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Antybiotykooporność drobnoustrojów wchodzących w skład kultur starterowych i ochronnych – możliwość horyzontalnego transferu genów oraz wpływ czynników środowiskowych na ekspresję oporności na antybiotyki

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Antibiotic resistance of microorganisms from starter and protective cultures - the possibility of horizontal gene transfer and the impact of environmental factors on the expression of antibiotic resistance

Doctoral thesis done at the Chair of Industrial and Food Microbiology, Faculty of Food Science, University of Warmia and Mazury in Olsztyn

written under the supervision of
Associate Professor Anna Zadernowska, PhD D.Sc.
and
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Chciałabym podziękować wszystkim tym, bez których praca ta nie powstałaby.

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oraz

Narodowego Centrum Nauki w ramach w ramach konkursu PRELUDIUM-20 na realizację projektu badawczego pt. „Wpływ obróbki wysokociśnieniowej na ekspresję antybiotykooporności oraz potencjał przenoszenia genów oporności na antybiotyki u szczepów z kultur starterowych” (nr projektu: 2021/41/N/NZ9/00675)



Podstawę niniejszej pracy stanowi cykl powiązanych tematycznie pięciu artykułów naukowych opublikowanych w czasopismach wymienionych w wykazie czasopism naukowych i recenzowanych materiałów z konferencji międzynarodowych MEiN:

P1. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424 **(100 pkt. MEiN$_{2020}$; IF$_{2020}$ 3,714, liczba cytowań – bez autocytowań - Web of Science - 18)**

Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu tematu pracy i przygotowaniu konspektu publikacji, samodzielnym zebraniu literatury i przygotowaniu wstępnej wersji manuskryptu oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 65%.

P2. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zadernowska A. 2022. Microorganisms from starter and protective cultures - Occurrence of antibiotic resistance and conjugal transfer of *tet* genes *in vitro* and during food fermentation. LWT–Food Science and Technology, 153, 112490. https://doi.org/10.1016/j.lwt.2021.112490 **(100 pkt. MEiN$_{2022}$; IF$_{2022}$ 4,006, liczba cytowań – bez autocytowań - Web of Science - 6)**

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P3. **Zarzecka U.,** Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090 **(140 pkt. MEiN$_{2022}$; IF$_{2022}$ 5,516, liczba cytowań – bez autocytowań - Web of Science - 3)**

Mój wkład w powstanie publikacji polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu literatury, udziale w walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu

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P4. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. Effect of high pressure processing on changes in antibiotic resistance genes expression among strains from commercial starter cultures. Food Microbiology, 110, 104169. https://doi.org/10.1016/j.fm.2022.104169 **(140 pkt. MEiN$_{2023}$; IF$_{2023}$ 6,374, liczba cytowań – bez autocytowań - Web of Science - 1)**
Mój wkład w powstanie publikacji polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu literatury, walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu, udziale w redakcji manuskryptu po recenzji oraz pełnieniu roli autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 60%.

P5. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. High-pressure processing effect on conjugal antibiotic resistance genes transfer *in vitro* and in the food matrix among strains from starter cultures. International Journal of Food Microbiology, 388, 110104. https://doi.org/10.1016/j.ijfoodmicro.2023.110104 **(100 pkt. MEiN$_{2023}$; IF$_{2023}$ 5,911, liczba cytowań – bez autocytowań - Web of Science - 2).**
Mój wkład w powstanie publikacji polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu literatury, walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu, udziale w redakcji manuskryptu po recenzji oraz pełnieniu roli autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 65%.

Sumaryczny Impact Factor (IF) w/w publikacji zgodnie z bazą JCR wynosi: **25,521** (zgodnie z rokiem opublikowania).
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Pełne teksty wymienionych powyżej prac (wraz z oświadczeniami współautorów publikacji) stanowią załączniki do niniejszej pracy.

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- **Zarzecka U.** Antybiotykooporność pałeczek *Listeria monocytogenes* izolowanych z żywności. (Antibiotic resistance of *Listeria monocytogenes* isolated from food. XLVI Międzynarodowe Seminarium Kół Naukowych, Olsztyn (Poland), 24.04.2017.

4) Udział w stażach naukowych

- 03.02.2023 – 04.03.2023 – udział w 30-dniowym stażu naukowym w Departamento de Higiene y Tecnología de los Alimentos, Universidad de León, Campus de Vegazana s/n, 24071 – León. Opiekun stażu: prof. María Mercedes López Fernández (dofinansowanie: program „PROM – Stypendium wymiany międzynarodowej dla doktorantów i kadry akademickiej" Narodowej Agencji Wymiany Akademickiej).

5) Udział w kołach i towarzystwach naukowych

- Polskie Towarzystwo Technologów Żywności

- Polskie Towarzystwo Mikrobiologów
- Naukowe Koło Mikrobiologii Żywności „Kocuria”

6) Udział w szkoleniach

- 19.02.2020 - 21.02.2020 - Szkolenie: Podstawy Real-Time PCR i wyznaczanie poziomu ekspresji genów metodą ΔΔCt i Pfaffl’a – PolyGen Sp. z o.o., Wrocław.

