymi próby mięsa wołowego i wieprzowego).

Wszystkie izolaty o profilu D (9 izolatów), charakteryzujące się między innymi wrażliwością na chlorek benzalkoniowy, zostały wyizolowane z prób ryby wędzonej. Podobnie, wszystkie izolaty o profilu E (8 izolatów), które wykazały obniżoną wrażliwość na chlorek benzalkoniowy także zostały wyizolowane tylko z jednego źródła, jakim były próby środowiskowe. Analogicznie wszystkie izolaty profili H (3 izolaty) i J (3 izolaty) pochodziły odpowiednio z parówek oraz z boczku i charakteryzowały się obniżoną wrażliwością na chlorek benzalkoniowy. Sugeruje to wystąpienie lokalnego, być może przejściowego, zanieczyszczenia środowiska produkcyjnego w przypadku izolatów o tych profilach.



Rycina 6. Wykres słupkowy prezentujący źródła pochodzenia skolekcjonowanych izolatów w połączeniu z informacją o grupie geno- i fenotypowej. Odcieniami koloru czerwonego, pomarańczowego i żółtego oznaczono izolaty o obniżonej wrażliwości na chlorek benzalkoniowy, odcieniami niebieskiego i zielonego oznaczono izolaty o standardowej wrażliwości, natomiast kolorem fioletowym zaznaczono izolaty o wrażliwości pośredniej. W celu zwiększenia czytelności grafiki, oznaczono na niej zbiorczo grupy, w których znajdowało się od 1 do maksymalnie 2 izolatów. Należą do nich grupy M, N, S, U i X (oznaczone M/…/X), w których znajdowały się izolaty wrażliwe na chlorek benzalkoniowy, grupy K, L, O, P, T, Y i Z (oznaczone K/…/Z), w których znajdowały się izolaty o obniżonej wrażliwości na chlorek benzalkoniowy oraz grupy Q, R i W (oznaczone Q/R/W), w których znajdowały się izolaty o pośredniej wrażliwości na chlorek benzalkoniowy.

3.2. Odniesienie do sformułowanych hipotez

Przed realizacją pracy doktorskiej postawiono 3 hipotezy dotyczące różnorodności izolatów *L. monocytogenes* pochodzących z przemysłu mięsnego. Zostały one zweryfikowane na podstawie oryginalnych badań, w oparciu wyniki otrzymane w ramach realizacji celów od **C1** do **C8**.

Pierwsza z hipotez (**H1**) odnosiła się do różnorodności genetycznej izolatów *L. monocytogenes*. Hipoteza ta została zweryfikowana na podstawie analiz RAPD-PCR i REP-PCR oraz w oparciu o analizy genoserotyów. Już w oparciu o wyniki tych analiz różnorodność genetyczna izolatów została potwierdzona. Co więcej, dalsze analizy ujawniły szczegółowe różnice między izolatami, takie jak polimorfizm długości genów (w przypadku *actA* oraz *inlB*) lub różnice w obecności niektórych badanych genów (*ilsA*, *bcrABC*, *emrC* i *qacH*). Należy jednak zaznaczyć, że w niektórych przypadkach nie znaleziono różnic między badanymi izolatami, na przykład w przypadku 44 izolatów pochodzących z boczku. Podsumowując, pierwsza hipoteza, brzmiąca „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego są różnorodne genetycznie" została częściowo potwierdzona.

Druga hipoteza (**H2**) odnosiła się do różnego stopnia wrażliwości izolatów *L. monocytogenes* na środki o charakterze przeciwmikrobiologicznym. Hipotezę tę zweryfikowano w oparciu o badania antybiotykooporności na 10 antybiotyków, a także na podstawie badania wrażliwości izolatów na chlorek benzalkoniowy, będący dezynfektantem. Wszystkie izolaty wykazały wrażliwość na wszystkie zbadane antybiotyki, wykazując jedynie różny stopień wrażliwości na cyprofloksacynę (u 10 izolatów zaobserwowano pośrednią wrażliwość między opornością a wrażliwością, a 143 izolatów było wrażliwych). Uzyskany stopień antybiotykooporności izolatów jest niski, szczególnie w kontekście ich odzwierzęcego pochodzenia. Izolaty wykazały natomiast różnorodność w kontekście wrażliwości na chlorek benzalkoniowy (71 izolatów zostało uznanych za wrażliwe, 3 wykazało wrażliwość pośrednią, a 79 uznano za posiadające obniżoną wrażliwość). Podsumowując, wyłącznie w oparciu o wyniki antybiotykooporności hipoteza ta nie mogłaby zostać potwierdzona, natomiast na podstawie wyników wrażliwości izolatów na chlorek benzalkoniowy potwierdzono drugą hipotezę, mówiącą, że „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się pod kątem ich wrażliwości na substancje o charakterze przeciwdrobnoustrojowym".

Ostatnia hipoteza (**H3**) odnosiła się do różnic częstotliwości występowania genów warunkujących wirulencję u zebranych izolatów. W czasie badań wykazano, że izolaty przedstawiają różne warianty genu *actA*, a także że dwa izolaty posiadają skrócony gen *inlB*.

Izolaty należące do serogrupy IIa, w przeciwieństwie do izolatów innych serogrup, posiadały gen *flaA*, natomiast jest on genem typowym dla izolatów tej serogrupy. Genem różnicującym badane izolaty w kontekście występowania w ich genomach genów warunkujących wirulencję był gen *ilsA*. Podsumowując, stopień różnorodności izolatów w tym kontekście był niewielki, jednak hipoteza trzecia, brzmiąca „izolaty *L. monocytogenes* pochodzące z przemysłu mięsnego różnią się częstotliwością występowania genów warunkujących wirulencję" także została potwierdzona.

3.3. Wnioski i ustalenia wynikające z przeprowadzonych badań i analiz

Izolaty *L. monocytogenes* pojawiające się w produktach mięsnych i środowisku przetwórstwa są różnorodne genetycznie oraz posiadają różnice fenotypowe objawiające się różnym stopniem tolerancji środków o charakterze przeciwmikrobiologicznym.

Niniejsza praca wykazała, że stopień antybiotykooporności wśród skolekcjonowanych izolatów jest niski, co jest zjawiskiem pozytywnym. Mimo ogólnego niskiego stopnia antybiotykooporności w badanej puli, izolaty o zredukowanej wrażliwości na chlorek benzalkoniowy wykazywały obniżoną wrażliwość na cyprofloksacynę częściej niż izolaty wrażliwe na ten dezynfektant. Sugeruje to istnienie oporności krzyżowej, co jest zjawiskiem niekorzystnym w kontekście bezpieczeństwa konsumentów.

Co więcej, mimo niskiego stopnia antybiotykooporności, częstotliwość występowania genów wirulencji u zebranych izolatów *L. monocytogenes* była wysoka (wszystkie wykazały się między innymi obecnością wszystkich genów z LIPI-1 oraz wszystkich zbadanych genów kodujących internaliny). Wszystkie izolaty posiadały także aktywność beta-hemolityczną, która wskazuje, że białka kodowane przez geny *hly* i *prfA* są funkcjonalne. Oznacza to, że izolaty te stanowią zagrożenie dla zdrowia konsumentów, a opracowanie metod ich efektywnej eliminacji ze środowiska produkcyjnego jest kluczowe w celu zapewnienia bezpiecznej żywności.

Ze względu na powszechne i częste stosowanie IV-rzędowych soli amoniowych w przemyśle spożywczym, geny odpowiedzialne za oporność na te związki (według niniejszej pracy szczególnie *emrC*) są rozpowszechnione wśród *L. monocytogenes* pochodzących z produktów mięsnych i środowiska produkcyjnego. Może to powodować zredukowanie efektywności eliminacji tych bakterii w procesach dezynfekcji, w konsekwencji prowadząc do zasiedlenia zakładu produkcyjnego i wtórnych skażeń wyrobów gotowych. Stanowi to duże zagrożenie dla zdrowia konsumentów, szczególnie biorąc pod uwagę wysoką częstotliwość występowania genów warunkujących wirulencję u badanych izolatów.

Co więcej, izolaty przystosowane do środowiska produkcyjnego (w znaczeniu wykazania obniżonej wrażliwości na chlorek benzalkoniowy) nie wykazują mniejszej częstości występowania genów warunkujących wirulencję niż izolaty wrażliwe na chlorek benzalkoniowy. Dodatkowo, izolaty o obniżonej wrażliwości na cyprofloksacynę częściej niż izolaty wrażliwe posiadają gen *ilsA*, różnicujący izolaty badanej puli pod kątem posiadanych genów warunkujących wirulencję.

Ze względu na potwierdzone w niniejszych badaniach coraz powszechniejsze zjawisko obniżonej wrażliwości na środki dezynfekcyjne, warto zwrócić uwagę na nowoczesne środki kontroli *L. monocytogenes.* Alternatywami wobec stosowania klasycznych środków dezynfekujących byłoby np. wykorzystanie bakteriofagów jako naturalnego środka służącego do kontroli *L. monocytogenes* w przemyśle spożywczym (Kawacka i in., 2020a). Niekonwencjonalną metodą jest również zastosowanie naturalnych substancji pochodzenia roślinnego, na przykład takich jak tymol, eugenol (znajdujących się w tymianku i oregano), cytral (z trawy cytrynowej) lub aldehyd cynamonowy (z cynamonu), które wykazują duży potencjał w inhibicji *L. monocytogenes* (Kawacka i in., 2020b). Postulowane jest także, że obiecującym, nowoczesnym środkiem, który może służyć do utrzymania higieny oraz dezynfekcji zakładów przemysłu spożywczego są bakteriocyny, czyli związki syntetyzowane przez bakterie fermentacji mlekowej, mające zdolność do inhibicji wzrostu bakterii psujących żywność oraz patogenów, w tym również bakterii *L. monocytogenes* (Camargo i in., 2018; Kumariya i in., 2019).

Przeprowadzone analizy wykazały konieczność bieżącego monitorowania podatności izolatów *L. monocytogenes* z przemysłu mięsnego na związki o charakterze przeciw-mikrobiologicznym. Umożliwiałoby to podjęcie działań naprawczych w momencie pojawienia się izolatów o obniżonej wrażliwości na wykorzystywane w zakładzie środki. Zmiana preparatów na takie, które w badaniach laboratoryjnych wykazują skuteczność przeciwko badanym izolatom mogłaby przyczynić się do znacznego ograniczenia skażeń wtórnych, a tym samym do polepszenia jakości mikrobiologicznej produktów spożywczych.

Częstość występowania poszczególnych badanych genów w genomach *L. monocytogenes* oraz doniesień o oporności lub obniżonej wrażliwości izolatów na dezynfektanty lub antybiotyki różni się w poszczególnych publikacjach. Dzieje się tak, ponieważ każda kolekcja pochodzi z innych, często odległych, obszarów geograficznych, była izolowana z różnych źródeł oraz została zebrana w różnych ramach czasowych. Wszystkie publikowane wyniki są cenne i składają się na bardziej kompletny obraz, który pozwala śledzić globalne trendy ewolucji cech patogenu, jakim jest *L. monocytogenes*.

W związku z wyżej wymienionymi aspektami istnieje konieczność ciągłego monitorowania *L. monocytogenes*, między innymi pod kątem rozpowszechniającego się zjawiska obniżonej wrażliwości na środki dezynfekcyjne, w tym IV-rzędowe sole amoniowe, wśród izolatów pochodzących z przemysłu spożywczego. Wyniki sygnalizują także potrzebę wprowadzenia środków zapobiegających zasiedlaniu środowiska produkcyjnego, tym samym ograniczając wtórne skażenia produktów spożywczych.

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GENOSEROTYPING OF *LISTERIA MONOCYTOGENES* STRAINS ORIGINATING FROM MEAT PRODUCTS AND MEAT PROCESSING ENVIRONMENTS

S u m m a r y

Background. *Listeria monocytogenes* is a foodborne human pathogen and a causative factor of listeriosis, which is an illness with a high mortality rate. Serotyping is a method for differentiating *L. monocytogenes* isolates based on unique combinations of somatic (O) and flagellar (H) antigens on the surface of their cells. Standard serotyping involves agglutination methods, which require using antisera. However, there are also genoserotyping methods which allow to categorise *L. monocytogenes* isolates into particular groups of serotypes (referred to as serogroups) based on genetic analyses. Differentiating *L. monocytogenes* isolates is an important issue in terms of food safety, surveillance and traceability of contamination sources. In this work, we present results of the genoserotyping of 153 *L. monocytogenes* isolates originating from meat products and meat processing environments at Polish processing plants. Two protocols were used for genoserotyping analyses: the first one allows to differentiate between four most common serotypes (1/2a, 1/2b, 1/2c and 4b) and the second one allows to distinguish hipervirulent serovar 4h from other serotypes.

Results and conclusion. Results achieved using both methods were consistent and all isolates were categorised into corresponding serogroups within the two methodologies. Most of the isolates (73.9 %) were characterised as members of the IIa serogroup (representing the 1/2a, 3a serovars). The IVb (4b, 4d, 4e) serogroup was the second most common (and comprised 18.3 % of isolates), followed by IIb (1/2b, 3b, 7) and IIc (1/2c, 3c), however, the last two groups were equally numerous (and each of them comprised 3.9 % of all isolates). None of the collected isolates belonged to the serogroup representing the 4a, 4c, 4ab and 4h serotypes.

Key words: serotyping, genoserotyping, serotypes, food safety, food surveillance

Introduction

Listeria monocytogenes is a bacterium that can be found in a variety of food products, including meat, fish, milk and vegetables [12, 18]. Heat treatment inactivates

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bacteria of that species, however, products can sometimes be contaminated after processing and due to that fact *L. monocytogenes* can be found in both raw and processed foods [2]. The bacterium can also settle in food processing environments, which may be a cause of food contamination [2, 18]. *L. monocytogenes* is tolerant to high salt concentration (up to 10 %) and low pH (in the range of 3 ÷ 9), as well as is able to proliferate at refrigeration temperatures (minimum 0 ÷ 1 °C), which makes the bacterium challenging to control in food products [2, 12].

L. monocytogenes consumed with contaminated food may cause an infection, called listeriosis, which is manifested as acute febrile gastroenteritis (with symptoms such as fever, diarrhoea, muscle pain and headache). However *L. monocytogenes* can cross intestinal barrier, blood-brain barrier and maternal-foetal barrier, leading to invasive listeriosis. Those types of infections can cause septicaemia, maternal-foetal infections (often resulting in abortions or stillbirths) or neurolisteriosis (e.g. in a form of meningitis) [18]. A mortality rate of listeriosis reaches 20 ÷ 30 % [2, 24].

Bacteria that belong to *L. monocytogenes* genus can be differentiated on the basis of their unique combinations of somatic (O) and flagellar (H) antigens on cells' surface [3, 11, 19]. Currently, there are 14 known serotypes (marked with the following symbols: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7, 4h) [9, 23], but only four of them (namely 4b, 1/2a, 1/2b, 1/2c) cause more than 95 % listerial cases and comprise more than 95 % isolates from food products [9]. Interestingly, the 1/2a isolate is the most frequently isolated from food, whereas the 4b serotype is responsible for the majority of infections [3, 20].

Standard agglutination methods with antisera can be used for stereotyping *L. monocytogenes* strains, however, the technique is limited by costs, availability and the need for technical expertise. Furthermore, the reproducibility of the results is not always satisfactory [9]. To increase the ease and accessibility of this classification system [3], some authors proposed PCR-based genoserotyping protocols. Genoserotyping is based on detecting particular genes in bacterial genome, which allows to categorise isolates into particular groups of serotypes (referred to as serogroups).

The first PCR-based genoserotyping methodology, established by Borucki and Call [3], included four pairs of primers and allowed to categorise bacteria into five serotype groups. However, PCR reactions need to be held separately, not in multiplex PCR, which increases analysis time. First multiplex-PCR serotyping protocol was proposed by Doumith et al. [9]. It allows to differentiate 4 major *L. monocytogenes* serovars (namely 4b, 1/2a, 1/2b, 1/2c) and therefore the technique has discriminatory power similar to traditional serotyping (as 95 % of isolates belongs to one of those four serotypes). Specifically, the methodology allows to differentiate serogroups: IIa (including the 1/2a, 3a serovars), IIb (1/2b, 3b, 7), IIc (1/2c, 3c), IVb (4b, 4d, 4e) and serogroup L (including all other *Listeria sensu stricto* species and remaining serotypes

of *L. monocytogenes*: 4a, 4c, 4ab and 4h) [5,13]. Although the protocol of Doumith et al. [9] is commonly accepted, the 4h serovar, which has been discovered recently, cannot be distinguished from other serovars by this protocol. The isolates of the 4h serovar are considered hipervirulent, posing a threat to food safety [23], and due to that fact Feng et al. [10] proposed an assay in which the 4h serovar is specifically distinguished from other serotypes [10]. There are also other protocols regarding genoserotyping of *L. monocytogenes* strains in multiplex-PCR reactions [6,8,15], a serotyping scheme using a combination of an antibody-based serogrouping and a multiplex PCR assay [4] and protocols for real-time PCR [1,21].

In general, the identification of genoserotypes is one of the methods of differentiating *L. monocytogenes* strains, which is an important issue in terms of food safety, food surveillance and traceability of contamination sources. Alía et al. [1] stated that carrying out the correct differentiation of *L. monocytogenes* serotypes is of utmost importance, as the epidemiology and persistence of this bacterium in meat processing plants may be related to its serotype [1].

Our hypothesis is that *L. monocytogenes* isolates originating from Polish meat products and meat processing environments present diverse genoserotypes. The aim of this research was to determine the genoserotypes of *L. monocytogenes* strains of that origin and additionally to compare the results obtained with two genoserotyping protocols – the one that is commonly accepted, proposed by Doumith et al. [9], and the modern one, put forward by Feng et al. [10].

Materials and methods

Microorganisms

Strains of putative *Listeria* spp. originating from Polish meat products (raw and processed ones) and meat processing environments in Poland were obtained during routine microbiological quality analyses. Environmental samples were obtained from surfaces both being in contact with food (e.g. knife surface, workers' gloves or transport boxes) and not coming into contact with food (e.g. shoe soles, floors or walls of a facility). The samples were collected between October 2020 and November 2021. Microorganisms were isolated on plates with selective agar media (OXFORD or ALOA). Bacteria from one colony of typical appearance were restreaked on Brain Heart Infusion (BHI) (POCH, Poland) agar plate and incubated at 37 °C for 24 hours prior to DNA isolation. Overall, 153 *L. monocytogenes* isolates were obtained from the samples delivered, 45 of which originated from the environment and 108 originated from food products. Details regarding the origin of the samples are presented in Fig. 1.

DNA isolation

Microorganisms were removed from 24-hour BHI agar plate culture with a sterile inoculation loop and subjected to DNA isolation procedure. Isolation was performed with a commercially available kit Genomic Mini (A&A Biotechnology, Poland), in line with the manufacturer's instructions. DNA concentration was measured with a Spectrophotometer ND-1000 (NanoDrop, USA).

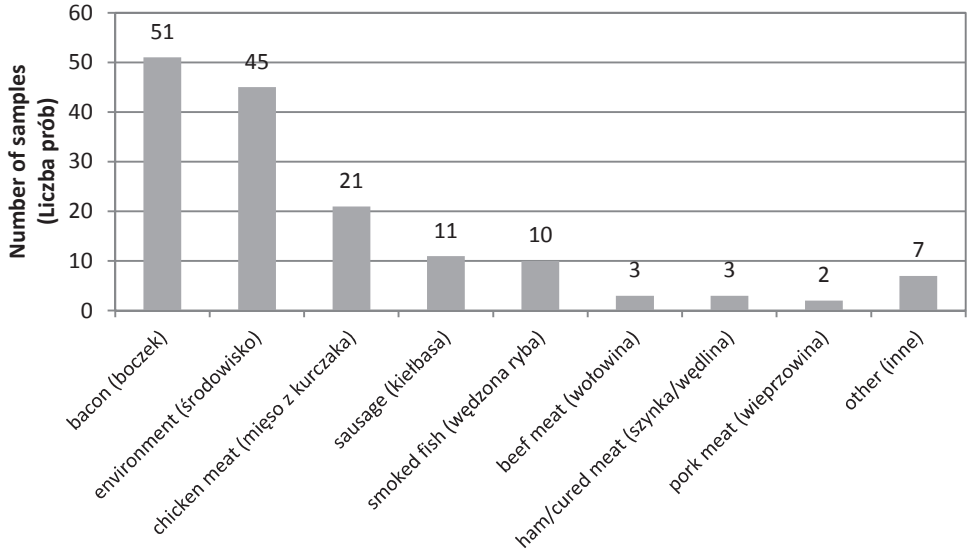

Fig. 1. Bar chart showing the sources of the samples included in the study
Rys. 1. Wykres słupkowy obrazujący źródła pochodzenia prób uwzględnionych w badaniu

Confirmation of species affiliation

Species were confirmed as *L. monocytogenes,* using two genetic methods, namely RFLP-PCR by Paillard et al. [16] and multiplex PCR by Li et al. [14], according to the protocols described in the references, with minor modifications. PCRs were performed in 10 µL final volume using RUN polymerase (A&A Biotechnology, Poland). Restriction digestions were performed using HhaI and XmnI (ThermoFisher Scientific, USA) enzymes with a corresponding Tango buffer. Only microorganisms confirmed as *L. monocytogenes* with both techniques were included in the study.