7) Udział w projektach badawczych

- 03.01.2022 – 02.01.2023 – Kierownik projektu badawczego: „Wpływ obróbki wysokociśnieniowej na ekspresję antybiotykooporności oraz potencjał przenoszenia genów oporności na antybiotyki u szczepów z kultur starterowych” finansowanego przez Narodowe Centrum Nauki (PRELUDIUM-20; Grant nr 2021/41/N/NZ9/00675)
- 02.2019 - 03.2019 - wykonawca w projekcie „Warunki stresowe podczas otrzymywania produktów sous-vide i ceviche z ryb i krewetek oraz przeżywalność i odpowiedź komórkowa bakterii wielolekoopornych” finansowanym przez Ministerstwo Nauki i Szkolnictwa Wyższego (kierownik projektu: mgr inż. Arkadiusz Zakrzewski)
- 01.06.2019 – 31.05.2021 - stypendysta w projekcie „Wpływ technologii płotków i obróbki sous-vide na ekspresję czynników wirulencji i genów oporności na antybiotyki u *Listeria monocytogenes* i *Enterococcus* sp.” finansowanym przez Narodowe Centrum Nauki (OPUS-15; numer projektu 2018/29/B/NZ9/00645), kierownik projektu: prof. Anna Zadernowska

8) Wykonane recenzje artykułów

Wykonanie 21 recenzji artykułów w następujących czasopismach:
- Applied Sciences (MDPI) – 4
- Infection and Drug Resistance (DovePress) – 3
- Animals (MDPI) – 2
- Antibiotics (MDPI) – 2
- Food Science & Nutrition (Wiley) – 2
- Archives of Microbiology (Springer) – 1
- Biology (MDPI) – 1
- Foods (MDPI) – 1
- Food Frontiers (Wiley) - 1
- International Journal of Environmental Research and Public Health (MDPI) – 1
- Journal of Consumer Protection and Food Safety (Springer) – 1
- Microbiology Research (MDPI) - 1
- Pharmaceutics (MDPI) – 1

CAŁOŚCIOWE ZESTAWIENIE DOROBKU NAUKOWEGO

(na dzień 20.09.2023)

Autorstwo/Współautorstwo w pracach naukowych			
	Liczba publikacji	**∑ IF (zgodny z rokiem opublikowania)**	**∑ punktów MEiN**
Publikacje w recenzowanych czasopismach naukowych	**14**	**52,702**	**1290**
LWT – Food Science and Technology	3	**11,726**	**300**
Food Microbiology	3	**17,406**	**420**
International Journal of Food Microbiology	1	**5,911**	**100**
Animals	1	**3,231**	**100**
Pathogens	1	**4,531**	**100**
Foods	1	**5,561**	**100**
International Journal of Environmental Health Research	1	**1,914**	**70**
Journal of the Science of Food and Agriculture	1	**2,422**	**100**
Przemysł Spożywczy	1	**-**	**-**
Kosmos-Problemy Nauk Biologicznych	1	**-**	**-**
Rozdziały w monografiach pokonferencyjnych	**11**	**-**	**320**
Inne aktywności naukowe			
Doniesienia na konferencjach naukowych	**18**		
	Krajowe: 13		
	Międzynarodowe: 5		
Udział w stażach naukowych	**1**		
Udział w kołach i towarzystwach naukowych	**3**		
Udział w szkoleniach	**1**		
Udział w projektach badawczych	**3**		
Wykonane recenzje artykułów	**21**		
Wskaźniki oceny dorobku naukowego (Web of Science)			
H-indeks	**5**		
Łączna liczba cytowań (bez autocytowań)	**52**		

WYKAZ STOSOWANYCH SKRÓTÓW

ARGs – geny oporności na antybiotyki (ang. antibiotic resistance genes)

BHI – podłoże mózgowo-sercowe (ang. brain-hear infusion)

CFU – jednostki tworzące kolonię (ang. colony forming units)

ERIC-PCR – Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction

FAO – Organizacja Narodów Zjednoczonych ds. Wyżywienia i Rolnictwa (ang. Food and Agriculture Organization of the United Nations)

FDA – Agencja Żywności i Leków (ang. U.S. Food and Drug Administration)

GRAS – status Generalnie Uznanych za Bezpieczne (ang. Generally Recognized as Safe)

HG – housekeeping gene

HGT – horyzontalny transfer genów (ang. horizontal gene transfer)

LAB – bakterie fermentacji mlekowej (ang. lactic acid bacteria)

MALDI-TOF – Spektrometria masowa z desorpcją/jonizacją laserową wspomaganą matrycą z analizatorem czasu przelotu (ang. Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry)

MIC – minimalne stężenie hamujące (ang. minimum inhibitory concentration)

MRS – podłoże wg de Man, Rogosa i Sharpe

OIE – Światowa Organizacja Zdrowia Zwierząt (ang. The World Organisation for Animal Health)

PPS – płyn fizjologiczny z peptonem (ang. physiological peptone saline)

QPS – kwalifikowane domniemanie bezpieczeństwa (ang. Qualified Presumption of Safety)

TBE – Tris-borate EDTA

UPGMA – metoda grup nieważonych par ze średnią arytmetyczną (ang. unweighted pair group method with arithmetic mean)

WHO – Światowa Organizacja Zdrowia (ang. World Health Organization)

STRESZCZENIE

Według WHO oporność na antybiotyki jest poważnym zagrożeniem i jednym z największych problemów zdrowia publicznego. Przez wiele lat problem antybiotykooporności wiązany był jedynie ze środowiskiem szpitalnym. Z czasem wykazano, że źródłem oporności na antybiotyki mogą być także szczepy pochodzące z innych środowisk, między innymi z żywności. Wiele szczepów stosowanych przemysłowo w produkcji żywności zostało uznanych za jej tradycyjne składniki, dzięki czemu uzyskały status bezpiecznych i zostały prawnie dopuszczone do stosowania w produkcji żywności na terenie Unii Europejskiej bez konieczności przeprowadzenia badań potwierdzających ich bezpieczeństwo. Ponieważ analiza występowania genów oporności jest stosowana stosunkowo od niedawna, nie ma informacji o ich występowaniu u szczepów stosowanych w produkcji żywności od wielu lat.

Celem pracy była analiza występowania i możliwości horyzontalnego przenoszenia genów oporności na antybiotyki wśród szczepów wchodzących w skład komercyjnych kultur starterowych i ochronnych. Analizie poddano także wpływ czynników stresowych związanych z produkcją i przechowywaniem żywności na zmiany w ekspresji genów kodujących oporność na antybiotyki, jak i częstość transferu genów.

Uzyskane wyniki wykazały, że szczepy wchodzące w skład komercyjnych kultur starterowych i ochronnych mogą być nosicielami genów nabytej oporności na antybiotyki. Najwyższe minimalne stężenia hamujące odnotowano dla tetracykliny (maks. >256 μg/ml), kanamycyny (maks. 48 μg/ml) i chloramfenikolu (maks. 32 μg/ml). Najczęściej występującymi genami oporności były *aph(3')-IIIa*, kodujący oporność na kanamycynę (n = 35; 60,3%) i gen oporności na chloramfenikol *cat* (n = 32; 55,2%). Szczepy wykazały także zdolność horyzontalnego przenoszenia genów oporności zarówno *in vitro* jak i *in situ*. Najczęściej dochodziło do transferu genów oporności na tetracykliny *tet*M i *tet*K.

Po zastosowaniu różnych wariantów obróbki wysokociśnieniowej oraz po ekspozycji na stres kwasowy i osmotyczny ekspresja genów kodujących oporność na aminoglikozydy (*aac(6')Ie-aph(2'')Ia* i *aph(3')-IIIa*), chloramfenikol (*cat*), ampicylinę (*bla*Z) i/lub tetracykliny (*tet*M, *tet*W) zwiększała się. Przechowywanie chłodnicze wywołało obniżenie poziomu ekspresji genów *aac(6')Ie-aph(2'')Ia*, *aph(3')-IIIa* i *tet*M. Wyniki uzyskane w analizie ekspresji genów korelowały z wynikami uzyskanymi

w analizie fenotypowej. Po ekspozycji na czynniki stresowe zaobserwowano także zmiany w częstości transferu badanych genów.

Uzyskane wyniki wskazują, że szczepy wchodzące w skład kultur starterowych i ochronnych stosowanych w przemyśle spożywczym mogą być rezerwuarami genów oporności na antybiotyki i brać udział w ich rozprzestrzenianiu. Ponadto wykazano, że warunki panujące podczas produkcji, utrwalania bądź przechowywania żywności mogą przyczyniać się do zmian w oporności na antybiotyki wśród szczepów, które przetrwały proces oraz do zmian w częstości transferu genów, co prowadzi do szybszego rozprzestrzeniania oporności w środowisku. Należy zauważyć, że chociaż antybiotykooporność bakterii pochodzących z kultur starterowych i ochronnych nie stanowi bezpośredniego zagrożenia dla konsumentów, jest to zjawisko budzące niepokój, którego nie można zignorować. Wskazuje to na konieczność weryfikacji szczepów celowo stosowanych w przemyśle spożywczym pod kątem ich oporności na antybiotyki, aby nie stawały się one środowiskowym rezerwuarem genów oporności.

Słowa kluczowe: kultury starterowe, kultury ochronne, czynniki stresowe, oporność na antybiotyki, ekspresja genów, horyzontalny transfer genów

ABSTRACT

According to the WHO, antibiotic resistance is a serious threat and one of the biggest public health problems. For many years, the problem of antibiotic resistance was associated only with the hospital environment. Over time, it has been shown that strains from other environments, including food can also be the source of antibiotic resistance. Many strains used industrially in food production have been recognized as its traditional ingredients, thanks to which they have obtained the status of safe and have been legally approved for use in food production in the European Union without the need to conduct tests confirming their safety. Since the analysis of the occurrence of resistance genes has been used relatively recently, there is no information about their occurrence in strains used in food production for many years.