Genoserotyping

Genoserotyping was performed with multiplex PCR reactions, according to two protocols proposed by Doumith et al. [9] and Feng et al. [10], with minor modifica-

tions. Primers, whose sequences are presented in Table 1, were synthesised to order by Genomed company.

In genoserotyping protocol according to Doumith et al. [9], the multiplex mixture contained 1U of *Taq* RUN polymerase (A&A Biotechnology) and compatible reaction buffer at recommended concentration, 0.2 mM dNTPs (A&A Biotechnology) and 1.5 µM of *lmo1118*, 1.0 µM of: *lmo0737*, ORF2819 and ORF2110, and 0.2 µM of *prs* final concentration for each primer. DNA was added in the amount of 10 ng per reaction. The mixture was filled with water to reach the final volume of 10 µL. Negative control sample with PCR-grade water used instead of DNA was employed in each reaction set. PCR was performed in T-Gradient thermocycler (Biometra) with the following programme: initial denaturation at 94 °C for 3 min; 35 cycles of: 94 °C for 24 s, 53 °C for 69 s, 72 °C for 69 s; and a final step of 72 °C for 7 min.

In the genoserotyping protocol proposed by Feng et al. [10], the multiplex mixture was prepared in a similar way as described above, however, different primers were used, namely: *LMxysn_1095*, *lmo1083* and *smcL*, all at final concentration of 0.25 µM. DNA isolated from *L. ivanovii* ATCC 19119 was used as a positive control for *smcL* amplicon, as well as PCR-grade water was used as a negative control sample in each reaction set. PCR was performed with the following programme: initial denaturation at 95 °C for 5 min; 30 cycles: at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final step at 72 °C for 10 min.

Table 1. Primers used in the study and their sequences
Tabela 1. Startery użyte w badaniu i ich sekwencje

Primers' name Nazwa starterów	Primers' sequence (5' → 3') Sekwencja starterów (5' → 3')	Product size (bp) Rozmiar produktu (pz)	Reference Źródło
lmo0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	Doumith et al. protocol [9]
lmo1118	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906	
ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	
ORF2110	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	
prs	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	370	
LMxysn_1095	F: AATACTTGGACAGACGGAACGC R: TCATCTGGCTCTTTTAGAACCG	602	Feng et al. protocol [10]
lmo1083	F: CACAAATGGTCTTGACGGGG R: TTTGCGCGTGATTTTAGTGG	390	
smcL	F: CACAGACCATTGTGGTGACTTG R: CGGTGCTTTCATTTTTTTACTC	889	

Reaction products were separated on 2 % agarose gel (dyed with ethidium bromide) in 1 × TAE buffer and visualised using Gel Doc Imaging System (Bio-Rad).

Data analysis

Microsoft Excel 2007 software was used to analyse the data and prepare the charts.

Results and discussion

In this study, we present the results of the genoserotyping of 153 *L. monocytogenes* isolates originating from meat products and processing environments. Experiments were performed in line with two genoserotyping protocols. The results achieved by employing both methodologies, which are presented in Fig. 2, were consistent and coherent in the case of all isolates, meaning that all of the samples were categorised into corresponding serogroups within the two methodologies. Hypothesis that isolates originating from Polish meat products and meat processing environments present diverse genoserotypes was confirmed by both methodologies.

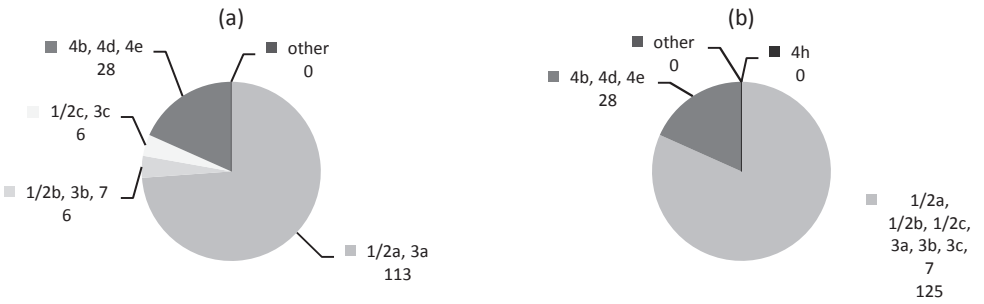

Fig. 2. Pie chart representing the results of genoserotyping acc. to: (a) Doumith et al. [9]; (b) Feng et al. [10] protocol

Rys. 2. Wykres kołowy przedstawiający wyniki genoserotypowania otrzymane według protokołu: (a) Doumith i wsp. [9]; (b) Feng i wsp. [10]

In the protocol according to Feng et al. [10], none of the isolates was identified as the 4h serovar, as well as none of the isolates was classified as a member of the serogroup consisting of the other serovars (4a, 4ab and 4c). Owing to that, in the case of our samples, the methodology of Doumith et al. [9] allowed to achieve more detailed results, as one serogroup (comprising the 1/2a, 1/2b, 1/2c, 3a, 3b, 3c and 7 serovars) from the protocol of Feng et al. [10] was further separated into three distinct groups in the protocol of Doumith et al. [9] (as can be seen in Fig. 2). Detailed results of serogrouping achieved in the protocol of Doumith et al. [9], with separation into samples from food products and processing environments, are presented in Table 2.

Most of the isolates included in the study (73.9 %) belonged to the IIa serogroup, whereas the IVb serogroup was the second most common (and comprised 18.3 % of all of the samples). Six isolates (3.9 %) were identified as belonging to the IIb serogroup, of which 4 were detected in food (these were samples from brawn (Polish *salceson*), pork meat, Vienna sausage and chicken meat) and 2 were detected in a processing environment (isolates originated from knife surface and shoe sole). All isolates (6 - 3.9 %) characterised as the IIc serogroup were identified in food products. Their origin was as follows: Vienna sausages (3 samples), chicken meat (2 samples) and white steamed sausage. None of the collected isolates belonged to the L serogroup, representing the remaining 4a, 4c, 4ab and 4h serovars.

Table 2. Results of serotyping acc. to protocol of Doumith et al. (2004)
Tabela 2. Wyniki serotypowania wg protokołu Doumith i wps. (2004)

Serogroup / Serogrupa		All isolates Wszystkie izolaty		Isolates from food products Izolaty pochodzące z żywności		Isolates from food processing environment Izolaty ze środowiska produkcyjnego żywności	
Symbol	Serovars Serowary	Number Liczba	%	Number Liczba	%	Number Liczba	%
IIa	1/2a, 3a	113	73.9	82	75.9	31	68.9
IIb	1/2b, 3b, 7	6	3.9	4	3.7	2	4.4
IIc	1/2c, 3c	6	3.9	6	5.6	0	0.0
IVb	4b, 4d, 4e	28	18.3	16	14.8	12	26.7
L	4a, 4c	0	0.0	0	0.0	0	0.0
	Sum / Suma:	153		108		45	

Out of 51 isolates originating from bacon, 47 belonged to the IIa serogroup and 4 to the IVb serogroup. Chicken meat samples (21 in total) represented three distinct serotypes, of which 18 samples were characterised as the IIa serogroup, 2 isolates belonged to the IIc serogroup and 1 isolate belonged to the IIb serogroup. Sausages (11 samples) also contained diverse *L. monocytogenes* isolates, as 6 samples (3 from dried fermented sausages (Polish *kabanos*), 2 from Vienna sausages and one from Silesian sausage) were characterised as the IIa serogroup, 4 samples (3 from Vienna sausages and one from white steamed sausage) belonged to the IIc serogroup and one sample (from Vienna sausage) was classified under the IIb serogroup. In contrast to the abovementioned group of products, all 10 samples from smoked fish contained isolates characterised as the IVb serogroup.

In general, our findings are consistent with most of literature reports. The results of genostereotyping according to protocol of Feng et al. [10] have not been published to date, however, many authors have used the protocol of Doumith et al. [9]. For ex-

ample, Psareva et al. [17] examined *L. monocytogenes* isolates collected between 2001 and 2020, originating from meat, poultry, dairy, and fish products from the Central European part of Russia. Out of 40 samples, 22 (55.0 %) belonged to the IIa serogroup; 11 (27.5 %) belonged to the IVb group; 4 (10.0 %) isolates belonged to the IIc group and 3 (7.5 %) to the IIb group [17], which makes the prevalence of particular serogroups similar to those achieved in our research.

On the other hand, Wang et al. [22] performed traditional serotyping of *L. monocytogenes* isolates from pork samples available at supermarkets in Wuhan, China. The 1/2a serovar comprised 45 out of 63 of all samples (71.4 %), the 1/2c serovar comprised 12 samples (19.0 %) and the 1/2b serovar comprised 6 samples (9.5 %). Other serovars were not detected [22]. That study demonstrated that the 1/2a serotype was the most commonly prevalent in food products, however, the authors did not detect any isolate which belonged to the 4b, 4d or 4e serovars, whereas in our case, the IVb serogroup was the second most common. Similarly to findings of Wang et al. [22], according to Coban et al. [7], who used the protocol of Doumith et al. [9], the IVb serogroup was the least numerous (1 sample – 1.0 %) in their study. The authors serogrouped 103 isolates originating from poultry samples from the retail markets and slaughterhouses in Turkey. However, the majority of *L. monocytogenes* strains (78 samples – 75.7 %) were characterised as the IIa serogroup (which is in agreement with our findings), followed by the IIc serogroup (15 samples – 14.5 %) and the IIb group (6 samples – 5.8 %) [7].

Chen et al. [5] performed genoserotyping of 102 isolates from food samples purchased at retail in U.S. FoodNet sites using the protocol of Doumith et al. [9]. The IIa serogroup was the most prevalent, with 46 isolates (45.1 %), while the second most common serogroup was IIb, with 28 samples (27.5 %), followed by the IVb group, with 20 (19.6 %) samples, and the IIc group, with 5 (4.9 %) samples. Interestingly, the authors also identified 3 isolates (2.9 %) which were characterised as the L serogroup (comprising the remaining serovars: 4a, 4ab, 4c and 4h) [5]. Microorganisms of that serogroup were not identified in this study.

In contrast to the aforementioned reports in which the IIa serogroup was the most common among isolates, Simonavičienė et al. [20], who used the protocol of Doumith et al. [9], have found that the IVb segrogroup was the most prevalent in cold smoked fish (salmon) products from Lithuania. Out of 37 *L. monocytogenes* isolates, 35 (90.5 %) belonged to the IVb serogroup, whereas only 2 samples (5.4 %) were identified as members of the IIa serogroup [20]. However, these results are also in agreement with our findings, as only one serogroup, namely IVb, was identified in all 10 samples originating from smoked fish included in our study.

In conclusion, scientific data is not entirely consistent as regards the detailed proportions and prevalence rate of particular serogroups of *L. monocytogenes* isolates,

however, in most cases the IIa serogroup (or the 1/2a serovar) is the most prevalent, which was also a result of our findings. In general, the 1/2a serotype is commonly considered a serotype the most frequently isolated from food products [3]. The discrepancies in the proportions of particular serogroups probably result from examining a different type of samples, originating from different sources, obtained at distant geographical locations and collected during different time frames.

Conclusions

1. *L. monocytogenes* isolates occasionally appearing in meat products and a processing environment were genetically diverse, as they were assigned to different serogroups. The prevalence rate of particular serogroups (and thus serotypes) is not equally distributed among isolates.
2. The IIa serogroup (comprising the 1/2a, 3a serovars) was the most common in our study and comprised 73.9 % of all samples. The IVb serogroup (comprising the 4b, 4d, 4e isolates) was the second most common and comprised 18.3 % of all isolates. The IVb serogroup was also the only one detected in all 10 samples of smoked fish included in our study.
3. Both genoserotyping protocols proposed by Doumith et al. [9] and Feng et al. [10] are useful tools to differentiate *L. monocytogenes* isolates. The methodologies present results that were consistent (in the case of all tested isolates) and complementary, as the protocol by Feng et al. [10] allows to distinguish the 4h serovar from other serovars, whereas the protocol by Doumith et al. [9] separates 7 serovars which fall into one group in the protocol by Feng et al. [10], into three serogroups.

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GENOSEROTYPOWANIE IZOLATÓW *LISTERIA MONOCYTOGENES* POCHODZĄCYCH Z PRODUKTÓW MIĘSNYCH I ŚRODOWISKA PRODUKCYJNEGO

S t r e s z c z e n i e

Wprowadzenie. *Listeria monocytogenes* jest ludzkim patogenem związanym z żywnością oraz czynnikiem wywołujący listeriozę, czyli chorobę o wysokim odsetku śmiertelności. Serotypowanie jest metodą różnicowania izolatów *L. monocytogenes* w oparciu o unikatowe kombinacje antygenów somatycznych (O) i rzęskowych (H) na powierzchni ich komórek. Klasyczne serotypowanie jest wykonywane z wykorzystaniem metod aglutynacyjnych, które wymagają użycia przeciwciał. Istnieją jednak metody genoserotypowania, które pozwalają zakwalifikować izolaty *L. monocytogenes* do poszczególnych grup serotypów (nazywanych serogrupami) na podstawie analiz genetycznych. Różnicowanie izolatów *L. monocytogenes* jest ważnym zagadnieniem w kontekście bezpieczeństwa żywności, kontroli i śledzenia źródeł skażeń. W niniejszej pracy przedstawiamy wyniki genoserotypowania 153 izolatów *L. monocytogenes* pochodzących z produktów mięsnych i środowiska produkcyjnego w polskich zakładach przetwórstwa. Do przeprowadzenia analiz genoseroptyu wykorzystano dwie metodyki: pierwsza pozwala na rozróżnienie czterech najczęściej występujących serotypów (1/2a, 1/2b, 1/2c oraz 4b), natomiast druga pozwala rozróżnić hiperwirulentny serowar 4h od innych serotypów.

Wyniki i wnioski. Otrzymane oboma metodami wyniki były zgodne i wszystkie izolaty zostały zakwalifikowane do odpowiadających sobie serogrup w obrębie obu metodyk. Większość izolatów (73.9 %) została scharakteryzowana jako należąca do serogrupy IIa (reprezentującej serowary 1/2a, 3a). Serogrupa IVb (4b, 4d, 4e) była drugą najbardziej liczną (zawierała 18.3 % izolatów), a następnie IIb (1/2b, 3b, 7) oraz IIc (1/2c, 3c), przy czym ostatnie dwie grupy były równoliczne (i każda z nich zawierała 3.9 % wszystkich izolatów). Żaden z zebranych izolatów nie należał do serogrupy reprezentującej serotypy 4a, 4c, 4ab i 4h.

Słowa kluczowe: serotypowanie, genoserotypowanie, serotypy, bezpieczeństwo żywności, kontrola żywności

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Listeria monocytogenes Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin

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Abstract: Antibiotic resistance is a global health problem, causing not only an increased mortality rate of bacterial infections but also economic losses due to, among other reasons, the need for longer hospital stays. *Listeria monocytogenes* is one of the foodborne pathogens with the ability to induce a serious illness called listeriosis, with approximately 20–30% fatal outcomes. The treatment regimen of listeriosis in humans includes the administration of antibiotics (in most cases, ampicillin or trimethoprim with sulfamethoxazole in case of allergies to β-lactams), so the resistance of this pathogen to antibiotics can potentially lead to increased mortality. The antibiotic sensitivity status of *n* = 153 *L. monocytogenes* isolates originating from meat food samples (raw and processed) and meat-processing environment (both contacting and non-contacting with food) collected between October 2020 and November 2021 in Poland was examined in this study. Susceptibility to antibiotics was determined using the disc diffusion method on Mueller–Hinton agar plates. All collected samples were susceptible to 9 antibiotics: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin (10 IU), streptomycin (10 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), tetracycline (30 μg) and vancomycin (30 μg). Some of the isolates (n = 10; 6.5%) showed reduced susceptibility to ciprofloxacin (5 μg), which was classified as an intermediate response. All these ten isolates were collected from surfaces contacting with food in food-processing facilities.

Keywords: food safety; food pathogens; antibiotics; biodiversity; fingerprinting

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1. Introduction

1.1. Antibiotics, Antibiotic Resistance

Antimicrobials are medicines used to prevent and treat infections in humans, animals, and plants. Antibiotics are one of the types of antimicrobials (among antivirals, antifungals, and antiparasitics) used specifically to prevent or treat bacterial infections [1]. World Health Organization, Geneva, Switzerland (WHO) states that antimicrobial resistance (AMR) is a global health and development threat and a factor of significant economic losses. Not only is AMR a reason for death and disability in affected people, but it also creates the need for longer hospital stays and access to more expensive medicines [1]. In general, antibiotic resistance is a natural phenomenon that occurs when bacteria are exposed to antibiotics, which causes selective pressure. Susceptible bacteria are killed or inhibited, whereas resistant bacteria have greater chances of surviving [2]. Factors responsible for the rapid rise of resistant infections include poor public health infrastructure and a high burden of disease, widespread antibiotic use in animal farming, and the unregulated sale of cheap antibiotics [3].

According to some authors, undertaking a coordinated and comprehensive multinational response to address AMR is critical [4]. Recent systematic analysis of antibiotic

resistance indicated that AMR is an issue in all regions of the world and, as a health problem, it is as large as HIV or malaria, potentially even greater [5]. It was estimated that by the year 2050, as many as 10 million people will die each year due to the rise of drug-resistant infections if appropriate actions are not taken [6]. Although this particular estimation has been quoted repeatedly and has reached more than 3000 citations [7], it was also criticized for insufficient scientific data and inaccuracy [8]. However, critics still claim that the AMR burden is likely to increase over time and that urgent action to overcome AMR is required [8]. Proposed solutions aiming to tackle AMR include: raising global awareness, improving hygiene to prevent the spread of infections, reducing unnecessary usage of antimicrobials, improving global surveillance of drug resistance, promoting rapid diagnostic to cut unnecessary use of antibiotics, promoting development and use of vaccines, improving the numbers and salaries of the people working with infectious diseases, establishing a Global Innovation Fund for early-stage and non-commercial research, promoting investment in new drugs, as well as improving existing ones and building a global coalition for real action [1,6,9–11]. A reduction of nontherapeutic use of antibiotics in animal agriculture is also crucial [12], as there is a direct link between antibiotic use in farms and the spread of antibiotic resistance to human populations [13]. What is more, the practice of using antibiotic formulations licensed for humans in animals should also be addressed in order to prevent the spread of antibiotic resistance between humans and animals [14].

1.2. Listeria monocytogenes and Food Safety

Listeria monocytogenes is a rod-shaped Gram-positive bacterium common in the environment; found in water, soil, and feces. Due to its ubiquitous nature, the existence of this bacterium in processing environments can occur, in some cases leading to bacterial contamination of final food products. *L. monocytogenes* can endure various stresses, such as sanitizers and pH. It can proliferate in refrigeration conditions, as well as survive mild heating (45 °C). In humans, after ingestion of contaminated foods, *L. monocytogenes* can cause life-threatening infections with symptoms such as meningitis, encephalitis, spontaneous abortion, or miscarriage due to its ability to cross the intestinal barrier, the blood–brain barrier, and the fetoplacental barrier. Usually, healthy individuals are not likely to experience severe infections. However, people with compromised immune systems (including the elderly, newborns, and pregnant women) are especially prone to life-threatening outcomes. *L. monocytogenes* is one of the leading causes of death from foodborne pathogens [15–17]. Many sources state that the mortality rate of listeriosis falls between 20% to 30% [17–22]. In the recent prospective observational cohort study, which included 818 cases from 372 centers, authors found that the overall 3-month mortality was 46% for bacteremia and 30% for neurolisteriosis, with only 39% of patients with neurolisteriosis making a full recovery [23].

L. monocytogenes is an organism under the surveillance of institutions such as the Centers for Disease Control and Prevention, Atlanta, Georgia state U.S. (CDC) [24] and European Centre for Disease Control and Prevention, Solna, Sweden (ECDC) [25]. The bacterium caused 2183 cases of illness in the European Union (EU) in 2021, which led to 923 hospitalizations and 196 deaths. The mortality rate in UE in 2021 was 13.7%, similar to that in 2020 (13.0%) and lower than in 2019 (17.6%) [26]. *L. monocytogenes* caused 23 outbreaks in UE in 2021, out of which 8 were classified as high-evidence outbreaks caused by: fish and fish products (4 cases), meat and meat products (3 cases), and broiler meat (1 case) [26]. In the United States, there were 4 identified outbreaks in 2021, caused by Queso Fresco (the outbreak caused 13 illnesses and 1 death), fully cooked chicken (3 illnesses, one death), and packed salads, which caused two independent outbreaks (10 illnesses and 1 death for one outbreak; 18 illnesses and 3 deaths for the second outbreak) [24].

1.3. Listeriosis and Antibiotics

CDC state that most people recover from intestinal illness without antibiotic treatment. Recommendations for individuals outside the high-risk groups include drinking extra fluids in cases when diarrhea occurs. However, antibiotics are needed for patients who have an invasive illness or a greater chance of becoming severely ill [27].

Among antibiotics used to treat listeriosis, ampicillin is typically the antibiotic of choice for human infections [28–32], interchangeably with amoxicillin, according to some sources [28]. Frequently gentamycin is recommended along with ampicillin as a treatment regimen due to its synergistic effects in vivo [28–31]. However, such a combination is not proven to improve patient survival rates [32]. A recent prospective observational cohort study has suggested that combined amoxicillin and gentamicin should be considered the first-line choice in invasive listeriosis [23]. In case of allergies to β-lactam antibiotics, when ampicillin cannot be administrated, trimethoprim with sulfamethoxazole is the generally recommended alternative to ampicillin [30–32]. Erythromycin and vancomycin are also listed as second-line antibiotics [29]. In animal infections, penicillin or oxytetracycline are recommended drugs [33].

1.4. Determination of Antibiotic Resistance in L. monocytogenes

The method typically applied by authors to determine antibiotic resistance of *L. monocytogenes* is disc diffusion [34–43], with differences in the procedures such as exact antibiotics used in the study, the content of the antibiotic in impregnated discs, divergent media, and varying incubation conditions. What is more, authors often decide to apply different criteria for interpreting the results of their experiments in terms of classifying particular strains as resistant, intermediate, or sensitive to antibiotics.

Clinical and Laboratory Standards Institute, Wayne, Pennsylvania state U.S. (CLSI) does not provide any standards for interpreting zones of inhibition achieved with the disc diffusion method of antibiotic susceptibility testing for *L. monocytogenes* [44]. The methodology recommended by CLSI for this organism includes the determination of Minimal Inhibitory Concentration (MIC) in cation-adjusted Mueller–Hinton broth supplemented with lysed horse blood (2.5–5% v/v). Given interpretive criteria include four antibiotics: penicillin, ampicillin, trimethoprim-sulfamethoxazole, and meropenem [45]. However, Bowker et al. presented results of the experiments suggesting that Mueller–Hinton agar with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F) is a suitable medium for antimicrobial susceptibility testing of *L. monocytogenes* for disk diffusion method, as authors have observed a correlation between MIC values and the zones of inhibitions [46]. Because of such observations, European Committee on Antimicrobial Susceptibility Testing, Basel, Switzerland (EUCAST) provides standards for interpreting zones of inhibition achieved in the disc diffusion method of antibiotic susceptibility testing of *L. monocytogenes*. Recommendations include using MH-F and incubation with 5% CO_2 at 35 ± 1 °C for 18 ± 2 h. Criteria for interpreting the resistance status of isolates include five antibiotics: benzylpenicillin, ampicillin iv, meropenem, erythromycin, and trimethoprim-sulfamethoxazole at given disc contents [47].

Even though recent studies of antibiotic resistance of *L. monocytogenes* from food samples are available in the literature, the presented study will provide a positive addition to the information which is already published (even in cases when samples were collected in relatively close areas [41,42]), due to a continuous possibility of emerging changes in antimicrobial resistance patterns of microorganisms. What is more, every microbial collection is unique, and thus every study adds value to the bigger, global picture of antibiotic resistance. Due to these reasons, conducting studies on antibiotic resistance of pathogens repetitively by many researchers is justified and even necessary to monitor potential changes since unknown antimicrobial resistance patterns can be discovered in every study.

2. Materials and Methods

2.1. Bacterial Isolates

Bacterial isolates (n = 153) of *L. monocytogenes* originating from meat food samples (both raw and processed) and meat-processing environment (both being with contact and non-contacting with produced food) collected between October 2020 and November 2021 in Poland were used in the study. Species affiliation of collected isolates was confirmed with two methodologies, namely RFLP-PCR by Paillard et al. (2003) [48] and multiplex PCR by Li et al. (2021) [49]. The exact methodology of the sample collection and identification process is reported elsewhere [50]. Isolates preserved in Brain Heart Infusion (BHI) glycerol stocks stored at −80 °C constituted material for the study.

2.2. Antimicrobial Susceptibility Testing—Disc Diffusion Method

Isolates from glycerol stock were cultured on a BHI agar plate, incubated for 18 h, and subsequently stored at 4 °C for up to two weeks for further use. Control strains used in the study were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. Single colonies from the agar plate were used to inoculate 5 mL of liquid BHI medium (Oxoid) prewarmed to room temperature (20 ± 2 °C) and incubated at 37 °C for 18 h. Inoculated media were then centrifuged, and supernatants were discarded. Sediment was used to prepare 0.5 ± 0.05 McFarland standard suspension in sterile distilled water. A sterile cotton swab was immersed in the prepared solution and used to inoculate (by smearing in three directions) a petri dish with Mueller Hinton Agar (Oxoid). Discs impregnated with antibiotics (Oxoid) were then aseptically placed on inoculated plates (up to 5 discs on one plate) and incubated at 37 °C for 18 ± 2 h. Zones of inhibition were measured after incubation in opened plates and with reflected light and expressed in mm. The singular zone of inhibition was measured three times, and a mean value was calculated. Experiments were done in two separate technical repeats, from which mean values of the inhibition zones were calculated. Antibiotic discs used in the study included: ampicillin (AMP; 10 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), erythromycin (E; 15 µg), gentamicin (CN; 10 µg), penicillin (P; 10 IU), streptomycin (S; 10 µg), sulfamethoxazole/trimethoprim (SXT; 1.25/23.75 µg), tetracycline (TE; 30 µg) and vancomycin (VA; 30 µg). Zones of inhibition were interpreted according to CLSI standards [44] using the interpretive criteria for *Enterococcus* spp. when possible (in the case when the criteria were available for tested antibiotics) and Enterobacterales in case of remaining antibiotics: gentamycin, streptomycin, and sulfamethoxazole/trimethoprim. Microsoft Excel software was used to analyze and visualize the data.

2.3. Fingerprinting—RAPD- and REP-PCR

For selected isolates, RAPD- and REP-PCR fingerprinting techniques were used. RAPD-PCR was performed with OMP-01 primer (5′-GTTGGTGGCT-3′) in the reaction with a final concentration of 0.3 µM primer and 200 µM dNTP, along with 0.3 µL RUN polymerase (A&A Biotechnology, Gdańsk, Poland), compatible RUN buffer at suggested concentration, 1 µL of template DNA and water to achieve the final volume of 10 µL. The reaction temperature profile was as follows: (95 °C for 5 min, 35 °C for 2 min, 72 °C for 1 min) × 45, 72 °C for 10 min [51]. REP-PCR was performed with REP 1R-I (5′-IIIICGICGICATCIGGC-3′) and REP 2-I (5′-ICGICTTATCIGGCCTAC-3′) primers in the reaction with a final concentration of 1 µM of each primer, 200 µM dNTP, 250 µM $MgCl_2$, along with 0.2 µL RUN polymerase, compatible RUN buffer at suggested concentration, 1 µL of template DNA and water to achieve the final volume of 10 µL. The reaction temperature profile was as follows: 95 °C for 5 min (90 °C for 30 s, 40 °C for 1 min, 72 °C for 1 min) × 30, 72 °C for 8 min [52]. PCR reactions were performed in a T-Gradient thermocycler (Biometra, Göttingen, Germany). Separation of amplified products was performed in 2100 Bioanalyzer Instrument (Agilent, Santa Clara, California State U.S.) using compatible DNA 12000 Kit (Agilent), according to manufacturer instructions.

The results achieved with both techniques in the form of generated gel images were connected together in order to create longer separation lines and provide more data. Analysis of aligned gels was performed with GelClust software, v.1.0.0.0 [53] with parameters set as sensitivity; 43, step; 8, error; 3, clustering method; UPGMA and distance coefficient; Jaccard. During the band marking step, manual adjustments were made if necessary.

3. Results

3.1. Antibiotic Susceptibility

Antibiotic susceptibility tests of 153 *L. monocytogenes* isolates (originating from meat products and processing environment) using the disc diffusion method were performed. Among ten antibiotics selected for this study, those commonly used to treat listeriosis were included. Results of performed experiments, along with applied interpretive criteria, are presented in Table 1.

Table 1. Results of antibiotic susceptibility testing of *L. monocytogenes* isolates with disc diffusion method and interpretative criteria applied.

Antibiotic	Sensitive Isolates	Intermediate Isolates	Resistant Isolates
Ampicillin (10 µg)	≥17 mm 153 (100%)	N/A [1] -	≤16 mm 0 (0%)
Chloramphenicol (30 µg)	≥18 mm 153 (100%)	13–17 mm 0 (0%)	≤12 mm 0 (0%)
Ciprofloxacin (5 µg)	≥21 mm 143 (93.5%)	16–20 mm 10 (6.5%)	≤15 mm 0 (0%)
Erythromycin (15 µg)	≥23 mm 153 (100%)	14–22 mm 0 (0%)	≤13 mm 0 (0%)
Gentamicin (10 µg)	≥15 mm 153 (100%)	13–14 mm 0 (0%)	≤12 mm 0 (0%)
Penicillin (10 IU µg)	≥15 mm 153 (100%)	N/A -	≤14 mm 0 (0%)
Streptomycin (10 µg)	≥15 mm 153 (100%)	12–14 mm 0 (0%)	≤11 mm 0 (0%)
Sulfamethoxazole/trimethoprim (1.25/23.75 µg)	≥16 mm 153 (100%)	11–15 mm 0 (0%)	≤10 mm 0 (0%)
Tetracycline (30 µg)	≥19 mm 153 (100%)	15–18 mm 0 (0%)	≤14 mm 0 (0%)
Vancomycin (30 µg)	≥17 mm 153 (100%)	15–16 mm 0 (0%)	≤14 mm 0 (0%)

[1] Not apply.

Almost all (n = 143, 93.5%) collected bacteria were susceptible to all tested antibiotics. None of the isolates was resistant to more than one antibiotic. The only antibiotic to which collected isolates showed reduced susceptibility was ciprofloxacin (5 µg), to which ten isolates (6.5%) were not fully susceptible. However, according to applied criteria, they were not resistant either and were classified as intermediate.

Histograms showing the distribution of inhibition zones in the sample collection are presented in Figure 1. All tested antibiotics, with the exception of ciprofloxacin, present a unimodal distribution of zones of inhibition. In the case of ciprofloxacin, there is a visible non-symmetric bimodal distribution, with a subpopulation of samples (indicated as orange bars in Figure 1C) with reduced zones of inhibition. All ten isolates classified as intermediate in terms of their susceptibility to ciprofloxacin with predetermined criteria are also clearly separated from other samples on the histogram.

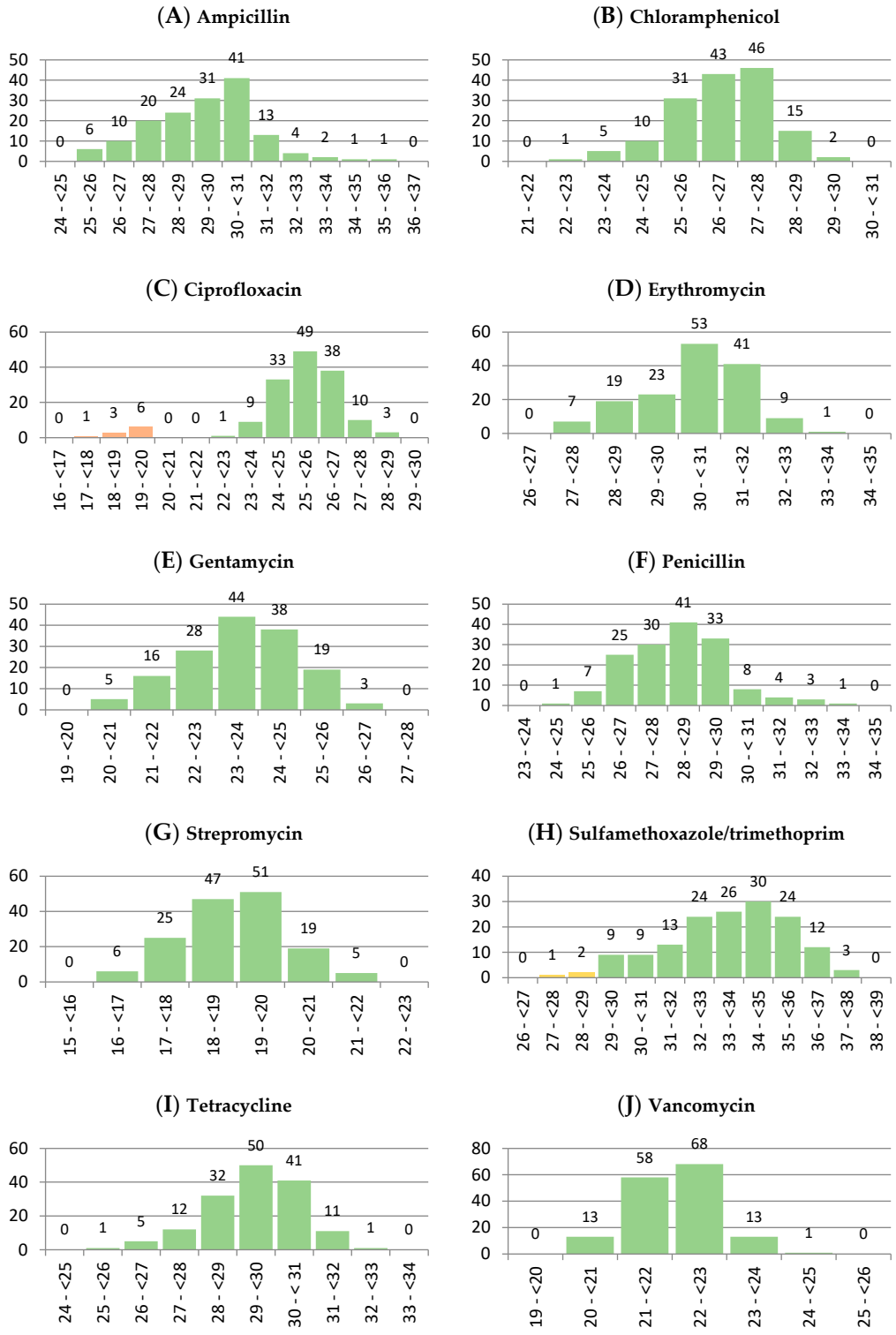


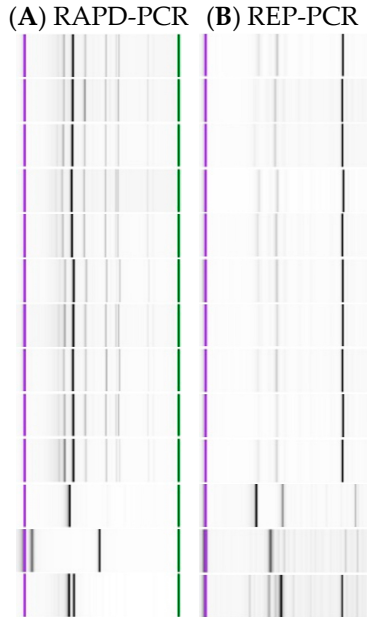
Figure 1. Histograms showing the distribution of inhibition zones (expressed in mm) of the *L. monocytogenes* isolates for the following antibiotics: (**A**): ampicillin (10 µg), (**B**): chloramphenicol (30 µg), (**C**): ciprofloxacin (5 µg), (**D**): erythromycin (15 µg), (**E**): gentamicin (10 µg), (**F**): penicillin (10 IU µg), (**G**): streptomycin (10 µg), (**H**): sulfamethoxazole/trimethoprim (1.25/23.75 µg), (**I**): tetracycline (30 µg), (**J**): vancomycin (30 µg).

All of the ten isolates with reduced susceptibility to ciprofloxacin were collected from the surface swaps of the food-processing environment, including the meat injector (99B), transport box (81), ham wrapper (100A), exit door from the smoking chamber (45), hands

of the worker weighting smoked mackerel fillets (in the smoked mackerel packing hall) (58B), gloves of the worker filleting the mackerel (98B), gloves of the worker working in smoking chambers area (67), swap from three boards (in the smoked mackerel packing hall) (96B), swap from four boxes (in the hall of cutting and packing smoked salmon) (65) and swap from four metal mats (in the hall of cutting and packing smoked salmon) (97B). The first three isolates were sampled on the same day, in the same processing plant, as well as the last seven isolates. All ten isolates present the same genoserotype VIb (representing serotypes 4b, 4d, and 4e), established with Doumith et al. (2004) protocol [54] (data not shown, see reference [50]).

3.2. Fingerprinting Results

RAPD- and REP-PCR fingerprinting of the ten isolates with reduced susceptibility to ciprofloxacin was performed. The patterns of the bands for those samples, as well as three other isolates sensitive to ciprofloxacin, are presented in Figure 2 below.



Isolate	Source of the isolate
99B	meat injector
81	transport box
100A	ham wrapper
45	exit door from the smoking chamber
58B	hands o/t/w [1] weighting smoked mackerel
98B	gloves o/t/w filleting the mackerel
67	gloves o/t/w working in the smoking area
96B	swap from three boards
65	swap from four boxes
97B	swap from four metal mats
289 [2]	marinated herring fillets
280 [2]	floor drain
52 [2]	chicken without viscera

[1] o/t/w/—of the worker; [2] ciprofloxacin sensitive isolates

Figure 2. Patterns achieved with the fingerprinting methods (**A**): RAPD-PCR (**B**): REP-PCR.

Achieved band patterns for the ten isolates with reduced susceptibility to ciprofloxacin are very similar within both methodologies, sometimes with differences in the intensity of particular bands, which may be a measurement error (the Agilent 1200 Kit used in this study has the quantitation accuracy of 25%). All of the bands are discernible in all ten samples, with only slight differences in the product sizing, which also can be caused by a measurement error (the kit used provides ± 15% sizing accuracy). In comparison, patterns for three other isolates, which showed clear differences within their separation lanes, are also included. A phylogenetic tree created with GelClust software is presented in Figure 3.

In the phylogenetic tree, the ten samples with reduced susceptibility to ciprofloxacin are clustered together, with the distance coefficient within the sample group falling between 0.097 in the case of the most similar samples (which originated from a swap from four boxes and gloves of the worker working in smoking chambers area) and 0.245 in case of the samples which are the most distant within this cluster. On the other hand, the three isolates sensitive to ciprofloxacin are clearly separated from the cluster of samples with reduced susceptibility to the antibiotic. The distance coefficient between the two created clusters reached 0.424, which indicates that the samples intermediate to ciprofloxacin are more distinct from the sensitive samples than they are distinct from themselves within their cluster.

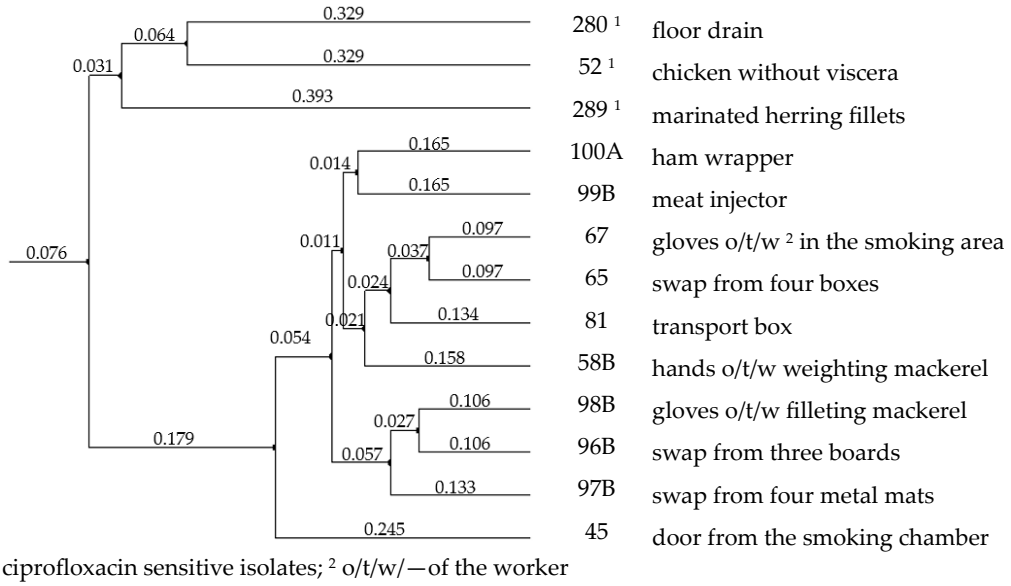


Figure 3. Phylogenetic tree generated based on aligned RAPD- and REP-PCR band patterns of the isolates.

Based on these results, we conclude that all ten isolates with reduced ciprofloxacin sensitivity are probably a contamination of the processing facility, which occurred on food-contacting surfaces during processing.