The aim of the study was to analyze the occurrence and possibility of horizontal transfer of antibiotic resistance genes among strains from commercial starter and protective cultures. The impact of stress factors associated with food production and storage on changes in the expression of genes encoding resistance to antibiotics, as well as the frequency of gene transfer, was also analyzed.

The obtained results showed that the strains from the commercial starter and protective cultures may be carriers of acquired antibiotic resistance genes. The highest Minimum Inhibitory Concentrations (MICs) were reported for tetracycline (max. >256 μg/ml), kanamycin (max. 48 μg/ml) and chloramphenicol (max. 32 μg/ml). The most common resistance genes were *aph(3')-IIIa*, encoding resistance to kanamycin (n = 35; 60.3%) and chloramphenicol resistance *cat* (n = 32; 55.2%). The strains also demonstrated the ability to horizontally transfer resistance genes both *in vitro* and *in situ*. The *tet*M and *tet*K genes for resistance to tetracyclines were transferred most frequently.

After applying various variants of high-pressure treatment and after exposure to acid and osmotic stress, expression of genes encoding resistance to aminoglycosides (*aac(6')Ie-aph(2'')Ia* and *aph(3')-IIIa*), chloramphenicol (*cat*), ampicillin (*blaZ*) and/or tetracyclines (*tet*M, *tet*W) increased. Refrigerated storage resulted in a downregulation of *aac(6')Ie-aph(2'')Ia*, *aph(3')-IIIa* and *tet*M genes. The results obtained in the analysis of gene expression correlated with the results obtained in the phenotypic analysis. Changes in the frequency of transfer of the studied genes were also observed after exposure to stress factors.

The obtained results indicate that the strains included in the starter and protective cultures used in the food industry may be reservoirs of antibiotic resistance genes and participate in their spread. In addition, it has been shown that the conditions prevailing during the production, preservation or storage of food can contribute to changes in antibiotic resistance among strains that survived the process and to changes in the frequency of gene transfer, which leads to faster spread of resistance in the environment. It should be noted that while antibiotic resistance in starter and guard cultures is not an immediate threat to consumers, it is a cause for concern that cannot be ignored. This indicates the need to verify the strains used in the food industry in terms of their antibiotic resistance, so that they do not become an environmental reservoir of resistance genes.
Keywords: starter cultures, protective cultures, stress factors, antibiotic resistance, gene expression, horizontal gene transfer

1. WSTĘP – ZAGADNIENIE W ŚWIETLE LITERATURY

1.1. ANTYBIOTYKOOPORNOŚĆ JAKO WSPÓŁCZESNY PROBLEM ZDROWIA PUBLICZNEGO

Według danych Światowej Organizacji Zdrowia (ang. World Health Organization – WHO) oporność drobnoustrojów na antybiotyki stanowi ogromny problem i staje się jednym z najpoważniejszych zagrożeń zdrowia publicznego. WHO ostrzega też przede wszystkim przed brakiem nowych antybiotyków do zwalczania narastającej oporności. Większość dostępnych obecnie antybiotyków to modyfikacje już istniejących, które w tym przypadku są jedynie rozwiązaniami doraźnymi. Bez pilnych działań zmierzamy w kierunku ery postantybiotykowej, w której powszechne infekcje i drobne urazy staną się śmiertelne (WHO, 2022). Światowa Organizacja Zdrowia wraz z Organizacja Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa (ang. Food and Agriculture Organization of the United Nations - FAO) wskazują, że oporność drobnoustrojów na antybiotyki jest problemem, który nie dotyczy, jak dawniej sądzono, jedynie środowiska szpitalnego. Należy więc patrzeć na ten problem globalnie, uwzględniając wszystkie możliwe obszary, w tym także łańcuch żywnościowy (Samtiya i in., 2022). Badania wskazują na związek między masowym nadużywaniem antybiotyków w produkcji rolnej a wzrostem oporności na antybiotyki. Wskazuje się, że bezpośrednią przyczyną pojawienia się oporności na antybiotyki w środowisku mogło być nadmierne i niekontrolowane stosowanie środków przeciwdrobnoustrojowych, biocydów i metali ciężkich w sektorze spożywczym i rolnym. Oporność na antybiotyki rozprzestrzeniła się szybko w wyniku globalizacji dostaw żywności, zwiększonej liczby ludności na obszarach miejskich i podróży międzynarodowych (Holmes i in., 2016; Molnar, 2019).

W kontekście narastającego problemu oporności na antybiotyki FAO i WHO wraz ze Światową Organizacją Zdrowia Zwierząt (World Organisation for Animal Health - OIE) zaproponowały podejście „One Health", dążące do równoważenia i optymalizacji zdrowia ludzi, zwierząt i środowiska (Aslam i in., 2021; McEwen i Collignon, 2018).