4. Discussion

Authors apply different criteria to interpret the data achieved with the disc diffusion method when testing antibiotic resistance of *L. monocytogenes*. For example, Ebakota et al. (2018) decided to use the disc diffusion method using the Mueller–Hinton Agar medium and applied criteria as sensitive when the zone of inhibition reached >20 mm, intermediate in case of zones in a range of 15–19 mm and resistant when the zones were ≤14 mm (regardless of the antibiotic used) [40], whereas for example Maurice Bilung et al. (2018), who also used disc diffusion method on Mueller–Hinton agar plate, decided to apply CLSI criteria for staphylococci [34]. Some other authors state that they interpreted the values of zones of inhibition according to CLSI. However, the exact interpretation criteria are not given in the publications [37,39], whereas others apply methodology adapted from CLSI (with non-modified Mueller–Hinton agar medium) and decide to interpret the data in accordance with EUCAST guidelines (regardless of the fact that EUCAST recommends using MH-F medium) [41].

The chosen interpretative criteria applied herein were taken from CLSI guidelines, as a methodology closest to CLSI recommendations was also used in this study. Due to a lack of criteria for *L. monocytogenes*, zones of inhibition dedicated for *Enterococcus* spp. were used and completed with guidelines for Enterobacterales in cases where ranges for *Enterococcus* spp. were not provided. Predetermined criteria did not cause a separation of samples that had an appearance of one population in created histograms. What is more, a subpopulation of samples clearly distinguished on the histogram in the case of ciprofloxacin (Figure 1C) was also separated with criteria adapted from CLSI. Interestingly, in the case of erythromycin and trimethoprim/sulfamethoxazole, the EUCAST recommendations include using the same disc content of antibiotics (15 µg and 1.25/23.75 µg, respectively) [47] as used in this study. For those concentrations, CLSI provides ranges of zones of inhibition of quality control strains [44], which predetermine the choice of the disc content.

Although EUCAST methodology requires using a modified medium (Mueller–Hinton agar + 5% defibrinated horse blood and 20 mg/L β-NAD in contrast to Mueller–Hinton agar), as well as different incubation conditions (with 5% CO_2 in contrast to ambient air), their criteria also seem to be interesting for interpreting the data achieved with the

methodology used in this study. In case of applying those criteria (which are: 25 mm or more for erythromycin and 29 mm or more for trimethoprim/sulfamethoxazole for the isolate to be considered sensitive), the boundary between resistant and sensitive bacteria would be closer (compared to the criteria used herein) to zones of inhibition achieved in this study in case of erythromycin (the lowest zones of inhibition observed in this study are in the range of 27 to 28 mm), but the classification of the strains as sensitive would remain unchanged. However, three isolates (out of 153 tested) would have to be classified as resistant to trimethoprim/sulfamethoxazole (indicated as yellow bars in Figure 1H). Interestingly, two of these three isolates are also on the list of ten with reduced susceptibility to ciprofloxacin. These isolates originate from the hands of the workers weighting smoked mackerel fillets (in the smoked mackerel packing hall) (58B) and meat injector (99B), whereas the third one originates from Vienna type sausage (210) (and has a genoserotype IIa representing serotypes 1/2a, 3a [50]).

However, in the disc diffusion method, even a 1 mm difference in the measurement can result in a different interpretation of the tested isolate as either sensitive or resistant. One should be aware that the results of the experiment may vary, e.g., due to differences in diffusion of the antimicrobial agent in the media caused by variations of conditions such as water activity, not to mention the final measurement error itself. For example, in one study, 128 isolates of *L. monocytogenes* tested against erythromycin presented a range of zones of inhibition between 27 and 38 mm in one laboratory. However, the range widened to 21 to 39 mm when tested at five sites, even using the same methodology. Similarly, in the same study, 126 isolates of *L. monocytogenes* presented ranges of inhibition caused by trimethoprim/sulfamethoxazole varying from 18 to 38 mm in one laboratory with clear separation of the resistant subpopulation from the sensitive strains in the form of bimodal distribution on a histogram, whereas when tested at five laboratories, the distribution of the results widened to the range of 12 to 40 mm with no more clear separation of resistant and sensitive isolates on the histogram, but rather a skewed left distribution was achieved [46]. Even strains recommended by CLSI guidelines to perform quality control experiments are given wide zones of inhibition. For example, *Escherichia coli* ATCC 25922 should create a zone of inhibition ranging from 29 to 38 mm (which is more than 31% difference between the lower value and the upper value) when tested with ciprofloxacin, whereas other control strain, *Staphylococcus aureus* ATCC 25923, tested with the same antibiotic, should create a 22 to 30 mm zone (which is more than 36% difference between the readings of the extremes) [44] and both extreme values still indicate that the experiments are valid. Due to these factors, applying an intermediate range to interpretative criteria is, in our opinion, a justified attempt so that a small difference in a final measurement would not drastically affect the interpretation.

Due to the abovementioned limitations, other approaches in determining antibiotic resistance could be used, including the determination of the minimum inhibitory concentrations, e.g., by broth dilution methods [55,56]. However, the disc diffusion method is still recommended for testing many pathogens by institutions such as CLSI and EUCAST, and it is routinely applied in accredited laboratories worldwide, as well as is referred to in numerous scientific papers. Hence the general value of this methodology should not be neglected.

In terms of comparing results achieved in this study to previous findings, in a recent paper published in 2022, 40 *L. monocytogenes* strains (isolated from food manufactured in Poland and Polish food processing environment) were tested for their sensitivity to 12 antibiotics, using disc diffusion method on Mueller–Hinton agar. Similarly to the results achieved herein, the authors also classified 100% of their samples as sensitive to ampicillin, chloramphenicol, erythromycin, tetracycline, and vancomycin. What is more, the authors identified two isolates (5%) with intermediate sensitivity to ciprofloxacin, which is a very similar rate to achieved herein (which is 6.5%). Five isolates (12.5%) were classified as resistant to trimethoprim/sulfamethoxazole, and one isolate (2.5%) was resistant to penicillin. In the case of gentamycin, one isolate (2.5%) was classified as intermediate [41].

In a different study (published in 2021) from the European Union, namely Italy, out of 98 *L. monocytogenes* samples originating from Italian slaughterhouses, processing plants, and fresh hams produced by these facilities, all isolates were susceptible to vancomycin, ampicillin, gentamicin, penicillin and streptomycin, which is in agreement with our findings. What is more, some of the isolates were also resistant to ciprofloxacin. However, the prevalence of such isolates was higher than in our publication and reached 42 samples (43%). Authors also identified isolates resistant to erythromycin (2 isolates), tetracycline (3 samples), and trimethoprim/sulfamethoxazole (3 samples) [42], whereas herein all isolates were susceptible to these antimicrobials.

In the study from Chile, published in 2022, all 14 tested *L. monocytogenes* isolates originating from various ready-to-eat food products were susceptible to chloramphenicol, erythromycin, penicillin, trimethoprim/sulfamethoxazole, tetracycline, and vancomycin [35], which is in agreement with our results. However, contrary to previously presented papers, all of the isolates were also classified as sensitive to ciprofloxacin. Interestingly, three isolates (21%) in the paper were classified as resistant to ampicillin, out of which two originated from plant-based foods, whereas the third one originated from cooked sausage [35]. The relatively high rate of ampicillin-resistant isolates is contrary to the results of our paper and is especially concerning given the fact that ampicillin is the antibiotic of choice in treating listeriosis.

The prevalence of resistant isolates was also higher than in our report in the case of a study published in 2021, which included 177 *L. monocytogenes* samples isolated in South Africa. Isolates originated from raw seafood, raw meats, and ready-to-eat foods, processing environment, and clinical samples. The authors determined the resistance of the isolates to 5 antibiotics, using the disc diffusion method on MH-F agar. Considering only 157 non-clinical samples included in the study, the authors identified 45 isolates (29%) resistant to erythromycin, 19 isolates (12%) resistant to tetracycline, 16 (10%) isolates resistant to chloramphenicol, and four isolates (3%) resistant to gentamycin [36], whereas all samples included in our study were sensitive to these antibiotics. Although different breakpoint zone criteria were used for interpreting the data in our paper, even a direct adaptation of the criteria from the discussed study would not have changed the results achieved by classifying isolates as sensitive in any case. On the other hand, similarly to our findings, all of the isolates from the discussed study (including clinical ones) were sensitive to ampicillin, which is an antibiotic of choice against *L. monocytogenes,* also in South African clinics and hospitals [36].

Contrary to our report and many abovementioned findings, all isolates (100%) from one study published in 2020 were resistant to ampicillin, as well as to penicillin. The research included 53 *L. monocytogenes* strains originating from imported beef cattle in Jordan. The authors used the disc diffusion method on Mueller–Hinton agar medium complemented with 5% defibrinated sheep blood to determine antibiotic resistance of the samples. Moreover, more than 90% of isolates were classified as resistant to clindamycin, tetracycline, and erythromycin, more than 80% were resistant to quinupristin/dalfopristin and linezolid, more than 70% showed resistance to streptomycin, teicoplanin, kanamycin, vancomycin, and ciprofloxacin, more than half of the isolates were resistant to ceftriaxone and gentamicin, as well as more than 40% of the isolates were resistant to chloramphenicol [43]. The prevalence of resistant and multi-drug resistant isolates is exceptionally high in this discussed paper. However, it is worth mentioning that for both ampicillin and penicillin, the author used $\leq$28 mm as a breakpoint criterium between sensitive and resistant bacteria (in the study, the same disc content of antibiotic was used as in our paper). Adapting these criteria to our study would lead to the reclassification of some isolates as resistant, namely 43 (28%) and 69 (45%) of isolates in cases of ampicillin and penicillin, respectively. However, in the case of chloramphenicol, gentamicin, erythromycin, streptomycin, tetracycline, and vancomycin (in every case, the same disc content of antibiotic was used herein and in discussed publication), a direct adaptation of the criteria set would not have affected the

interpretation of the results. What is more, in the case of ciprofloxacin, the ten isolates that were considered intermediate would still be classified as sensitive.

Although there are many similarities between the results achieved in our paper and the studies from the same country (Poland) examining samples collected in a close timeframe [41], as well as from other European Union country (Italy) [42], in general, lower occurrence of antibiotic resistance was observed in our collection of *L. monocytogenes* isolates than in other presented studies. Additional fingerprinting analyses performed in our report showed similarities within the isolates with an intermediate response to ciprofloxacin. However, further investigation is needed to uncover whether the ten ciprofloxacin intermediate isolates are clones of the same origin or rather a genotype-specific for the ciprofloxacin-intermediate phenotype of *L. monocytogenes*.

5. Conclusions

L. monocytogenes isolates present different patterns of antibiotic resistance in different studies, depending on their source (in terms of types of products) as well as origin (in terms of geographical locations). Many similarities in the results can be observed in this study and in reports from a close area (the same country—Poland), as well as another European Union country (Italy), whereas papers from South Africa and Southwest Asia (Jordan) presented overall higher percentage of resistant isolates. Those area-dependent differences may originate from different practices of using antibiotics regionally (both in animal agriculture and in human medicine), as well as from existing regulations preventing the overuse of antibiotics in some areas. Applying different interpretative criteria to achieved results is also, to some extent, a contributor to differences in antimicrobial patterns presented in scientific papers.

Limitations of our study include shortcomings of the disc diffusion method itself, especially the fact that even a slight difference in the measurement of the zone of inhibition can result in different classifications of the tested isolate as either sensitive or resistant.

The future perspective of our study includes additional investigation to disclose whether isolates with intermediate response to ciprofloxacin are clones of the same origin. More sensitive approaches than applied RAPD- and REP-PCR would include, for example, Pulsed Field Gel Electrophoresis or Whole Genome Sequencing, which would probably provide an answer to this question.

Improving global surveillance of drug resistance is one of the proposed solutions aiming to reduce the negative effects of antimicrobial resistance. Hence conducting research in this area repetitively by many scientists worldwide is crucial to monitor potential changes in antibiotic resistance patterns of pathogens globally.

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Review

Nonhemolytic *Listeria monocytogenes*—Prevalence Rate, Reasons Underlying Atypical Phenotype, and Methods for Accurate Hemolysis Assessment

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Abstract: *Listeria monocytogenes* is a foodborne pathogen that typically presents β-hemolytic activity. However, there are literature reports indicating that *L. monocytogenes* strains are sometimes non-hemolytic or their zones of hemolysis are perceivable only after removal of the colonies from the agar plate. Nonhemolytic *L. monocytogenes* are most commonly encountered in food products, but some have also been detected in clinical samples. Usually, atypical bacteria of this species belong to serotype 1/2a. Mutations of the *prfA* gene sequence are the most common reason for changed phenotype, and mutations of the *hly* gene are the second most common cause. There are also reports that the methodology used for detecting hemolysis may influence the results. Sheep or horse blood, although most commonly used in modern studies, may not allow for the production of clear hemolytic zones on blood agar, whereas other types of blood (guinea pig, rabbit, piglet, and human) are more suitable according to some studies. Furthermore, the standard blood agar plate technique is less sensitive than its modifications such as bilayer or top-layer (overlay) techniques. The microplate technique (employing erythrocyte suspensions) is probably the most informative when assessing listerial hemolysis and is the least susceptible to subjective interpretation.

Keywords: CAMP test; food safety; species identification

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1. Introduction

Listeria monocytogenes is a ubiquitous bacterium that is sporadically found in various food products, both processed and fresh or raw. It is also tolerant to high salt concentrations, low pH and low temperatures with the ability to replicate under refrigeration conditions. Once ingested with contaminated food, the bacteria can cause an illness, called listeriosis, with symptoms ranging from mild gastroenteritis to bacteremia, septicemia, meningitis, and abortions or stillbirths in the case of pregnant individuals [1,2]. Fully developed listeriosis has a mortality rate described as high, relatively higher compared to other foodborne illnesses [1–4]. In a prospective cohort study performed in France, called the MONALISA study, the 3 month mortality rate was estimated to be 46% for bacteraemia and 30% for neurolisteriosis cases [5]. However in other studies, the mortality rate is often lower, for example, it reached 16.8% among patients admitted to Spanish hospitals between 2001 and 2016 [6] or 18.3% among patients administrated to the hospital in a case-control study conducted between 2010 and 2019 in Japan [7]. European Union (EU) case fatality reached 15.6%, 13.6%, and 17.6% in 2017, 2018, and 2019, respectively, which makes listeriosis one of the most serious foodborne diseases under EU surveillance [8]. The EU notification rate of confirmed listeriosis cases was 0.46 cases per 100,000 population in 2019 [8], whereas the death rate from listeriosis was estimated to be approximately 0.13 patients per 100,000 population in a study performed in Spain [6].

β-Hemolysis, which is the ability to completely lyse red blood cells (erythrocytes) [9], is considered a species characteristic of *L. monocytogenes*. It is an important phenotypic

criterion to differentiate *L. monocytogenes* from *L. innocua* [10–14]. Modern books and publications still state that *L. monocytogenes* is β-hemolytic without pointing to possible exceptions [4,15,16]. There are also many publications discussing the diverse aspects of *L. monocytogenes* and, in some of them, the lack of β-hemolysis presented by collected isolates would probably be considered an exclusive criterion, as authors confirmed species identification (apart from testing other traits) with β-hemolytic tests [17–20]. However, Lindbäck et al. (2011) pointed out that *L. monocytogenes* may easily be overlooked when the identification of *Listeria* species is based upon hemolysis, as there are some literature reports discussing atypical *L. monocytogenes* isolates that show not only weak but also complete lack of hemolysis [12,21], and there are atypical *L. innocua* isolates that exhibit hemolysis [22,23]. Furthermore, there are findings suggesting that isolates presenting hemolytic activity may be determined as non-hemolytic, because different methodologies have variable sensitivities [24]. The issue was also addressed many years earlier by authors who suggested that hemolysis of *Listeria* spp. is often weak or questionable, which as a consequence leads to subjective interpretation [25].

The aim of this review was to gather reports on atypical nonhemolytic *Listeria monocytogenes* strains in order to estimate the prevalence rate among diverse samples, present reasons underlying the atypical phenotype, and to summarize and organize reports about techniques used for assessing listerial hemolysis.

2. Hemolytic Phenotype of *L. monocytogenes*

The hemolytic activity of *L. monocytogenes* is determined by hemolysin, specifically listeriolysin O (LLO), which is a pore-forming protein (sometimes referred to as Hly) encoded by the *hly* gene. Upstream of *hly*, there is also a *prfA* gene that encodes the master virulence regulator PrfA, required for, apart from many other genes, *hly* expression [26–28]. In general, nonhemolytic *L. monocytogenes* strains were considered less pathogenic than typical *L. monocytogenes*, owing to the fact that production of LLO was proved to be essential for virulence of these bacteria, as a strict relation between hemolytic activity and virulence potential was found [29]. Moreover, many nonpathogenic isolates show weak or a lack of hemolysis [30,31]. Due to the fact of these reports, a classically hemolytic phenotype was considered to be a virulence marker of *L. monocytogenes* [32,33]. However, listeriolysin is not the only factor involved in virulence [31], and even though to our knowledge it is still the most important virulence agent [27], we now know that during infection *L. monocytogenes* can spread to distant organs even in the absence of LLO expression [27,28]. It was also reported that strains responsible for clinical cases do not always present high hemolytic activity [31], which indicates the importance of monitoring (e.g., in food industry) both hemolytic and nonhemolytic strains of *L. monocytogenes*.

Interestingly, although a diminished hemolytic phenotype may, to some extent, be related to hypovirulence, it does not necessarily indicate that *L. monocytogenes* strains presenting increased hemolytic activity are hypervirulent. In contrast, the self-limiting expression of virulence factors, in the case of *L. monocytogenes*, restricts host cell damage, which prolongs the intracellular life of the pathogen, thereby promoting a persistent infection state [28]. Prevention of excessive LLO-induced cell damage allows to avoid premature destruction of the replicative niche of the pathogen, which is an important aspect of the cytosolic phase of listerial infection [27]. For example, in one study, the authors analyzed *L. monocytogenes* strains in which the virulence potential was attenuated. Within the studied group, there was one strain with higher than wild-type hemolytic activity. That trait alone was a probable cause of virulence attenuation in the case of the isolate. According to authors, overexpression of the hemolysin gene causing excessive host cell membrane damage led to exposure of the bacterium to the extracellular milieu, which subsequently resulted in smaller plaque formation in the plaque assay and a virulence-attenuated phenotype in an animal infection model [34].

3. Nonhemolytic Phenotype of *L. monocytogenes*—Prevalence Rate

As mentioned earlier, *L. monocytogenes*, in general, is considered β-hemolytic, and its hemolytic activity is often used as a criterion to confirm species identification. However, there are findings indicating that nonhemolytic *L. monocytogenes* are also sporadically encountered, especially in samples from food and food-processing environments. Nonetheless, these bacteria were also found in clinical samples.

In a recent study, the hemolytic activity among *L. monocytogenes* was screened by [26]. The authors included 57,820 *L. monocytogenes* isolates, which strongly outnumbers every other report of that type made to date. The prevalence rate of nonhemolytic *L. monocytogenes* isolates according to that study was 0.1% [26]. On the other hand, there are also reports indicating that nonhemolytic *L. monocytogenes* isolates constitute more than 20% and up to 85.7% of all samples collected by other authors [10,21,35–37]; however, in some cases, there is the possibility that bacterial clones (well established in a facility or particular environmental niche) were collected in multiple samples or that the results were overestimated due to the small number of specimens included in the study. Summarized literature reports about nonhemolytic or weakly hemolytic *L. monocytogenes* are presented in Table 1.

Table 1. Nonhemolytic or weakly hemolytic *L. monocytogenes* strains reported in the literature.

Number of *L. monocytogenes* Isolates in the Study	Origin of the Isolates in the Study	Number of Nonhemolytic Isolates (%)	Number of Isolates with Weak Hemolysis (%)	Reference
57,820	Food, clinical, veterinary, environmental, and other	60 (0.1%)	N/A [1]	[26]
1	Dog urinary tract infection	N/A	1 (100%)	[38]
3	Pet food	1 (33.3%)	N/A	[36]
26	Pork, slaughterhouses, markets, and human infections	N/A	6 (23.1%)	[39]
Not specified [2]	Equipment and products from one plant producing smoked salmon	42 (–)	N/A	Yndestad and Hauge (2006), as cited in [21]
38	Seawater, sediment, and shellfish	8 (21.1%)	N/A	[35]
7	Milk products from one manufacturer	6 (85.7%)	N/A	[10]
181	Human clinical, animal clinical, food, and environment	4 (2.2%)	N/A	[40]
12	Smoked fish	1 (8.3%)	N/A	[41]
27 [3]	Meat and poultry or obtained from a culture collection	12 (44.4%)	1 (3.7%)	[37]

[1] N/A—not applied, isolates of that type were not discriminated in the reference; [2] 90 samples were taken, but the precise number of collected *L. monocytogenes* isolates was not specified; [3] only isolates identified as *L. monocytogenes* with both an API system and an ACCU-Probe were included.