1.2. KULTURY STARTEROWE I OCHRONNE W PRZEMYŚLE SPOŻYWCZYM

Od tysięcy lat ludzkość wykorzystuje właściwości drobnoustrojów w celu otrzymywania produktów spożywczych o określonych cechach organoleptycznych oraz do konserwowania żywności. Wraz z rozwojem przemysłu spożywczego opracowano kultury wyselekcjonowanych szczepów drobnoustrojów do przemysłowego

wykorzystania. Spośród nich najczęściej wymienia się kultury starterowe oraz kultury ochronne. Wykorzystanie przemysłowych kultur drobnoustrojów zintensyfikowało się w ostatnich dziesięcioleciach (Laulund i in., 2017).

Kultury starterowe wprowadzane są na etapie produkcji żywności w celu uzyskania produktu o pożądanych właściwościach i cechach organoleptycznych. Bez ich udziału otrzymanie właściwego produktu nie byłoby możliwe, ponieważ mikroorganizmy te biorą udział w najważniejszych przemianach, mają za zadanie, m.in. przeprowadzenie fermentacji cukrów, wytworzenie substancji smakowo-zapachowych, biorą też udział w prowadzeniu przemian proteolitycznych i lipolitycznych. Niektórzy autorzy jako funkcję kultur starterowych wskazują także wydłużenie okresu przydatności do spożycia (García-Díez i Saraiva, 2021).

Kultury ochronne definiuje się jako żywe mikroorganizmy celowo dodawane do żywności w celu kontroli jej stanu bakteriologicznego bez zmiany jej właściwości technologicznych i sensorycznych. W skład kultur ochronnych wchodzą głównie bakterie fermentacji mlekowej (ang. Lactic acid bacteria – LAB). Wykazano bowiem, że mogą one hamować wzrost wielu mikroorganizmów chorobotwórczych, takich jak *Listeria monocytogenes*, *Clostridium botulinum*, czy *Staphylococcus aureus*. Działanie antagonistyczne wobec innych drobnoustrojów wynika w głównej mierze z konkurencji o związki pokarmowe oraz produkcji przez LAB metabolitów o właściwościach przeciwbakteryjnych i przeciwgrzybicznych, takich jak etanol czy nadtlenek wodoru, a także syntetyzowanych rybosomalnie peptydów o właściwościach przeciwdrobnoustrojowych – bakteriocyn (Ben Said i in., 2019; Dokka i in., 2018; Silva i in., 2018).

Stosowanie kultur starterowych jest praktykowane w produkcji żywności od lat, ponieważ pozwala na uzyskanie produktu o pożądanych cechach w kontrolowanych warunkach, z kolei stosowanie kultur ochronnych stało się bardziej powszechne w ostatnich latach. Wzrost zainteresowania ich stosowaniem wynika z dążenia do tzw. czystej etykiety i związanej z tym rezygnacji ze stosowania konserwantów, co pociąga za sobą konieczność zapewnienia bezpieczeństwa żywności w inny, naturalny sposób. W tym miejscu zaproponowano wprowadzanie szczepów bakterii fermentacji mlekowej, bezpiecznych dla konsumenta, jednak działających antagonistycznie wobec innych mikroorganizmów, w tym chorobotwórczych (Bintsis, 2018; Zadernowska i in., 2017).

1.3. UREGULOWANIA PRAWNE DOPUSZCZAJĄCE DROBNOUSTROJE DO STOSOWANIA W ŻYWNOŚCI

Z uwagi na fakt, że drobnoustroje wchodzące w skład kultur przemysłowych trafiają do żywności w dużej liczbie, konieczne jest upewnienie się, że nie stanowią one bezpośredniego bądź pośredniego zagrożenia dla zdrowia konsumenta. Przemysłowe kultury drobnoustrojów podobnie jak inne składniki żywności, muszą spełniać wymagania określone w Prawie Żywnościowym, czyli Rozporządzeniu Unii Europejskiej nr 178/2002, którego Artykuł 14 mówi, że zabronione jest wprowadzanie do obrotu żywności, która może być niebezpieczna dla konsumenta, a zapewnienie bezpieczeństwa żywności jest obowiązkiem podmiotu wprowadzającego żywność na rynek. W tym celu niezbędne było opracowanie norm prawnych dotyczących kryteriów dopuszczających drobnoustroje do stosowania w żywności (Herody i in., 2010; van der Meulen, 2013).

W Stanach Zjednoczonych do stosowania w żywności dopuszcza się szczepy mające status GRAS (ang. Generally Recognized As Safe). System ten wprowadzono w 1958 roku. Zgodnie z poprawką do ustawy o żywności, lekach i kosmetykach z 1958 r. dotyczącej dodatków do żywności wszystkie składniki egzogenne celowo wprowadzane do łańcucha żywnościowego, w tym kultury drobnoustrojów, są dodatkami do żywności, dlatego przed wprowadzeniem do obrotu podlegają zatwierdzeniu przez Agencję Żywności i Leków (ang. Food and Drug Administration - FDA). Jednak gdy dany dodatek ma status GRAS lub posiada historię bezpiecznego stosowania w żywności przed 1958 r. nie wymaga zatwierdzenia przez FDA przed wprowadzeniem na rynek (Russo i in., 2017).

Z kolei w roku 2007 Europejski Urząd ds. Bezpieczeństwa Żywności EFSA (ang. European Food Safety Authority) nakreślił schemat oparty na koncepcji kwalifikowanego domniemania bezpieczeństwa (ang. Qualified Presumption of Safety – QPS). Jest to narzędzie służące do szybkiej oceny ryzyka w odniesieniu do produktów zawierających mikroorganizmy. Aby otrzymać status QPS szczep musi być zidentyfikowany pod względem taksonomicznym i niechorobotwórczy, co musi zostać poparte dostępną wiedzą literaturową wystarczającą do potwierdzenia bezpieczeństwa. Ponadto jasno zdefiniowane musi zostać przeznaczenie szczepu (Laulund i in., 2017).

Wiele szczepów LAB ze względu na ich długą historię bezpiecznego stosowania w produkcji żywności zostało uznanych za tradycyjne składniki żywności. Dzięki temu szczepy te zostały prawnie dopuszczone do stosowania w produkcji żywności bez

konieczności przeprowadzenia badań potwierdzających ich bezpieczeństwo, decydujących o pozwoleniu na stosowanie do żywności. Problematyczny może być jednak fakt, że szczepy, którym w przeszłości przyznano status bezpiecznych nie muszą być regularnie sprawdzane pod kątem występowania nabytej oporności na antybiotyki. Ponieważ analiza występowania genetycznych determinant oporności jest stosowana stosunkowo od niedawna, nie ma żadnych informacji o występowaniu genów oporności i mobilnych elementów genetycznych u szczepów stosowanych w produkcji żywności od wielu lat. Szczepy te potencjalnie mogą jednak stać się rezerwuarami genów oporności i przyczyniać się do ich rozpowszechniania (Laulund i in., 2017).

1.4. ANTYBIOTYKOOPORNOŚĆ WŚRÓD BAKTERII FERMENTACJI MLEKOWEJ

W ostatnich latach badania dotyczące selekcji i rozprzestrzeniania oporności na antybiotyki koncentrowały się głównie na klinicznie istotnych gatunkach bakterii. Obecnie wiadomo już, że także bakterie związane z żywnością mogą być nosicielami genetycznych determinant oporności. Spowodowało to wzrost zainteresowania tematyką oporności na antybiotyki wśród drobnoustrojów związanych z żywnością i zintensyfikowanie badań w tym zakresie, skupiano się jednak głównie na drobnoustrojach patogennych bądź potencjalnie patogennych. Jednak, jak wskazują dane literaturowe, oporność na antybiotyki może występować też u mikroorganizmów niechorobotwórczych dla człowieka, m.in. szczepów celowo dodawanych do żywności w postaci kultur przemysłowych. Oporność naturalna, a także będąca wynikiem spontanicznych mutacji nie wzbudza niepokoju, ponieważ charakteryzuje się niewielkim ryzykiem horyzontalnego przenoszenia. Obecnie jednak coraz częściej podaje się, że szczepy uznawane za bezpieczne, takie jak bakterie fermentacji mlekowej, mogą wykazywać duże zdolności adaptacyjne, co przejawia się zdolnością do nabywania oporności przez pozyskiwanie plazmidów i/lub transpozonów zawierających geny oporności, które następnie na drodze horyzontalnej mogą być przekazywane innym szczepom (Erginkaya i in., 2018; Verraes i in., 2013).

Oporność na antybiotyki wśród bakterii fermentacji mlekowej została najdokładniej udokumentowana u szczepów z rodzaju *Lactobacillus*. Według literatury są one naturalnie oporne na glikopeptydy (np. wankomycyna), chinolony (np. ciprofloksacyna i norfloksacyna) oraz niskie stężenia aminoglikozydów (np. gentamycyny, streptomycyny, kanamycyny). Jednak wykazują także potencjał do nabywania oporności na inne antybiotyki, głównie antybiotyki hamujące syntezę

białek, takie jak chloramfenikol, tetracyklina oraz makrolidy (np. erytromycyna). Oporność na chloramfenikol wynika najczęściej z obecności genu *cat*. Z kolei oporność na erytromycynę jest często związana z obecnością genów *erm*(A), *erm*(B) i *erm*(C). Nabyta oporność na tetracykliny u szczepów *Lactobacillus* zwykle kodowana jest przez geny *tet*(M), *tet*(S) i *tet*(W) (Federici i in., 2014; Georgieva i in., 2015; Sharma i in., 2016).