Out of 57,820 *L. monocytogenes* samples from various sources (including food, clinical, veterinary, and environmental) collected between 1987 and 2008, 60 were identified as nonhemolytic. Most of the atypical isolates originated from food and food production environments (35 samples) and only three originated from human clinical cases, whereas the rest (22 samples) originated from unknown but nonhuman sources [26]. In another study, where 181 *L. monocytogenes* samples originating from human clinical, animal clinical, food, and environmental sources were included, the authors found four isolates that showed no hemolysis on blood agar plates. Three of them were collected from food sources and the fourth one was the strain type *L. monocytogenes* NCTC 10357 [40].

Examination of 26 *L. monocytogenes* strains originating from pork, slaughterhouses, markets, and human infections revealed that six isolates gave "weak positive" results in

hemolysis assays; however, the authors did not precisely define the criteria for such a designation (other isolates gained either "positive" or "strong positive" results). All six isolates were collected at the same market (four from poultry and two from the floor) within an unspecified time frame by the authors. Furthermore, all isolates belonged to the 1/2a serovar [39], which altogether indicates that it potentially could be one persistent strain established in the facility. Similarly, in one fish processing plant, many nonhemolytic *L. monocytogenes* isolates were detected. Samples from equipment and final products were taken twice with a 6 month time span in between samplings. Out of 90 samples in total, 42 were positive for nonhemolytic *L. monocytogenes* (Yndestad and Hauge (2006), as cited in [21]). There was also a paper in which the authors reported that six out of seven *L. monocytogenes* isolates collected from milk products during routine sampling presented a nonhemolytic phenotype. All milk products were produced by the same manufacturer and all six strains belonged to the same serovar 1/2a [10].

In another report on a strain with a nonhemolytic phenotype that was presumptively persistent in an environmental niche related to marine samples (i.e., seawater, sediment and, shellfish), samples were taken from 18 sites located along an approximately 500 km of the Atlantic coast. Samples were collected over a period of two years. *L. monocytogenes* was present in 38 samples, and in eight cases the isolates were nonhemolytic. All eight isolates with that phenotype (although isolated within a significant time frame) shared the same pulsotypes via pulsed-field gel electrophoresis (PFGE) analysis and all belonged to serovar 1/2a [35].

From the abovementioned reports, it is apparent that nonhemolytic *L. monocytogenes* are most often found in food products and food-processing environments. One paper also reported that *L. monocytogenes* isolates lacking β-hemolysis were found in pet food. The isolate belonged to the 1/2a serotype [36]. On the other hand, there is also a report on *L. monocytogenes* from a diabetic dog with a urinary tract infection. The isolate showed "extremely weak" hemolysis, perceivable only after colonies were removed from the agar plat; the bacterium belonged to the 1/2a serovar [38].

Although most of the nonhemolytic *L. monocytogenes* strains belong to the same serovar 1/2a, it is worth mentioning that this is one of the serovars (along with 1/2b, 1/2c, and 4b) most frequently isolated in general [42–44].

4. Reasons Underlying Diminished Hemolysis

As mentioned earlier, the hemolytic phenotype of *L. monocytogenes* depends mostly on the *hly* and *prfA* genes. However, in the majority of cases, mutations within the *pfrA* gene are responsible for changes in phenotype, whereas *hly* mutations are detected less frequently.

In the largest study to date, performed by [26], which included 60 distinct nonhemolytic *L. monocytogenes* isolates, 56 of the strains had mutations in the central virulence regulator gene—*prfA*. Of those isolates, seven had PrfA protein with amino acid substitution and six had the protein enlarged, whereas in the rest (i.e., 43 strains) the protein was truncated. Two of the strains with mutated PrfA (both of them had a truncated version of the protein) presented mutations also in the *hly* gene, which subsequently resulted in Hly truncation. Three other nonhemolytic strains (without detected mutations in the *prfA* gene) showed mutations in the *hly* sequence: one had a truncated Hly protein and the other two had amino acid substitution in the Hly sequence). One strain presented complete loss of the *hly* gene and, interestingly, that isolate additionally showed no PrfA activity due to the missing *gshF* gene, critical for PrfA activation, even though the *prfA* sequence mutations within the gene of that strain have not been discovered. The research also revealed that atypical nonhemolytic strains are phylogenetically diverse, and the authors concluded that loss of hemolytic activity is caused by independent events across the *L. monocytogenes* population [26].

Mutation within the *prfA* gene was also reported in the case of *L. monocytogenes* strain isolated from a dog with a urinary tract infection, where nucleotide replacement led to substitution of glycine to aspartic acid at residue 145 within the critical helix-turn-helix

motif of PrfA. The authors concluded that further research was necessary to determine whether that mutation was indeed responsible for the reduction in the hemolytic activity of that strain [38]. Similarly, whole genome sequencing of the nonhemolytic isolate originating from pet food revealed a single base pair deletion in the *prfA* gene, which led to truncation of the PrfA protein [36].

All six samples isolated from the same market with "weak positive" results of β-hemolysis tests had mutations in both the *prfA* and *hly* genes. In the *prfA* sequence, a deletion of five nucleotides led to truncation of the PrfA protein. The isolates also presented amino acids substitutions in the Hly protein sequence [39].

There is also a report suggesting that truncation of PrfA (and the subsequent lack of hemolytic activity) can be spontaneously reversible due to the fact of slipped-strand mispairing [21]. Two nonhemolytic *L. monocytogenes* strains were collected in the spring and autumn from the same fish processing plant in Norway and had indistinguishable PFGE patterns. When the atypical strains were injected intraperitoneally into mice, they caused death in 60% of the animals. Interestingly, from both the liver and spleen of all of the deceased mice, the authors recovered *L. monocytogenes* isolates that were both hemolytic and nonhemolytic. The PFGE patterns of the isolates of both phenotypes were indistinguishable from each other and also from the mother strain, which was originally injected into the mice. Sequencing of *prfA* genes was performed in both hemolytic and nonhemolytic strains. It revealed a duplication of seven base pairs in the nonhemolytic strain when compared to the hemolytic strain and control EGDe strain. The duplication changed a reading frame and resulted in a truncated PrfA protein, which led to a subsequent lack of hemolytic activity. The authors concluded that slipped-strand mispairing was a mechanism that resulted in excising the repeat and suggested that the aforementioned mechanism regulated phase-variable expression of virulence in *L. monocytogenes* [21]. Interestingly, a seven bp repeat was also observed in three other strains isolated in France ([31,45,46] as cited in [21]), which suggest that isolates with the 7 bp repeat mutation can potentially be spread worldwide.

On the other hand, there is a report of the spontaneous loss of hemolytic activity in *L. monocytogenes* ATCC 35152 that was originally hemolytic. On blood agar plates, the hemolytic and nonhemolytic colonies occurred in ratios of approximately 3:1 to 2:1 and had stable phenotypes when they were restreaked on fresh blood agar. Investigations in other laboratories supported that, indeed, *L. monocytogenes* ATCC 35152 had two phenotypes [47], further supported by a publication by other authors [48]. However, the molecular mechanism responsible for the switch from a hemolytic to a nonhemolytic phenotype has not been determined.

Apart from Hly and PrfA, it was also demonstrated that cold shock proteins (Csps) are important factors in terms of hemolysin expression and exhibiting a hemolytic phenotype. Although deletions of single *cps* genes did not influence hemolysis on blood agar plates, the *L. monocytogenes* EGDe strain lacking all three listerial Csps (Δ*cspABD*) caused diminished hemolysis on agar blood plates, which was further confirmed by analyses on bacterial supernatants, as the strain caused four-fold less hemolysis than the wild-type strain. Double deletion mutants harboring only either *cspA* (Δ*cspBD*) or *cspD* (Δ*cspAB*) caused less hemolysis than the parental wild-type strain, whereas mutants harboring only *cspB* (Δ*cspAD*) showed a hemolysis phenotype that was not significantly lower or even marginally better compared to the wild-type strain. According to the authors of the study, CspB seems to be the most significant for the hemolytic phenotype, as it is sufficient to maintain wild-type levels of LLO activity and gene expression [49].

5. Impact of Methodology on the Hemolytic Phenotype

Apart from molecular mechanisms responsible for atypical phenotype, there are also methodological details that may influence the results of hemolysis assessment. In general, hemolysis of *Listeria* spp. is often weak or questionable which, as a consequence, may lead to subjective interpretation of the results [25]. Moreover, different methodologies have variable sensitivity [24], and sometimes obtained results may appear elusive, and

they are not always consistent within methodologies. For example, there is a report on *L. monocytogenes* strains that demonstrated variable phenotypes, sometimes appearing as hemolytic and sometimes as nonhemolytic when stabbed on a blood agar plate "with usually no discernible pattern emerging" [50].

There are many protocols designed and used for the assessment of hemolytic activity of *L. monocytogenes* and other *Listeria* species. However, most of these methodologies can be classified into five general types, which then vary in the details. The methodologies used for assessing listerial hemolysis are not systemized in the literature and sometimes referred to with different names. Herein, we aimed to classify the methodologies into groups and summarize the reports on their effectiveness.

5.1. Hemolysis Assays

5.1.1. Blood Agar Technique

The most basic method is the blood agar technique, currently most often applied by authors who perform hemolysis assays of *Listeria* spp. [21,23,26,35,38,39,49,51,52]. Blood or blood cells are added to agar medium and, thus, blood is equally distributed on the agar plate. After solidification of the medium, bacteria are streaked on the agar surface, incubated, and screened for hemolytic zones. The incubation time is usually 24 h [26,47,49–51,53], sometimes prolonged to 48 h [21,26,47,50] at temperatures ranging from 35–37 °C [21,26,38,47,49,51,53]. Usually, Columbia blood agar plates are used [23,26,38,49], but in earlier studies, authors reported using Tryptic soy agar [30,50,53] or other media, e.g., heart infusion agar [47]. The concentration of defibrinated blood is usually 5% (*v*/*v*) in the agar medium [30,38,39,47,53,54].

Positive control strains *L. monocytogenes* CLIP 74910 [23,26] and *L. monocytogenes* 10403S [52] as well as negative control strains *L. innocua* CLIP 74915 [23,26] and *L. innocua* ATCC 33090 [52] were used in some studies.

The methodology allows for not only reading the results as positive or negative, but the authors were able to differentiate (e.g., strong, moderate, weak, or very weak) hemolysis based on the hemolytic zone size [39,55].

However, previous studies showed that it was sometimes necessary to remove bacterial colonies from the agar surface in order to see hemolytic zones that appeared only in the contact area [38,55], although one author, who also observed the phenomenon, decided to refer to those types of colonies as nonhemolytic as opposed to those giving clear zones of hemolysis around bacterial colonies [47]. To prevent achieving that type of equivocal results and to provide a clear-cut reading of hemolytic activity, a modification of the standard method was proposed. The methodology requires the use of an exceptionally thin layer of blood agar medium (8 mL) and inoculating the plate on a small area with a heavy cell mass. The incubation time is shorter than normal and lasts 6 h [56]. Utilization of a thinner layer of blood agar medium allowed for observation of a narrow zone of hemolysis around colonies of *L. monocytogenes*, which previously gave ambiguous results in hemolytic activity assays [56].

5.1.2. CAMP Test

Another method to assess the hemolytic activity of *Listeria* spp. is to use the CAMP test, which similarly to the aforementioned technique, requires the use of blood agar plates. The test was based on the principle that hemolysis of *L. monocytogenes* is enhanced in the vicinity of *Staphylococcus aureus* and precisely its β toxin [13,57]. The name "CAMP test" finds its origin in the first letters of the original authors (Christie, Atkins, and Munch-Petersen) of the note about the phenomenon of *Staphylococcus* β toxin, which showed the ability to enhance or induce hemolysis around colonies of some *streptococci* isolates on blood agar plates [58,59].

Darling (1975) [59] proposed a standardization of the CAMP test procedure. He recommended using sheep blood agar plates on which β-toxin-producing *S. aureus* is streaked in a straight line across the center of the plate. Then, strains of the microorganisms to be

tested are streaked in straight 2–3 cm lines at a right angle to the *S. aureus* streak but with caution not to touch the *Staphylococcus* line. Plates are incubated at 37 °C. Enhancement of the hemolysis of the tested isolate in the vicinity of *S. aureus* and the appearance of the "arrowhead" shape of hemolysis, pointing towards the *Staphylococcus* streak, indicate positive CAMP test results [59]. Currently, when *Listeria* isolates are tested, it is proposed to make two parallel streaks of *S. aureus* and additionally *Rhodococcus equi* with an approximately 3–4 cm space between the lines. Then, tested isolates are inoculated with a streak at a right angle to both previously made lines, but not touching them (leaving approximately a 1–2 mm space between streaks). *L. monocytogenes* hemolysis is enhanced by *S. aureus*, but *L. innocua* hemolysis is enhanced by *R. equi*, which allows for species differentiation [13,16]. There are also variations of the CAMP test, where commercially available discs soaked with *Staphylococcus* β toxin are used instead of the streaking bacterial culture of *S. aureus* [13,37].

Because of the enhancement of hemolysis exhibited by *L. monocytogenes*, a CAMP test with *S. aureus* can sometimes be used to resolve questionable hemolysis results [15]. However, the CAMP test may also generate false-positive and false-negative results due to the subjectivity of the interpretation [60] and provide ambiguous "plus–minus"-type reactions [37]. Some scientists proposed using other techniques (especially the microplate technique) as a reliable methodology to assess the hemolytic character of *Listeria* [60] or to change the type of blood to guinea pig which, according to authors, renders hemolysis-enhancing methods, such as the CAMP test, unnecessary [48].

5.1.3. Top-Layer (Overlay) Technique

The top-layer technique, also referred to as the red blood cells top-layer technique [24] or overlay technique [61] has been reported as a more sensitive alternative for the standard blood agar technique [61]. This methodology was originally designed for detection of listerial hemolysis directly on selective plating media, which simplifies and rationalizes screening for *L. monocytogenes* colonies among those that grow on selective plating media [61,62].

In this technique, selective plating media (without blood addition) are streak inoculated with bacteria and incubated at 37 °C for 48 h. After incubation, plates are maintained at 4 °C for 2 h, and then a thin (8 mL) top layer of nonselective (BHI) medium with red blood cells is gently poured on the top of the base layer. Plates are then incubated again for 14 h at 30 °C and screened for hemolysis [61,62].

Other authors reported also using nonselective media as a base layer (namely, a brain heart infusion (BHI) agar or Columbia agar base, either with or without the addition of potassium tellurite) [24,32], increasing the top-layer volume up to 15 mL [32] and changing the conditions of second incubation to 24 h at room temperature [32].

There is also a variation of the top-layer technique where wells are made in the base layer using a drill bit, filter-sterilized bacterial culture supernatants are then introduced into the wells (in portions), and plates are stored to allow supernatant absorption. After a given time, the non-absorbed supernatant is removed, and a top-layer is added. Plates are then incubated and hemolysis is expressed as a diameter of the zones of hemolysis [32].

With the top-layer technique, *Listeria* gave much clearer hemolytic areas on every tested selective plating medium using sheep red blood cells compared to when sheep red blood cells or sheep blood were incorporated into the base medium as traditionally performed [61,62]. *Listeria* agar selective medium modified (LSAMM) was suggested to be the most suitable selective plating medium to be used with the overlay technique, as the hemolytic zones that were big, sharp, and easily recognizable [61,62].

When nonselective agar media were employed as a base layer, *L. monocytogenes* displayed bigger zones of hemolysis on BHI agar than on Columbia agar base when sheep blood was used [32], which was also confirmed in other study [24]. Hemolytic zones on both types of media with horse or human blood were comparable [24].

Similar to the standard blood agar technique, this method allows to estimate the hemolytic power of the isolates. The authors were able to differentiate (e.g., strong or

moderate) hemolysis [24]. However it was sometimes necessary to remove the colony from the medium to perceive hemolysis that occurred only in a contact area [24].

5.1.4. Bilayer Technique

The bilayer technique (sometimes referred to as the "blood agar" technique [24]), similarly to the top-layer technique, requires using plating agar media that consists of two layers: the base one without the addition of blood and the top one that contains red blood cells. The difference between the bilayer and top-layer methods is that in the bilayer technique, bacteria are inoculated on the top of the second layer.

The plate, as originally designed, consists of one layer (10 mL) of Columbia blood agar base (without blood) and a 5 mL top layer of the same agar supplemented with 5% horse blood [63]. Bacterial colonies are surface inoculated and incubated overnight (16–24 h) at 35 °C with 5% CO_2 [63].

Modifications of the procedure proposed by other authors include using more medium (15 mL) in the first layer [12], using less (4.5 mL) [48] or more (8 mL) [12] medium in the second layer, employing other types of base media [12,24] as well as other types of blood (sheep, guinea pig, or human) [24,48], and performing aerobic incubation [12,24,48] at 37 °C [48] for up to 48 h [12,24,48].

According to authors of original methodology, β-hemolysis zones of *Listeria* are not perceivable on bacterial colony counters with that technique, but they can be seen by tilting the plate at an angle oblique to a fluorescent desk lamp [63]. However, other authors did not report using a fluorescent desk lamp to read the results [12,24,48].

According to the authors that compared nine different media, blood agar base no. 2 and Columbia blood agar base were the best choices for detecting the hemolytic activity of *L. monocytogenes* when employing a bilayer technique [12].

Similar to the standard blood agar technique, this method allows to estimate the hemolytic power of the isolates. The authors were able to differentiate (e.g., strong or moderate) hemolysis [24]; however, it was, in some cases, necessary to remove the colony from the medium to determine the hemolytic activity [48].

5.1.5. Microplate Technique

The microplate technique (called also the microfuge-tube assay [37], microwell hemolysis test [48], or micro-technique [55]) was proposed as an alternative to other methods aiming to determinate listerial hemolytic activity. The methodology employs erythrocyte suspensions and eliminates interpretation difficulties that may be encountered when assessing hemolysis produced by *Listeria* spp. on blood agar plates [55]. It was originally used to determine the activity of purified *L. monocytogenes* hemolysin [64] and not the hemolytic activity of bacteria or bacterial cell supernatants, but other authors have employed the test for these purposes.

The technique is based on preparing serial dilutions (usually in a 96-well microtiter plate [24,31,45,49,50,55,65]) of bacterial cultures [24,34,55], bacterial supernatants [66–68], or bacterial filtrates [31,45,49,50,65] and then adding a standardized amount of blood or red blood cells suspensions to each dilution. Some authors use bacterial suspensions or bacterial cultures that have a standardized optical density (OD) before the hemolysis assessment [34,49,65,66]. Blood addition is followed by incubation, which is significantly shorter than in other methods, and lasts 30 min [69], 40 min [37,49], 45 min [24,66], 1 h [34,50,64], 3 h [31,45,65], 6–8 h [55], or 8 up to 10 h [67,68] depending on the author's choice. The incubation temperature is 37 °C for the majority of protocols [24,31,34,45,49,55,64–69].

After incubation (and sometimes centrifugation of well contents [31,37,45,65,66]), hemolysis is measured. Usually, it is performed by visual scoring [34,50,55,64,67,68], but it can also be assessed with a spectrophotometer [49,65,66].

Hemolytic activity is usually expressed in complete hemolytic units (CHUs), which are defined as the reciprocal of the highest dilution at which complete hemolysis occurred [34,55,64,69]; however, some authors decide to also determine the minimal hemol-

ysis unit (MHU), which is defined as the reciprocal of the highest dilution at which any hemolysis was detected [55,68]. There are also hemolytic units (HUs) based on the reciprocal of the highest dilution at which at least 50% hemolysis of the erythrocytes could be observed [31,45,50,66]. In the case of absorbance measurements, the results can be expressed as a percent of hemolysis in relation to a negative control which is set to 100% [49]. In one case, hemolytic units were expressed as a percentage of the hemolysis of the bacterial control strain (10403S) [34].

In some experiments based on this technique, the authors reported using additives that enhanced listerial hemolysis. Treating horse red blood cells with crude exosubstances of *S. aureus*, *R. equi*, *Pseudomonas fluorescens*, or *Acinetobacter calcoaceticus* prior the microplate assay led to enhancement of the hemolytic activity of *L. monocytogenes* isolates, which was expressed in an increase of CHUs or MHUs or both of the parameters [55]. Similarly, potassium tellurite in a range of concentrations from 0.004% to 0.02% added to the media enhanced hemolysis presented by *L. monocytogenes* cultures [32], as well as charcoal-treated broth utilization (compared to untreated medium) led to approximately 10 times higher hemolytic activity of *L. monocytogenes* strains [66].

Some authors reported using positive and negative controls with this technique. *L. innocua* ATCC 33090 [37], phosphate buffer saline used instead of bacterial supernatant [49], or uninoculated medium [65] served as negative controls. With *L. monocytogenes* NCTC 7973 [37], sodium dodecyl sulfate (0.1%) used instead of bacterial culture [65] or blood samples fully hemolyzed by 0.05% Triton X [34] served as positive controls.

The methodology also has a simplified version that does not require preparation of serial dilutions. Bacterial suspensions are directly placed in one well or tube and a standardized amount of blood is added. The degree of hemolysis is assessed after incubation. It allows for the differentiation (e.g., complete, strong, and moderate) hemolysis or negative results [24,55].

This methodology provides unequivocal results, which allow to clearly differentiate between weak and strong hemolytic strains [55], as the results can be presented in comparable units. This methodology provided clear-cut readings in cases where the CAMP test gave ambiguous results [37], and even the simplified version of the microplate technique was assessed as a reliable, simple, and fairly rapid method for clearly differentiating between hemolytic and nonhemolytic *Listeria* strains [24].

5.2. Blood Type Impact

The origin of red blood cells (RBCs) may have an impact on the results of hemolytic assessments. Literature reports are not consistent with the findings regarding which blood type is the best for those types of studies. The authors of more modern experiments usually use either horse [26,51,70] or sheep [38,39,52] RBCs when testing the hemolysis of bacteria from the *Listeria* genus; however, some authors decide to use alternatives such as bovine [49] or human [65] blood.

In one study, *L. monocytogenes* 15313 gave clear hemolytic zones when human (types A+, B−, AB−, O+), rabbit, piglet, and chicken blood types were used; on bovine blood, it gave a weak response, whereas no hemolytic reaction was observed with sheep or horse blood [71]. The lack of β-hemolysis of these strains on agar containing sheep or horse blood was also reported earlier by other authors [72]. In another report, *L. monocytogenes* isolates originating from food produced clear zones of hemolysis on guinea pig blood agar, while lytic zones on sheep blood agar did not extend beyond the edge of the colonies and could only be confirmed after removal of the colonies. According to the authors, zones of β-hemolysis on guinea pig blood are clearer than on other (i.e., cow, horse, sheep, and rabbit) types of tested blood [48].

In other paper comparison of RBCs types led to conclusion that hemolytic activity of analyzed isolates was stronger in the presence of RBCs from sheep or guinea pig compared to horse and human, where lytic zones were very small or even questionable [24]. Blood type had impact on the results only when some techniques were used (namely bilayer

technique or top-layer technique with selective media), whereas in other (microplate technique or top-layer technique with nonselective media) blood type had little or no impact [24].

Abovementioned findings are not in agreement with the paper in which *L. monocytogenes* strain SO93 had no hemolytic activity when sheep RBCs were used, but exhibit hemolysis on horse RBCs [45]. Similarly 24 *L. monocytogenes* strains were found to present stronger hemolytic activity on media containing horse blood than on those containing sheep blood [12].

6. Summary and Concluding Remarks

Although *L. monocytogenes* is considered β-hemolytic microorganism, there are reports about isolates of atypical phenotype. The largest study performed to date determined that prevalence rate of nonhemolytic *L. monocytogenes* reaches approximately 0.1% [26]. Nonhemolytic isolates are encountered in food and food processing environments in majority of cases, however some clinical isolates also presented diminished hemolysis. Usually weak or lack of hemolysis is caused by mutations within central virulence regulator *prfA* or hemolysin *hly* genes. There are however still some unexplained reports regarding nonhemolytic phenotype, e.g., in case of *L. monocytogenes* ATCC 35152 strain which spontaneously lost hemolytic activity [47].

Hemolysis of *L. monocytogenes* is sometimes difficult to assess and many authors have dedicated their time to eliminating ambiguous results that were obtained during the tests. Although many publications present inconsistent findings regarding, for example, medium that enables the easiest interpretation of the results or the most accurate blood type, there are many valuable protocols to be considered when testing the hemolytic abilities of *Listeria* spp. isolates. The microplate technique appears to be the least susceptible to subjective interpretation and provides results expressed in units that can be easily compared within specimens.

Due to the reports regarding sporadic nonhemolytic *L. monocytogenes* isolates (as well as sporadic hemolytic *L. innocua*) and considering the limitations of the methodologies available for assessing the listerial hemolytic phenotype, we conclude that species identification based on hemolytic abilities should be performed with exceptional caution in the case of the *Listeria* genus. Potential overlooking of *L. monocytogenes* isolates may, for example, lead to life-threatening infections in the case of food samples, as there are reports about weakly hemolytic isolates that caused clinical infections, and there are publications addressing the issue of isolates that switch their phenotype. However, rejection of nonhemolytic specimens from scientific studies addressing diverse aspects of *L. monocytogenes* is also very concerning, and it may be a reason for the possible underrepresentation of the isolates of that type in many publications.

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High Prevalence of Virulence-Associated Genes and Length Polymorphism in *actA* and *inlB* Genes Identified in *Listeria monocytogenes* Isolates from Meat Products and Meat-Processing Environments in Poland

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Abstract: *Listeria monocytogenes* is a human pathogen that has the ability to cause listeriosis, a disease with possible fatal outcomes. The typical route of infection is ingestion of the bacteria with contaminated food. In this study, 13 virulence-associated genes were examined with PCR in the genomes of 153 *L. monocytogenes* isolates collected from meat products and processing environments in Poland. All isolates possessed genes from LIPI-1—*hly*, *actA*, *plcA*, *plcB* and *mpl*—as well as four internalins: *inlA*, *inlB*, *inlC*, *inlJ*. Invasion-associated protein *iap*, as well as genes *prfA* and *sigB*, encoding regulatory proteins, were also detected in all isolates. Gene *flaA*, encoding flagellin, was detected in 113 (74%) isolates. This was the only gene that was not detected in all isolates, as its presence is serotype-dependent. Gene *actA* showed polymorphism with longer and shorter variants in PCR amplicons. Two isolates were characterized by truncated *inlB* genes, lacking 141 bp in their sequence, which was confirmed by gene sequencing. All isolates were positive in hemolysis assays, proving the synthesis of functional PrfA and Hly proteins. Four genotypes of *L. monocytogenes* based on *actA* polymorphism and two genotypes based on *inlB* polymorphism were distinguished within the isolates' collection.

Keywords: food safety; gene mutation; virulence; invasiveness; genotyping

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1. Introduction

Listeria monocytogenes is a Gram-positive, facultatively anaerobic rod adapted to various conditions. These ubiquitous bacteria are found throughout the environment. Many domestic animals, especially ruminants, are carriers of *L. monocytogenes*, which leads to the contamination of animal breeding areas, with the subsequent possibility of food contamination. The ingestion of *L. monocytogenes* with contaminated food may result in self-limiting gastroenteritis occurring with fever and diarrhea. Although usually listeriosis is mild, in severe cases symptoms include sepsis, meningitis, encephalitis, and spontaneous abortion. Overall mortality risk is estimated to be approximately 15% or higher, depending on patient status and comorbidities [1–6].

L. monocytogenes is an invasive intracellular pathogen. Its virulence depends on many adhesion and invasion factors that facilitate gastrointestinal tract colonization and crossing of the intestinal barrier [7]. Virulence factors also include proteins facilitating dissemination in the host, including brain and placenta colonization, cell-to-cell spread, the adhesion, and invasion of macrophages and escape from *L. monocytogenes*-containing vacuoles. Proteins enabling survival in the intestines, such as acid and bile tolerance proteins, are also virulence factors. The most important virulence-associated genes of *L. monocytogenes* are localized on Listeria pathogenicity island-1 (LIPI-1), namely, *hly*, *actA*, *plcA*, *plcB* and *mpl* [5,7–9]. All

virulence-associated genes from LIPI-1 are positively regulated by a pleiotropic transcriptional regulator PrfA (encoded by the *prfA* gene), which is considered the main positive regulatory factor of virulence genes in *L. monocytogenes* [9–11].

Other groups of key listerial virulence factors, outside of LIPI-1, include internalins. The most important ones are *inlA* and *inlB*, but many have been identified (including *inlC*, *inlJ*, *inlH*, *inlK*, *inlL*, *inlF* and *inlP*) [8,9,12,13]. Other known listerial virulence factors include, for example, invasion-associated protein (encoded by *iap*), flagellin (encoded by *flaA*), a general stress-response regulator, called sigma factor B, (encoded by *sigB*), Listeria-mucin-binding invasin A, bile salt hydrolase, and cell invasion LPXTG protein, ClpP, a heat shock protein that is involved in intracellular growth or fibronectin-binding protein [7,8,10,12], to just name a few. However, not all *L. monocytogenes* isolates harbor all discovered virulence genes [14–16].

A species-specific characteristic of *L. monocytogenes*, historically considered to be a virulence marker, is beta-hemolytic activity [17]. This trait is often used to confirm the species identification of the isolates [4,18,19]. However, there are also reports about isolates of this species that do not present hemolytic phenotype, mainly due to mutations within either the *hly* gene, or, more frequently, *prfA* mutations [20]. Furthermore, it has been suggested that the spontaneous loss of virulence in natural populations of *L. monocytogenes*, although rare, is possible due to the fact that some of the virulence genes are under purifying selection. This opens an evolutionary path for potential saprophytism for this pathogen [20]. Hence, tracking potential changes in the virulence-associated genes patterns in food isolates of *L. monocytogenes* is important, as a trend of reducing pathogenicity in this genus may be observed.

2. Aim of This Study

The aim of this study was to assess the diversity of *L. monocytogenes* isolates collected in recent years in Poland originating from meat products and meat processing facilities. The presence or absence (or polymorphic form) of selected virulence-associated genes was assessed and the hemolytic phenotype of those isolates was determined. An estimation of the virulence potential of the collected isolates based on the obtained results was an additional aim of this study.

3. Materials and Methods

3.1. Bacterial Isolates and Genetic Material

A collection of 153 *L. monocytogenes* isolates used in this study originated from both raw meat samples and processed meat products manufactured in Poland (n = 108), as well as from meat processing plants in Poland (n = 45), representing food processing and environmental surfaces. The isolates were collected between October 2020 and November 2021. The DNA of those microorganisms used for gene detection was isolated using a Genomic Mini kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. Bacterial isolates preserved in brain heart infusion broth (BHI; Oxoid, Warsaw, Poland) glycerol stocks stored at −80 °C were used for hemolysis assays. Isolates included in the study were confirmed as *L. monocytogenes* species with two separate genetic analyses, using a PCR-RFLP according to Paillard et al. (2003) [21] and multiplex PCR according to Li et al. (2021) [22] protocols. Details regarding the isolates' collection process, DNA isolation procedure, and the exact methodology for species affiliation were reported previously [23].

3.2. Detection of Virulence-Associated Genes

The presence of thirteen virulence-associated genes in genomes of *L. monocytogenes* isolates was analyzed in the study, using standard PCR for most of the genes or multiplex PCR for the simultaneous detection of the two genes *inlB* and *inlC*. If an amplicon for any gene was not detected in a multiplex reaction, then separate PCRs using only one pair of primers were performed for verification. If discrepancies occurred between multiplex and

singleplex, only the singleplex results were taken into account. Additionally, two pairs of primers (here referred to as *actA1* and *actA2*) were used to analyze the presence of the *actA* gene.

Reaction mixtures contained 0.2U of RUN polymerase (A&A Biotechnology), along with compatible reaction buffer at the recommended concentration, 0.2 mM of each dNTPs (A&A Biotechnology) and primers (concentrations are given in Table 1), which were ordered in Genomed S.A. (Warsaw, Poland). DNA was added in the amount of 10 ng per reaction. Reactions were performed in a final volume of 10 µL. Thermal cycling was performed in the T-Gradient thermocycler (Biometra, Göttingen, Germany) with annealing temperatures, as presented in Table 1. PCR products were separated by electrophoresis in an agarose gel-containing ethidium bromide and visualized using Gel Doc Imaging System (Bio-Rad, Hercules, CA, USA).

Table 1. Sequences of primers used in the study and cycling condition information.

Primers Name	Target	Primers' Sequence	Primer Concentration [µM]	Annealing Temperature [°C]	Amplicon Length (bp)	Reference
prfA	Listeriolysin positive regulatory protein	F: 5′-GATACAGAAACATCGGTTGGC-3′ R: 5′-GTGTAATCTTGATGCCATCAGG-3′	0.3	49	274	[24]
sigB	Sigma factor	F: 5′-TCATCGGTGTCACGGAAGAA-3′ R: 5′-TGACGTTGGATTCTAGACAC-3′	0.35	51	310	[25]
plcA	Phosphatidylinositol-specific phospholipase C	F: 5′-CTGCTTGAGCGTTCATGTCTCATCCCCC-3′ R: 5′-CATGGGTTTCACTCTCCTTCTAC-3′	0.5	60	1484	[26]
plcB	Phosphatidylicholin-specific phospholipase C	F: 5′-GCAAGTGTTCTAGTCTTTCCGG-3′ R: 5′- ACCTGCCAAAGTTTGCTGTGA-3′	0.5	55	795	[27]
hly	Listeriolysin O	F: 5′-GCAGTTGCAAGCGCTTGGAGTGAA-3′ R: 5′-GCAACGTATCCTCCAGAGTGATCG-3′	0.3	62	456	[28]
actA1	Actin polymerization protein	F: 5′- CGCCGCGGAAATTAAAAAAAGA-3′ R: 5′- ACGAAGGAACCGGGCTGCTAG-3′	0.4	62	839 (or 950)	[29]
actA2	Actin polymerization protein	F: 5′-GACGAAAATCCCGAAGTGAA-3′ R: 5′-CTAGCGAAGGTGCTGTTTCC-3′	1.0	63	268 (or 385)	[30]
mpl	Metalloprotease	F: 5′-GGCTCATTTCACTATGACGG-3′ R: 5′- GCTTCCCAAGCTTCAGCAACT-3′	0.5	60	143	[27]
inlA	Internalin A	F: 5′-ACGAGTAACGGGACAAATGC-3′ R: 5′-CCCGACAGTGGTGCTAGATT-3′	0.5	55	800	[31]
inlB	Internalin B	F: 5′- CATGGGAGAGTAACCCAACC-3′ R: 5′- GCGGTAACCCCTTTGTCATA-3′	0.75	57	500	[32]
inlC	Internalin C	F: 5′- CCCACAATCAAATAAGTGACCTT-3′ R: 5′- CTGGGTCTTTGACAGTATTTGTT-3′	1.25	57	400	[32]
inlJ	Internalin J	F: 5′-TGTAACCCCGCTTACACAGTT-3′ R: 5′-AGCGGCTTGGCAGTCTAATA-3′	0.5	55	238	[31]
iap	Invasion associated protein	F: 5′-ACAAGCTGCACCTGTTGCAG-3′ R: 5′-TGACAGCGTGTGTAGTAGCA-3′	0.3	56	131	[33] *
flaA	Flagellin	F: 5′-TTACTAGATCAAACTGCTCC-3′ R: 5′-AAGAAAAGCCCCTCGTCC-3′	1.0	54	538	[34]

* referred to as presumptive β-hemolysin gene in Reference [33].

At least one randomly chosen sample representing one amplicon size amplified with one primer pair was sequenced. PCR products were purified prior to sequencing directly from the PCR mixture or after separation in an agarose gel, using the A&A Biotechnology kits, with either Clean-Up Concentrator or Gel-Out Concentrator. Sequencing was performed by Genomed S.A. Obtained sequences were determined to be fragments of genes of interest using Blast software (BLASTN 2.14.1+) [35,36].

In the case of *inlB* gene, in order to obtain a full-length sequence for more reliable reads, sequences achieved with the forward primer were aligned with the sequence achieved using the reverse primer. BLAST [37] was used for this purpose. The obtained aligned

sequences (for isolates 50 and 235) were compared with each other as well as with the *inlB* gene sequence of *L. monocytogenes* EDG-e from the GenBank database (accession number NC_003210.1), also using BLAST.

3.3. Hemolysis Assay

Hemolysis assays were performed on a commercially available Columbia Agar with 5% Sheep Blood (Becton, Dickinson and Company, Heidelberg, Germany). Bacterial isolates from glycerol stocks were streaked into BHI agar plates and incubated for 18 h at 37 °C. After incubation, bacteria were re-streaked from BHI to the blood agar plate and incubated at 37 °C for 24 h and 48 h, with assessment after both time periods. *Listeria ivanovii* ATCC 19119 was used as a positive control and *Listeria innocua* food isolates 135, 145, and 159 were used as negative controls.

4. Results

4.1. Presence of Virulence-Associated Genes

In the case of ten tested virulence-associated genes, namely, *iap*, *sigB*, *prfA*, *hlyA*, *inlC*, *inlA*, *inlJ*, *plcA*, *plcB*, and *mpl*, all of the *L. monocytogenes* isolates included in the study were positive in PCR with amplicons of expected length, in accordance with the literature references. The results were further confirmed with sequencing and BLAST analyses. An amplicon of the *flaA* gene was present in 113 (74%) isolates, all of which belonged to genoserotype IIa (representing serotypes 1/2a, 3a) (results reported previously; see Reference [23]). Furthermore, all isolates representing genoserotype IIa harbored the *flaA* gene [23].

In the case of actA and inlB genes, polymorphism of amplicon sizes was detected within the isolates' collection. Summarized virulence detection results are presented in Table 2.

Table 2. Summarized results of virulence genes' detection.

Primer Name	Approx. Amplicon Size (bp)	Number of Isolates (%)
prfA	274	153 (100%)
sigB	310	153 (100%)
plcA	1484	153 (100%)
plcB	795	153 (100%)
hlyA	456	153 (100%)
actA1	839 950 no amplicon	15 (10%) 120 (78%) 18 (12%)
actA2	268 385	28 (18%) 125 (82%)
mpl	143	153 (100%)
inlA	800	153 (100%)
inlB	500 360	151 (99%) 2 (1%)
inlC	400	153 (100%)
inlJ	238	153 (100%)
iap	131	153 (100%)
flaA	538 no amplicon	113 (74%) 40 (26%)

In the case of /*actA*/ and /*inlB*/ genes, polymorphism of amplicon sizes was detected within the isolates' collection. Summarized virulence detection results are presented in Table 2 above.

4.2. *actA* Polymorphism

Typing *L. monocytogenes* using the two *actA* primer pairs allowed for four genotypes to be differentiated, which are presented in Table 3.

Table 3. Number of isolates presenting the amplicon variants with particular *actA* primer pairs.

	***actA1* Shorter Amplicon (839 bp)**	***actA1* Longer Amplicon (950 bp)**	***actA1* No Amplicon**
actA2 shorter amplicon (268 bp)	15	0	13
actA2 longer amplicon (385 bp)	0	120	5

Interestingly, there is no clear pattern between genoserotype, as determined using the protocol of Doumith et al. protocol [38] (results published previously; see Reference [23]), and the *actA* amplicon variants achieved in this study, which allowed for the further differentiation of genoserotyped isolates.

Polymorphic variants of the *actA* gene detected with *actA1* and *actA2* primer pairs are presented in Figure 1 below.

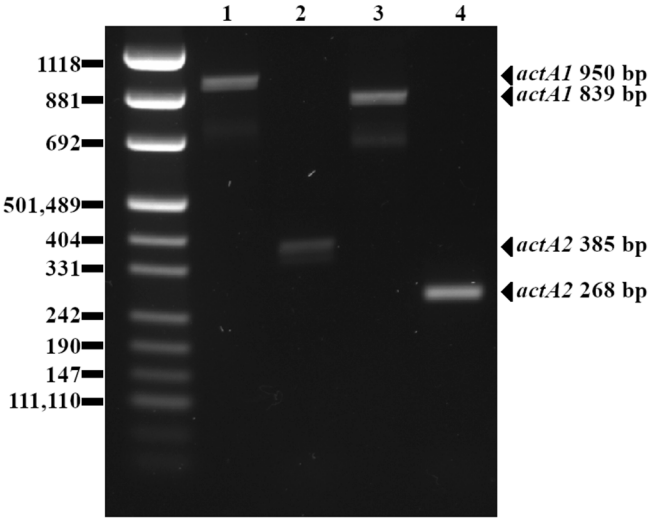

Figure 1. Agarose gel (2%) electrophoresis of DNA fragments generated with PCR using both *actA1* and *actA2* primers. Lanes 1 and 2: *L. monocytogenes* 234 (longer PCR amplicon achieved with *actA1* and *actA2*, respectively); Lanes 3 and 4: *L. monocytogenes* 45 (shorter PCR amplicon achieved with *actA1* and *actA2*, respectively).

4.3. *inlB* Polymorphism

Two isolates presented a shorter *inlB* amplicon than expected. Both of them are characterized by shorter variants of the *actA* gene, both belonged to the same rare genoserotype IIb (representing serotypes 1/2b, 3b, 7), and both shared the same antibiotic resistance profile, with susceptibility to all 10 tested antibiotics (results published previously; see Reference [39]), although the vast majority (95%) of isolates from this study were characterized by this particular susceptibility profile.

The two isolates with the truncated *inlB* gene originated from head cheese (processed meat product, also known as brawn) (isolate 50) and the Vienna-type sausage (isolate 235).

Sequences of the *inlB* genes of those two isolates, when aligned with the BLAST, showed 357/366 (99%) identities. When compared to the EGD-e strain, both isolates 50 and 235 lack the 141-bp long fragment (encoding the β-repeat sheet and partially encoding the GW1 domain of the InlB protein [40]) within the *inlB* gene sequence.

The detected variants of the *inlB* gene are presented in Figure 2 below.