Oporność na antybiotyki bakterii z rodzaju *Lactococcus* poznano słabiej. Podaje się, że większość gatunków należących do tego rodzaju jest naturalnie oporna na metronidazol, trimetoprim i cefoksytynę. Zgodnie z literaturą bakterie z rodzaju *Lactococcus* mogą nabywać oporność na różne antybiotyki, głównie makrolidy - erytromycynę i linkomycynę, tetracykliny i niektóre antybiotyki o szerokim spektrum działania, na przykład rifampicynę i chloramfenikol. Nieznacznie zmniejszoną wrażliwość obserwuje się również w przypadku chinolonów i antybiotyków β-laktamowych. Wśród różnych zidentyfikowanych genów oporności genem najczęściej stwierdzanym u *Lactococcus* jest gen *erm*(B), który ulega ekspresji konstytutywnej lub indukowalnej. Oporność na tetracykliny jest kodowana głównie przez gen *tet*(M) (Devirgiliis i in., 2013; Erginkaya i in., 2018).

Literatura wskazuje, że bakterie z rodzaju *Pediococcus* są naturalnie oporne na glikopeptydy, niskie stężenia aminoglikozydów, ciprofloksacynę oraz trimetoprim i sulfametoksazol. Szczepy należące do tego rodzaju mogą wykazywać także nabytą oporność, najczęściej wskazywana jest oporność na tetracykliny i makrolidy. Oporność na tetracykliny jest kodowana najczęściej przez geny *tet*(K), *tet*(O), *tet*(S) i *tet*(W), z kolei oporność na makrolidy przez gen *erm*(B) (Cordeiro i in., 2010; Kastner i in., 2006).

Z uwagi na fakt, że bakterie fermentacji mlekowej nie są organizmami chorobotwórczymi głównym zagrożeniem związanym z występowaniem u nich oporności na antybiotyki jest ryzyko horyzontalnego przeniesienia genów warunkujących oporność do bakterii patogennych. Co prawda w ostatnich latach zaczęły pojawiać się doniesienia dotyczące zakażeń wywołanych przez szczepy *Lactococcus* czy *Lactobacillus,* jednak dotyczyły one jedynie osób ze skrajnie obniżoną odpornością, dlatego nie można przyjąć, że drobnoustroje te stanowią bezpośrednie zagrożenie dla zdrowia konsumentów (Fraqueza, 2015). Literatura wskazuje, że bakterie fermentacji mlekowej mogą być nosicielami plazmidów o różnej wielkości, na których zlokalizowane są determinanty oporności. Ich obecność stwierdzano z porównywalną częstością wśród szczepów *Lactobacillus* i *Lactococcus* (Gevers i in., 2003; Patel i in., 2012). Innym

rodzajem mobilnych elementów genetycznych, których obecność stwierdza się wśród szczepów LAB, w tym także tych o znaczeniu przemysłowym są transpozony koniugacyjne, zarówno o szerokim, jak i wąskim zakresie gospodarzy. Występują one zarówno u szczepów *Lactococcus* (Tn*5276* i Tn*5301*) jak i *Lactobacillus* (Tn*916*/Tn*1545*). Życka-Krzesińska i in. (2015) na podstawie analizy obecności plazmidów i transpozonu Tn*916* u opornych na tetracyklinę szczepów *Lactococcus lactis* wykazali związek genu *tet*(M) występującego u szczepów z tego gatunku z transpozonem Tn*916*, który uczestniczy w przenoszeniu oporności na tetracykliny. Pozwala to sugerować, że bakterie z rodzaju *Lactococcus* mogą stanowić biologiczny rezerwuar genów oporności na tetracykliny. Dowiedziono również, że geny kodujące oporność na makrolidy oraz na tetracykliny są szeroko rozpowszechnione wśród drobnoustrojów właśnie ze względu na ich związek z mobilnymi elementami genetycznymi (Mendonça i in., 2016; Dec i in., 2017). Gen *tet*(M) jest związany z transpozonami koniugacyjnymi z rodziny Tn*916*/Tn*1545*, które mają wysoki potencjał przenoszenia horyzontalnego, *tet*(K) często znajduje się na małych przenośnych plazmidach, podczas gdy *tet*(O) może być zlokalizowany na plazmidzie koniugacyjnym lub na chromosomie. Z kolei wśród genów oporności na makrolidy najbardziej rozpowszechnione są geny *erm*(A), *erm*(B) i *erm*(C). Gen *erm*(B) jest związany z transpozonem koniugacyjnym Tn*916* lub transpozonami z rodziny Tn*916*-Tn*1545*, które charakteryzują się znacznym potencjałem horyzontalnego przenoszenia, z kolei geny *erm*(A) i *erm*(C) są zlokalizowane na chromosomie i na małych plazmidach (Nawaz i in., 2011; Preethi i in., 2017).