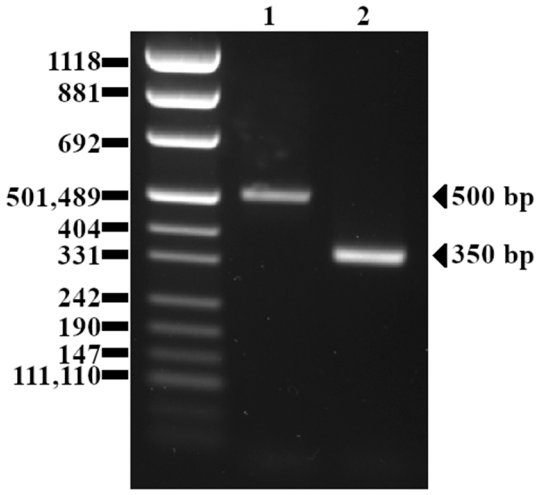

Figure 2. Agarose gel (2%) electrophoresis of DNA fragments generated with PCR using *inlB* primers. Lane 1: *L. monocytogenes* 99B (full-length PCR amplicon); Lane 2: *L. monocytogenes* 235 (with a deletion in *inlB* gene).

4.4. Hemolysis

All 153 tested *L. monocytogenes* isolates presented the hemolytic phenotype. The response was consistent after 24 h and 48 h incubation. Positive results for the hemolysis assay indicate that Hly (listeriolysin O) encoded by the *hly* gene is functional, and that the PrfA regulator, encoded by *prfA*, positively regulating *hly* expression, also remains active.

5. Discussion

5.1. General Prevalence of Virulence-Associated Genes

Recent studies of *L. monocytogenes* originating from Poland do not always confirm the prevalence of all tested virulence-associated genes in bacterial genomes [14,15]. For example *hlyA* and *prfA* were present in 100% of the 27 food isolates and 13 isolates from the food processing environments, whereas *inlB* and *sigB* genes were present in 26 (97.5%) and 20 (82.5%) of the samples, respectively [14]. Genes *plcB*, *hlyA*, *iap*, *actA*, *prfA*, and *sigB* were present in all seven isolates originating from fish housed in Poland, whereas *inlB* was detected in six (85.7%) isolates [15]. However, similarly to our results, internalin family member genes *inlA*, *inlB*, *inlC*, *inlE*, *inlF*, and *inlJ* and the pathogenicity island LIPI-1 were found in 48 (100%) of the tested isolates from different kinds of ready-to-eat (RTE) food of animal origin and from a food processing environment in Poland [40]. Interestingly, the authors also identified one isolate with a deletion of 141 nucleotides in the *inlB* gene [40].

In recent international studies, some authors report the presence of the tested virulence genes in 100% of the examined isolates (including, e.g., *hlyA*, *prfA*, *iap*, *inlA*, *inlB*, *mpl*, *plcA*, and *plcB* [41]), whereas sometimes particular genes were detected only in a subgroup of samples, e.g., only 70% and 80% of the isolates from bovine farms in India harbored *plcA* and *plcB* genes, even though *hlyA* and *iap* were present in all of them [16].

The only gene not detected in all isolates in our study is *flaA*, encoding flagellin, which is a protein specific to the 1/2a and 3a serotypes [34]. All isolates positive for the gene encoding flagellin (74%) belong to genoserotype IIa (representing serotypes 1/2a, 3a) [23], and all isolates that were negative belong to other genoserotypes, which is consistent with the theoretical expected results.

5.2. actA Polymorphism

A polymorphism within the *actA* gene of *L. monocytogenes* isolates has already been reported in the literature [42,43]. Furthermore, the partial sequencing of this gene has been used as a tool for subtyping *L. monoctogenes* isolates, enabling the division of the isolates into two [42] or three [43] lineages. In our study, we identified four genotypes based on *actA* typing using two sets of primers.

Interestingly, the *actA* gene is located on LIPI-1 between the *mpl* and *plcB* genes [44] and, in our study, both flanking genes were identified in all 153 isolates, without detecting any visible polymorphism. This raises questions about the origin of variability within this particular gene, especially considering that the genotypes established based on *actA* are not correlated with serotypes, which was proved by our study and also reported earlier by other researchers [45].

Although there are studies indicating that the in vitro virulence of *L. monocytogenes* is not determined by the *actA* polymorphism [46], some publications found that the *actA* polymorphism influences the virulence potential of the isolates [42,43,45]. In one study, isolates classified based on the *actA* polymorphism as lineage II showed significantly lower invasiveness on epithelial Caco-2 cells than lineage I isolates [43]. Similarly, in the paper where *L. monocytogenes* was subtyped with this method, isolates from lineage I all contained highly invasive isolates, as well as isolates with moderate and low invasiveness, whereas lineage II contained only low-invasive isolates. The invasiveness was established with a cell-invasion assay with the CX-1 human colon cancer cell line [42]. Earlier studies indicated that the deletion of one large unit within a proline-rich region of ActA resulted in a reduction in intracellular bacterial speed, as well as decreased virulence [45]. The primer pairs (*actA1* and *actA2*) used herein include regions translated to proline-rich regions of the protein.

5.3. inlB Polymorphism

Two isolates from the collection examined in this study presented a truncated *inlB* gene with a 141 bp deletion. Interestingly, Kurpas et al. (2020) also identified an isolate from Poland with 141 bp deletion in the same region of *inlB* gene [40].

There are also other reports about *L. monocytogenes* strains harboring mutations within this gene. For example, a food isolate from Mexican-style soft cheese *L. monocytogenes* F2365 harboring mutations resulting in premature stop codons in *inlB* was characterized by a reduced invasion efficiency in Caco-2 cells [47]. The same isolate with introduced point mutation resulting in rescued *inlB* expression showed approximately 9-fold and 1.5-fold higher invasion in HeLa and JEG-3 cells, respectively, when compared to the parental strain [48]. These findings are consistent with a study in which a constructed *inlB* deletion *L. monocytogenes* mutant showed significantly decreased invasiveness in a mouse as compared to wild-type isolate [49]. However, there is also a report indicating that the *L. monocytogenes* A23 strain with inactive internalin B protein remained virulent in a plaque assay on human adenocarcinoma cell line HT-29 [50]. Interestingly, one study demonstrated that InlB domain variants may differ in their ability to support intragastric infection (measured as bacterial loads in livers of intragastrically infected mice), even though, in a cell culture study (measuring the invasion rate of murine colon carcinoma C26 cells), the results were not clearly apparent [51].

5.4. Hemolysis

Hemolysis assay is a phenotypic criterion used for confirmation of the species affiliation of collected isolates [29,52–54]. However, there are reports stating that some *L. monocytogenes* isolates do not present with hemolysis, with a rate of approximately 0.1% according to a study which included 57,820 isolates of food, clinical, veterinary, environmental, and other origins [20]. A lack of hemolytic phenotype can indicate diminished virulence caused by either a nonfunctional Hly protein due to mutations or its hampered

expression due to mutations in the *prfA* gene, encoding the PrfA regulator that positively regulates Hly expression [20].

Hemolysis assays in the case of *L. monocytogenes* may provide interpretative difficulties. There is no consistency within the literature regarding the most sensitive method for hemolysis assessment or the blood type that would provide optimal test sensitivity [55]. Similarly to many other authors [20,56–63], we decided to apply the blood agar technique. Due to the fact that we observed weaker hemolysis in the case of *L. monocytogenes* strains than in the positive control, we do not recommend using *L. ivanovii* ATCC 19119 for that purpose, as this factor could contribute to false-negative reads in the case of *L. monocytogenes* isolates.

6. Summary and Conclusions

Although all 153 isolates possessed the 12 tested genes (*iap*, *sigB*, *prfA*, *hly*, *actA*, *inlB*, *inlC*, *inlA*, *inlJ*, *plcA*, *plcB*, and *mpl*), a PCR detection of virulence-associated genes allowed us to differentiate *L. monocytogenes* strains in our collection. The presence of the *flaA* gene was strictly serotype-dependent, whereas a polymorphism found in the *actA* gene could further differentiate strains pre-grouped into serogroups. Four major genotypes were identified based on *actA* typing. Two isolates were also differentiated from the rest of the collection due to a 141 bp deletion within the *inlB* gene sequence.

In terms of the prevalence of virulence-associated genes, our results are in agreement with the literature. Some authors report an absence of particular genes (e.g., *plcA*, *plcB*, *sigB*, *inlB*) in a subset of samples, whereas all of them were detected in all isolates included in our study. However, we did not observe that our results broke with any clear trend presented in the literature. The prevalence of particular genes in *L. monocytogenes* isolates depends on the strain collection itself and minor discrepancies are not only unsurprising but even expected. Due to this fact, the research on this topic is ongoing.

Based on the results and the available literature, we cannot draw conclusions regarding the potentially diminished virulence of some isolates in our isolates collection. However, isolates may differ in terms of their invasiveness due to the presence or absence of other virulence-associated genes that are not included in our study. Furthermore, gene mutations, even those that are undetectable with PCR, may lead to the diminished activity of a synthesized protein or the premature stopping of synthesis. In our study, we detected polymorphic forms in the case of *actA* and *inlB* genes, which may influence virulence potential. However, there is no literature consensus about the impact of those two genes on the invasiveness. The hemolytic phenotype observed in all isolates was confirmed to have hemolytic activity, proving that the Hly protein, as well as its regulator PrfA, are functional.

This study provided up-to-date knowledge about the high rate of prevalence of virulence-associated genes in the genomes of *L. monocytogenes* included in the study. Further research about the importance of detected *inlB* mutations in terms of the invasion rate should be performed in order to establish its influence on the virulence potential of isolates carrying this mutation.

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

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Gene *emrC* Associated with Resistance to Quaternary Ammonium Compounds Is Common among *Listeria monocytogenes* from Meat Products and Meat Processing Plants in Poland

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Abstract: (1) Background: *L. monocytogenes* is a food pathogen of great importance, characterized by a high mortality rate. Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC), are often used as disinfectants in food processing facilities. The effectiveness of disinfection procedures is crucial to food safety. (2) Methods: A collection of 153 isolates of *L. monocytogenes* from meat processing industry was analyzed for their sensitivity to BC using the agar diffusion method. Genes of interest were detected with PCR. (3) Results: Genes *emrC*, *bcrABC,* and *qacH* were found in 64 (41.8%), 6 (3.9%), and 1 isolate (0.7%), respectively, and 79 isolates (51.6%) were classified as having reduced sensitivity to BC. A strong correlation between carrying QACs resistance-related genes and phenotype was found (p-value < 0.0001). Among 51 isolates originating from bacon (collected over 13 months), 48 had the *emrC* gene, which could explain their persistent presence in a processing facility. Isolates with the *ilsA* gene (from LIPI-3) were significantly (p-value 0.006) less likely to carry QACs resistance-related genes. (4) Conclusions: Reduced sensitivity to QACs is common among *L. monocytogenes* from the meat processing industry. Persistent presence of these bacteria in a processing facility is presumably caused by *emrC*-induced QACs resistance.

Keywords: food safety; disinfection; genotypes; gene profiles; virulence; cross-resistance

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1. Introduction

1.1. Listeria Monocytogenes and Food Safety

Food is essential to life, and owing to this fact, food safety is considered to be a basic human right [1]. The major challenges of providing non-harmful food include its microbiological safety, especially in the context of bacteria, as they cause more foodborne incidents than other microbe groups, e.g., viruses or protozoa. Bacterial genera responsible for most foodborne infections include *Salmonella*, *Vibrio*, *E. coli*, *Shigella*, *Brucella*, *Campylobacter*, and *Listeria* [1]. Among those foodborne pathogens, *Listeria monocytogenes* presents the highest death toll, estimated to reach approximately 13–15% in the United States [2,3], or even up to 20–30% for those who contract listeriosis [4–7]. These bacteria are responsible for 19% of the total deaths caused by consumption of contaminated food products in the USA [8], and due to high mortality and high hospitalization rate of affected patients, *L. monocytogenes* causes tremendous annual economic losses, reaching almost USD 2.8 billion. That constitutes 18% of the total economic burden caused by major foodborne infectious agents associated with illnesses acquired through food products [2,3].

L. monocytogenes is a ubiquitous bacterium which can be found in water, soil, decaying vegetation, and even the human digestive tract [9]. It is characterized by easy adaptation to environmental conditions, including its ability to grow in a wide range of temperatures

(0 °C–45 °C) and pH (4.3–9.6), toleration of high salt concentrations (up to 10.0% NaCl), and low water activity (A_w to 0.90) [6]. These features facilitate survival and multiplication in food processing facilities [5], leading to its wide distribution and, in some cases, persistence in food processing environments [10]. Foods associated with high rates of *L. monocytogenes* infections include raw and processed food of both animal and plant origins, for example: raw sprouts, unpasteurized milk, soft cheeses, cold deli meats, cold hot dogs, as well as smoked seafood [9].

The presence of *L. monocytogenes* in food products is strictly regulated in many countries. In the United States, absence of *L. monocytogenes* in ready-to-eat food is required, whereas in European Union, as well as in Canada and Australia/New Zealand, absence is required in foods intended for infants and special medical purposes. In other foods, the bacteria may not exceed 100 CFU/g throughout its shelf-life [11]. Hence, effective and efficient elimination of *L. monocytogenes* from food processing environments is crucial in order to comply with legal regulations and to provide safe food products. Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC), are widely used for disinfection procedures in food processing facilities due to their efficacy against a variety of bacteria, fungi, and viruses; noncorrosive properties; and biodegradability [12–15].

However, resistance of *L. monocytogenes* to QACs can occur, as sanitizers may exert selective pressure, causing QACs-resistance associated genes to be maintained or acquired [5]. Among those genes, there are *bcrABC*, *qacH*, *emrE*, *emrC*, *qacC*, and *qacA*, which have been identified as the most common in the genomes of *L. monocytogenes* in the USA [16]. Proteins encoded by these five genes all belong to the small multidrug resistance family [12,14,17–19]. However, other factors, such as *mdrL* [20], which, on the other hand, falls into the major facilitator superfamily [17], or *fepR* [21], which is a transcriptional regulator [22], have also been identified as being involved in diminished BC sensitivity.

Furthermore, not only does QACs resistance reduce the effectiveness of disinfection procedures, but it is also a proven factor leading to cross-resistance of *L. monocytogenes* to antibiotics, such as chloramphenicol, ciprofloxacin, clindamycin, kanamycin, novobiocin, penicillin, streptomycin, and trimethoprim [23–25]. Hence, the cross-resistance phenomenon poses a threat to the effectiveness of antibiotic treatment of listeriosis. On the other hand, *L. monocytogenes* isolates well-adapted to food-processing environments, characterized by higher occurrence of genes involved in stress resistance and tolerance to disinfectants, are usually hypovirulent and less likely to cause an infection [26].

1.2. Aim of the Study

The main aim of this study was to analyze the occurrence of common genes responsible for resistance to QACs (*bcrABC*, *emrC*, and *qacH*) among 153 *L. monocytogenes* isolates originating from meat products and meat processing plants in Poland and to find differences between the isolates in the collection in terms of their sensitivity to BC.

The additional aim of this study was to correlate presence of Listeria Pathogenicity Island 3 (LIPI-3) in the genomes of collected bacteria to the QACs sensitivity phenotype and QACs resistance-related genes in order to verify a hypothesis of diminished virulence potential among QACs-adapted isolates.

2. Results

The results of sensitivity testing of the isolates to BC are presented in Figure 1 in the form of a histogram. The histogram shows the distribution of particular sizes of the clearing zones achieved around the spot of BC solution (6 mg/mL) placement in an agar diffusion assay. Additionally, five gene profiles achieved for the three QACs-resistance associated genes are indicated on the bars with colors, according to the legend.

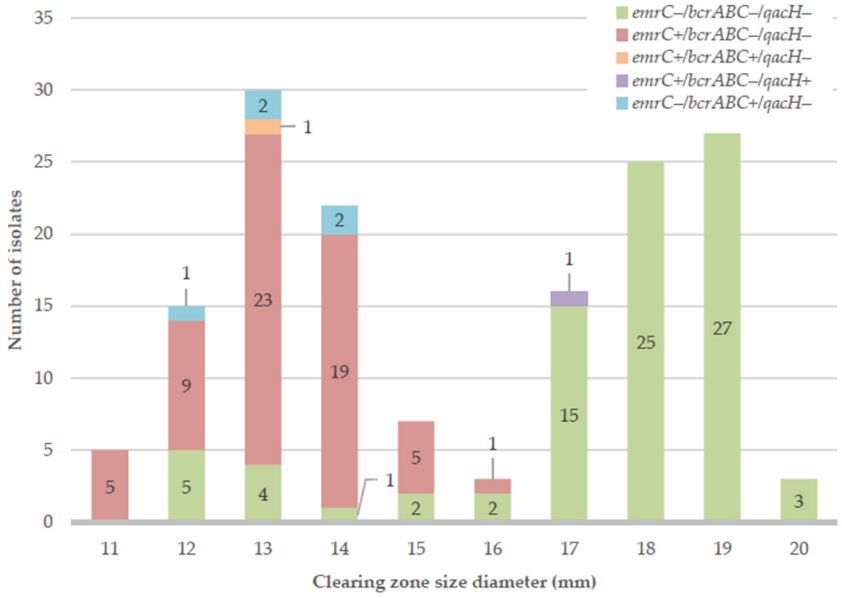

Figure 1. Histogram showing the distribution of the diameters of clearing zones around the BC solution (6 mg/mL) drop along with gene profile information.

In the histogram, there is a clear bimodal distribution, indicating the existence of two subpopulations within the collection of isolates. The subpopulation represented by the bars on the left side of the chart (from 11 to 15 or 16 mm) is characterized by a reduced susceptibility to BC compared to the subpopulation on the right side (from 16 or 17 mm to 20 mm). Based on the histogram distribution, the clearing zone diameter equal to or lower than 15 mm was a criterion applied to characterize isolates with reduced sensitivity to BC. Isolates with a zone diameter equal to or higher than 17 mm were considered sensitive, whereas isolates characterized by a clearing zone diameter of 16 mm were considered to present an intermediate response.

With those criteria applied, more than half, namely, 79 isolates (51.6%) of the total isolates showed reduced sensitivity to BC, which indicates prevalence of reduced sensitivity to QACs among *L. monocytogenes* isolates originating from the food industry. Summarized results of the phenotypic analysis are presented in Table 1.

Table 1. Classification of the response of the isolates to BC based on the agar diffusion analysis.

Response to BC	Number of Isolates (%)
Sensitivity	71 (46.4%)
Intermediate	3 (2.0%)
Reduced sensitivity	79 (51.6%)

In terms of genes related to QACs resistance, the gene *emrC* was the most prevalent and was present in 64 isolates (41.8%), followed by the *bcrABC* gene, which was present in 6 isolates (3.9%). The gene *qacH* was present in one isolate (0.7%). Overall, the collection was characterized by five different gene profiles based on the detection of those three genes. The most prevalent gene profile was *emrC−/bcrABC−/qacH−* (represented by green bars in Figure 1), and 84 isolates (54.9%) had that genotype. The second most prevalent was *emrC+/bcrABC−/qacH−* (represented by red bars), and 62 isolates (40.5%) were characterized by that genotype. The gene *bcrABC* alone was detected in five isolates (3.3%) (represented by turquoise bars), and in one isolate (0.7%) together with the *emrC* gene (represented by an orange bar). The gene *qacH* was present in only 1 isolate (0.7%), which also had the *emrC* gene (represented by a purple bar).

The correlation between carrying at least one of the QACs-resistance related genes and the presented phenotype is very strong (chi-square p-value < 0.0001). Among 71 isolates

considered to be sensitive, 70 isolates (98.6%) did not have any analyzed QACs resistance-related genes. Interestingly, the one remaining isolate (1.4%) carried not only one, but two genes (gene profile *emrC+/bcrABC−/qacH+*), which, on the contrary, would suggest a strong tolerance to QACs. This one isolate (represented by a purple bar in Figure 1) originated from a meat processing environment (conveyor belt).

Among 79 isolates characterized by a reduced sensitivity to BC, 67 (84.8%) had at least one QACs-resistance associated gene, and the remaining 12 isolates (15.2%) did not have any analyzed QACs resistance-related genes. In our earlier studies, we have demonstrated that 10 isolates in this collection were characterized by a reduced sensitivity to ciprofloxacin [27]. Interestingly, all 10 of those isolates fall within the group of those 12 isolates with reduced sensitivity to BC, but which do not harbor any analyzed QACs-resistance related genes. That suggests the existence of a common mechanism for QACs and ciprofloxacin resistance, which is not related to any of genes included in the study herein.

A clear pattern between an isolate origin and their QACs resistance gene profile can be observed. In the collection, there were 51 isolates originating from bacon (collected over the period of 13 months), and 48 of them had the *emrC* gene. All those 48 isolates had reduced sensitivity to BC in the phenotypic analysis. Resistance to QACs could be a potential reason for the persistent presence of *L. monocytogenes* in bacon samples, as their eradication from the processing plant could be impaired. Apart from bacon samples, 13 environmental samples (out of which 9 originate from sewage drain swabs) and 8 sausage samples had isolates with at least one QACs resistance-related gene. A bar chart showing the sources of the isolates along with their gene profiles is presented in Figure 2.

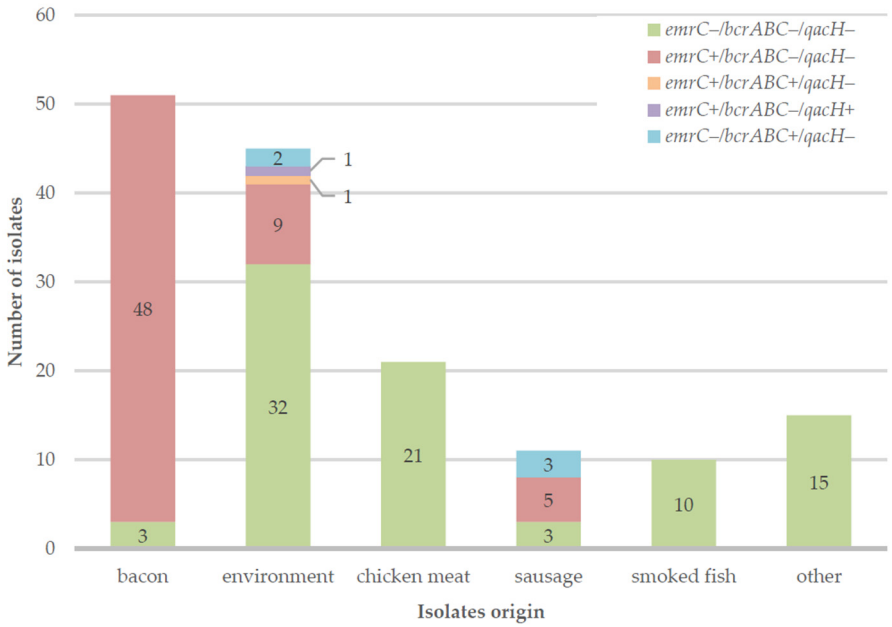

Figure 2. Bar chart showing the sources of the isolates included in the study along with gene profile information.

The *ilsA* gene, the first one from LIPI-3, was the only virulence-associated one included herein, as the collection has been pre-characterized in our previous work. We demonstrated that all 153 isolates harbored at least one variant of 12 analyzed virulence-associated genes, including all from LIPI-1, internalins, and others [28]. The *ilsA* gene analyzed herein was present in 18 isolates (11.8%).

Overall, the analyses of genes included in the study allowed us to differentiate seven groups of isolates with different gene profiles. Summarized results of the genetic analyses are presented in Table 2.

Table 2. Summary of gene profiles prevalence in analyzed collection.

Gene Profile				Number of Isolates (%)
emrC	***bcrABC***	***qacH***	***ilsA***	
−	−	−	−	69 (45.1%)
+	−	−	−	59 (38.6%)
−	−	−	+	15 (9.8%)
−	+	−	−	5 (3.3%)
+	−	−	+	3 (2.0)%
+	−	+	−	1 (0.7)%
+	+	−	−	1 (0.7)%

The *ilsA* gene was detected both in isolates without any QACs resistance genes (15 isolates) and in isolates with the *emrC* gene (3 isolates). Isolates carrying the *ilsA* gene were statistically significantly (chi-square *p*-value 0.006) less likely to carry any QACs resistance-related genes. This result supports a hypothesis about diminished virulence potential among QACs-adapted isolates. However, there was no statistical significance between carrying the *ilsA* gene and the presented phenotype (chi-square *p*-value 0.294), as the *ilsA* gene was detected in six isolates classified as sensitive, one isolate classified as intermediate, and eleven isolates with reduced sensitivity to BC.

3. Discussion

The most common QACs resistance-associated gene was *emrC*, detected in 64 (41.8%) isolates in this paper. In other studies, this gene was also identified, albeit less frequently. For example, analysis of a set of 25,083 publicly available *L. monocytogenes* genomes from the United States revealed that approximately 0.04% of isolates have that gene [16]. This analysis, however, included samples from various sources, including, e.g., human or aquatic animals, where selective pressure for QACs resistance genes is weaker than in food processing environments, where disinfection procedures are often performed. On the other hand, in an analysis of 4969 genomes of *L. monocytogenes* collected from United States food processing facilities, the gene *emrC* was not detected in any case [29]. However, in the study which included 13 *Listeria* isolates from a meat processing facility in Ireland, the gene *emrC* was present in two persistent isolates and one "presumed non-persistent" *L. monocytogenes* isolate [13]. Similarly, in a study from Poland, where 48 *L. monocytogenes* isolates from ready-to-eat meat and meat processing environments were examined, three isolates harbored *ermC* [30]. These results indicate that *emrC* is more common in Europe than in the United States. Overrepresentation of *emrC* in our collection is probably a result of issues with persistent presence of *L. monocytogenes* in a bacon-producing factory, presumably due to *emrC*-induced QACs resistance.

The gene *bcrABC*, which was present in six isolates (3.9%), was the second most common in our study. In many studies examining QACs resistance determinants, this gene is the most frequently identified. For example, in an already mentioned study which analyzed publicly available *L. monocytogenes* genomes from the United States, the *bcrABC* was identified in as much as 28.6% of samples [16], and in a study which included only food processing samples, it was present in nearly half (46%) of all isolates [29]. In Canadian studies, 41.5% (out of 1279 analyzed) *L. monocytogenes* isolates from various foods and food manufacturing environments harbored the *bcrABC* gene [31]. In Europe, *bcrABC* was identified in 3 out of 13 *Listeria* isolates from an Irish meat processing facility [13] and 1 out of 48 from Polish meat products and meat processing plants [30]. Contrarily to *emrC*, the results indicate more frequent presence of *bcrABC* in the United States than in Europe.

The gene *qacH* was the least frequent and was present in one isolate (0.7%) in our study. In some publications, this gene is reported to be identified very frequently, e.g., in 40% of the *Listeria* isolates from dairy products and the cattle environment in Egypt [32] or even up to 83% of *L. monocytogenes* in strains isolated from fish, fish products, and food-producing factories in Poland [33]. However, studies on bigger sample collections

have found that this gene is present in 1.76% of *L. monocytogenes* from United States [16], in approximately 5% of isolates from food processing facilities in the United States [29], and in 1.09% of *L. monocytogenes* contamination in food manufacturing environments in Canada [31]. Hence, the prevalence of *qacH* reported herein is low.

Among the 12 isolates (15.2%) with reduced sensitivity to BC, whose phenotypes were not explained by the detection of any QACs resistance-related genes, there were 10 isolates presenting reduced sensitivity to ciprofloxacin, which have been identified in earlier studies [27]. That suggests a cross-resistance between QACs and ciprofloxacin. Indeed, QACs adaptation is known to increase minimal inhibitory concentrations (MICs) of *L. monocytogenes* to ciprofloxacin, which has already been reported in literature [20,23,24]. Furthermore, to some extent, other genes, such as *emrE*, *qacC*, *qacA*, *mdrL*, and *fepR* [14,16,20,21], may have played a role in the diminished sensitivity of isolates to BC; however, those genes were not analyzed herein.

In terms of phenotypic analysis, in order to determine the response of *L. monocytogenes* to BC, authors usually evaluate the MICs of the isolates and interpret the results based on predetermined cutoff points. For example, in a study investigating *L. monocytogenes* from food and the food environment in Brazil, all 82 isolates were classified as having reduced susceptibility to BC, as their MICs varied from 16 to 128 μg/mL and the predetermined cutoff point was 10 μg/mL [34]. In another study, out of 77 *L. monocytogenes* from meat-processing facility, 17 were considered to be resistant to BC. The cutoff point of 12.5 μg/mL was applied based on the differences between MICs achieved in that study [35].

However, protocols other than assessing MICs are also applied. For example, an analysis of the sensitivity of isolates from six different turkey-processing plants in the United States was performed by spotting a bacterial suspension onto a blood agar containing BC at a concentration of 10 μg/mL. Based on this assay, 57 out of 123 isolates were identified as resistant [36]. A similar methodology was used to determine the sensitivity of 287 *L. monocytogenes* strains isolated from fish, fish products, and food-producing factories in Poland. Namely, bacterial suspensions were spotted on blood agar containing 5, 10, and 20 μg/mL of BC. Strains were classified as resistant if confluent growth was recorded on agar containing ≥10 μg/mL of BC. Based on the analysis, 40% of the isolates were considered resistant in that study [33].

In general, the common occurrence of isolates with reduced sensitivity to BC among *L. monocytogenes* originating from food products and processing environments has been observed by many authors. Although our results are not related to any particular concentrations, differences in the sensitivity of the collected isolates to BC were found. A high initial concentration of BC (6 mg/mL) allows the disinfectant to diffuse and create a concentration gradient in the agar medium. The achieved clearing zones enabled straightforward differentiation of the collected isolates.

In terms of the virulence of food-associated *L. monocytogenes* isolates, similarly to the prevalence of QACs resistance-related genes, it varies depending on the analyzed collection. The collection of the isolates analyzed herein was already studied in a publication in which their virulence potential was assessed based on the presence of genes of interest. The paper showed that all 153 isolates harbored a variant of 12 virulence-associated genes, including all from LIPI-1; four internalins, including *inlA*-*inlB* locus; and others [28]. Hence, the presence of *ilsA* from LIPI-3 was the only analysis included herein, and it allowed the virulence potential of the isolates to be differentiated to some extent. The prevalence of LIPI-3 in *L. monocytogenes* genomes varies greatly depending on the analyzed sample, sometimes even reaching 100% of isolates [37,38]. However, in the vast majority of papers, the results fall between 5% and 50% [6].

In conclusion, the prevalence of particular QACs resistance-related genes, as well as the prevalence of LIPI-3 in genomes of *L. monocytogenes*, are different in every publication, as every collection originates from different sources, different geographical areas, and is gathered during different time frames. All published results add up to a more complete picture, which allows for the tracking of global trends in terms of the prevalence of partic-

ular genes. Due to the frequent use of QACs in the food industry, genes responsible for QACs resistance are common among *L. monocytogenes* originating from food products and production environments. Based on the results published herein, such conclusions can also be drawn.

4. Materials and Methods

4.1. Isolates

A collection of 153 *L. monocytogenes* isolates was used in this study. Isolates originated from Polish meat processing plants (45 environment isolates) and meat products (108 isolates). Food isolates were isolated from bacon (51 isolates), chicken meat (21 isolates), sausage (11 isolates), smoked fish (10 isolates), and other sources (15 isolates), including, e.g., beef, pork, and fish. The isolates were collected over a period of 13 months, from October 2020 to November 2021. Isolates preserved as glycerol stocks stored at −80 °C were used for phenotypic analyses, and their DNA was used for genetic analyses. Detailed information about the collection process and DNA extraction procedure has already been published [39].

4.2. Phenotypic Analysis

The sensitivity of the collected isolates to BC was assessed using an agar diffusion method similar to a well-known disc diffusion method commonly applied to antibiotic sensitivity testing. However, the substance was placed directly onto the agar medium in the form of a drop of the tested solution without using discs containing the tested antimicrobial agent. This technique has already been described in the literature in the context of testing the sensitivity of *Listeria* spp. to bacteriocins [40]. The detailed methodology applied for this study is described below.

Isolates preserved in the form of glycerol stocks stored at −80 °C were used to inoculate brain heart infusion (BHI; Oxoid, Warsaw, Poland) agar medium with a sterile microbiological loop. Inoculated plates were then incubated at 37 °C for 16 h and stored at 7 °C for up to three weeks (21 days) for further use. A single colony was picked from the agar plate and used to inoculate 5 mL of BHI broth, which was then incubated at 37 °C for 18 h. After incubation, the culture was centrifuged (5 min with approx. 1700 g-force) and re-suspended in sterile deionized water (2.5 mL). This suspension was used to establish a 0.5 McFarland density in 3 mL of sterile deionized water, in which a cotton swab was then immersed and used to surface-inoculate a sterile BHI agar plate. The inoculation was performed by gently rubbing the whole agar surface in three directions.

A 10 µL drop of water-dissolved and filter-sterilized BC (Sigma-Aldrich, Poznań, Poland) solution with a concentration of 6 mg/mL was placed on the inoculated agar surface using an automatic pipette. A drop of water (10 µL) was applied as a negative control on the same petri dish, at a distance from the BC drop. Plates were left at room temperature (22 °C) for an hour to allow for the absorption of the drops into the agar medium. Then, plates were incubated at 37 °C for 18 h. After incubation, the diameter of the clearing zones around the spot of BC solution drop placement was measured in three technical replicates, and the average diameter was calculated. The experiment was performed in two independent replicates. The final result of this assay is an average diameter from two replications, expressed in mm and rounded out to the nearest integer. Sterility controls were performed with every batch of samples.

4.3. Genetic Analyses

Genetic analyses were performed with PCR using primers described in the literature [12,32,41,42]. Three genes associated with resistance and reduced sensitivity to QACs were analyzed, as well as the *ilsA* gene, the first gene on the LIPI-3. Primers used in the study were synthesized to order by Genomed S.A. (Warsaw, Poland). PCR reactions were performed in a T-Gradient thermocycler (Biometra, Göttingen, Germany) with the conditions given below in Table 3. The genes *ilsA*, *emrC*, and *bcrABC* were analyzed in reactions

using 0.2 U RUN polymerase (A&A Biotechnology, Gdańsk, Poland), with dedicated buffer, 0.2 mM nucleotide mix (A&A Biotechnology), and 0.5 µM of primers. The gene *qacH* was detected using StartWarm HS-PCR Mix (A&A Biotechnology) with 1.0 µM of primers. Matrix DNA was added in the amount of 10 ng, whereas in negative controls, water was used instead of the matrix DNA. Reactions were performed in 10 µL of final volume.

Table 3. Detailed information about PCR conditions.

Gene	Primers	Amplicon Size [bp]	Cycling Conditions	Reference
bcrABC	F: CATTAGAAGCAGTCGCAAAGCA R: GTTTTCGTGTCAGCAGATCTTTGA	1100	94 °C 5 min; (94 °C 30 s; 57 °C 50 s; 72 °C 60 s) × 30; 72 °C 5 min	[12]
emrC	F: TTATTCCATTTTATTACTGGCAATG R: CGTATTTATATTTAACACTAGCCA	387	94 °C 2 min; (94 °C 15 s; 50 °C 30 s; 72 °C 30 s) × 36; 72 °C 5 min	[41]
qacH	F: ATGTCATATCTATATTTAGC R: TCACTCTTCATTAATTGTAATAG	366	95 °C 5 min; (95 °C 25 s; 48 °C 40 s; 72 °C 40 s) × 35; 72 °C 5 min	[32]
ilsA	F: CGATTTCACAATGTGATAGGATG R: GCACATGCACCTCATAAC	280	94 °C 5 min; (94 °C 30 s; 52 °C 30 s; 72 °C 60 s) × 30; 72 °C 5 min	[42]

PCR products were separated in agarose gels and visualized with ethidium bromide. One randomly chosen sample for each gene was purified using the Clean-Up Concentrator kit (A&A Biotechnology) and sequenced by Genomed S.A. company. The sequences were then analyzed using BLAST 2.15.0 [43,44].

4.4. Data Analyses

Data were analyzed using MS Office Excel 2019 Software. The chi-square test was used to verify the significance of data independence.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13080749/s1.

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Uniwersytet Przyrodniczy w Poznaniu
ul. Wojska Polskiego 28, 60-637 Poznań

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2022, Genoserotyping of Listeria monocytogenes strains originaiting from meat products and meat processing environments, Żywność. Nauka. Technologia. Jakość, 29, 2 (131), 34 – 44* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, przygotowaniu projektu badań, opracowaniu krytycznego przeglądu literatury, przeprowadzeniu badań laboratoryjnych, w tym zbieraniu danych, walidacji danych, analizie wyników, interpretacji wyników i sformułowaniu wniosków, przeprowadzeniu obliczeń, przygotowaniu wykresów oraz opracowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 75%.

Data	Podpis
05.08.2024	Iwona Kawacka

Agnieszka Olejnik-Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
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Oświadczenie o współautorstwie

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Mój całkowity udział w powstaniu tej publikacji wyniósł 25%.

Data	Podpis
05/09/2024	AOSchmidt

Iwona Kawacka

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Bernadeta Pietrzak, Marcin Schmidt, Agnieszka Olejnik-Schmidt, 2023, Listeria monocytogenes Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin, Life, 13, 821* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, przygotowaniu projektu badań, opracowaniu krytycznego przeglądu literatury, przeprowadzeniu badań laboratoryjnych, w tym zbieraniu danych, walidacji danych, analizie wyników, interpretacji wyników i sformułowaniu wniosków, przeprowadzeniu obliczeń, przygotowaniu wykresów oraz opracowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 75%.

Data	Podpis
05.09.2024	Iwona Kawacka

Bernadeta Pietrzak

Katedra Biotechnologii i Mikrobiologii Żywności
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Bernadeta Pietrzak, Marcin Schmidt, Agnieszka Olejnik-Schmidt, 2023, Listeria monocytogenes Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin, Life, 13, 821* mój indywidualny udział w jej powstaniu polegał na zbieraniu danych po przeprowadzonych badaniach oraz recenzowaniu i edytowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 5%.

Data	Podpis
05.09.2024	Bernadeta Pietrzak

Marcin Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Bernadeta Pietrzak, Marcin Schmidt, Agnieszka Olejnik-Schmidt, 2023, Listeria monocytogenes Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin, Life, 13, 821* mój indywidualny udział w jej powstaniu polegał na ocenie poprawności analizy wyników oraz recenzowaniu i edytowaniu manuskryptu.

Mój całkowity udział w powstaniu tej publikacji wyniósł 5%.

Data	Podpis
05.09.2024	M Schm

Agnieszka Olejnik-Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Bernadeta Pietrzak, Marcin Schmidt, Agnieszka Olejnik-Schmidt, 2023, Listeria monocytogenes Isolates from Meat Products and Processing Environment in Poland Are Sensitive to Commonly Used Antibiotics, with Rare Cases of Reduced Sensitivity to Ciprofloxacin, Life, 13, 821* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, przygotowaniu projektu badań, ocenie poprawności analizy wyników oraz recenzowaniu i edytowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 15%.

Data	Podpis
05/09/2024	AOSchmidt

Iwona Kawacka

Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Agnieszka Olejnik-Schmidt, Marcin Schmidt 2022, Nonhemolytic Listeria monocytogenes—Prevalence Rate, Reasons Underlying Atypical Phenotype, and Methods for Accurate Hemolysis Assessment, Microorganisms, 10, 483* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, opracowaniu krytycznego przeglądu literatury, analizie danych oraz opracowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 80%.

Data	Podpis
05.09.2024	Iwona Kawacka

Agnieszka Olejnik-Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
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Oświadczenie o współautorstwie

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Mój całkowity udział w powstaniu tej publikacji wyniósł 15%.

Data	Podpis
05/09/2024	AOschmidt

Marcin Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
Wydział Nauk o Żywności i Żywieniu
Uniwersytet Przyrodniczy w Poznaniu
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Oświadczenie o współautorstwie

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Mój całkowity udział w powstaniu tej publikacji wyniósł 5%.

Data	Podpis
05.09.2024	.....

Iwona Kawacka

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2024, High Prevalence of Virulence-Associated Genes and Length Polymorphism in actA and inlB Genes Identified in Listeria monocytogenes Isolates from Meat Products and Meat-Processing Environments in Poland, Pathogens, 13, 444* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, przygotowaniu projektu badań, opracowaniu krytycznego przeglądu literatury, przeprowadzeniu badań laboratoryjnych, w tym zbieraniu danych, walidacji danych, analizie wyników, interpretacji wyników i sformułowaniu wniosków, przeprowadzeniu obliczeń oraz opracowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 75%.

Data	Podpis
05.09.2024	Iwona Kawacka

Agnieszka Olejnik-Schmidt

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Mój całkowity udział w powstaniu tej publikacji wyniósł 25%.

Data	Podpis
05/09/2024	AOSchmidt

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy *Iwona Kawacka, Agnieszka Olejnik-Schmidt, 2024, Gene emrC associated with resistance to quaternary ammonium compounds is common among Listeria monocytogenes from meat products and meat processing plants in Poland, Antibiotics, 13, 749* mój indywidualny udział w jej powstaniu polegał na autorstwie koncepcji, przygotowaniu projektu badań, opracowaniu krytycznego przeglądu literatury, przeprowadzeniu badań laboratoryjnych, w tym zbieraniu danych, walidacji danych, analizie wyników, interpretacji wyników i sformułowaniu wniosków, przygotowaniu wykresów, przeprowadzeniu obliczeń, analizie statystycznej oraz opracowaniu manuskryptu.
Mój całkowity udział w powstaniu tej publikacji wyniósł 80%.

Data	Podpis
05.09.2024	Iwona Kawacka

Agnieszka Olejnik-Schmidt

Katedra Biotechnologii i Mikrobiologii Żywności
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Mój całkowity udział w powstaniu tej publikacji wyniósł 20%.

Data	Podpis
05/09/2024	AOSchmidt