Przypuszcza się, że geny oporności na antybiotyki mogą być przenoszone przez bakterie fermentacji mlekowej w obrębie gatunku i rodzaju, ale postuluje się także możliwość przekazywania genów innym mikroorganizmom, zarówno komensalom przewodu pokarmowego, m.in. *Escherichia coli*, jak i mikroorganizmom chorobotwórczym takim jak *Staphylococcus aureus*, *Bacillus subtilis*, a nawet *Listeria monocytogenes* (Thumu i Halami, 2018). Możliwość zajścia międzygatunkowego transferu plazmidów niosących geny oporności między szczepami bakterii fermentacji mlekowej a komensalami przewodu pokarmowego została potwierdzona zarówno w warunkach *in vitro, in situ* w modelu żywności jak i *in vivo* na organizmach modelowych. Zgodnie z literaturą, najczęściej dochodzi do transferu genów oporności na tetracykliny i makrolidy (Preethi i in., 2017; S. C. R. Thumu i Halami, 2019). Warty zaakcentowania jest fakt, że bakterie dodawane do żywności na skalę przemysłową

zwykle występują w produkcie w dużej liczbie, po spożyciu produktu duża liczba żywych komórek dociera do jelita grubego, gdzie wchodzi w kontakt z bytującymi tam mikroorganizmami. Zgodnie z literaturą, duże zagęszczenie komórek w przewodzie pokarmowym człowieka zapewnia doskonałe warunki do poziomego transferu plazmidów koniugacyjnych i transpozonów zawierających geny kodujące oporność na środki przeciwdrobnoustrojowe (Devirgiliis i in., 2013; Nawaz i in., 2011). Mimo iż problematyka transferu genów oporności przez bakterie fermentacji mlekowej jest obecnie dość dobrze poznana, wciąż istnieje niewiele doniesień dotyczących koniugacyjnego przenoszenia determinantów oporności na antybiotyki z bakterii fermentacji mlekowej wykorzystywanych w kulturach przemysłowych do innych drobnoustrojów, co sugeruje potrzebę podjęcia badań w tym zakresie.

1.5. WPŁYW CZYNNIKÓW STRESOWYCH NA ANTYBIOTYKOOPORNOŚĆ DROBNOUSTROJÓW

Ważnym aspektem, który nie był dotychczas poruszany w badaniach naukowych, jest wpływ warunków produkcji i przechowywania żywności na antybiotykooporność szczepów stosowanych w kulturach starterowych lub ochronnych. Mikroorganizmy te są narażone na różne stresy środowiskowe podczas produkcji żywności. Mogą być one związane ze zmianami środowiska w trakcie fermentacji, m.in. obniżenie pH z powodu akumulacji kwasów organicznych lub może wynikać z procesów przetwarzania/utrwalania żywności. Najczęściej wymieniane to: niska temperatura podczas przechowywania w warunkach chłodniczych oraz dodatek NaCl. Obecnie wspomina się również o obróbce wysokociśnieniowej (HPP), obiecującej nowej metodzie nietermicznego utrwalania żywności (Mbye i in., 2020; Ma i in., 2019).

W odpowiedzi na różne warunki stresowe mikroorganizmy opracowały kompleksowe strategie zapobiegania uszkodzeniom powodowanym przez niekorzystne środowisko (Ma i in., 2020; Yang i in., 2021). Z przeprowadzonych dotychczas badań nad wpływem stresów związanych z przemysłem spożywczym na mikroorganizmy wynika, że adaptacja mikroorganizmów do niesprzyjających warunków zwykle wiąże się z indukcją dużej liczby genów, syntezą białek odpowiedzi stresowej i rozwojem oporności krzyżowej. na inne rodzaje stresu. Regulacja tych odpowiedzi jest złożona i ściśle związana ze szlakami metabolicznymi. Inne badania potwierdziły, że bakterie fermentacji mlekowej wykazują wiele reakcji fizjologicznych i adaptacyjnych w niesprzyjających warunkach środowiskowych (Wu i in., 2013). Zazwyczaj ta adaptacja

ma miejsce jako bakteryjna odpowiedź komórkowa na czynnik stresowy poprzez regulację mechanizmów molekularnych. Aktywacja tych mechanizmów może skutkować selekcją wariantów o zwiększonej tolerancji lub oporności na inne czynniki stresowe, w tym zmniejszoną wrażliwość na kilka antybiotyków, w tym antybiotyki ważne w praktyce klinicznej (Al-Nabulsi i in., 2015; Giacometti i in., 2021; McMahon i in., 2007a; Komora i in., 2017). Podaje się również, że ekspozycja na różne warunki stresowe może skutkować mobilizacją niektórych elementów genetycznych, a tym samym zwiększoną częstotliwością transferu genów (Giacometti i in., 2021; McMahon i in., 2007b). Wszystkie te badania przeprowadzono tylko dla mikroorganizmów chorobotwórczych. Nie ma badań dotyczących związku między reakcją na stres a opornością na antybiotyki wśród niepatogennych mikroorganizmów, takich jak bakterie fermentacji mlekowej.

Omówione zagadnienia teoretyczne zostały omówione w publikacji:

P1. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424

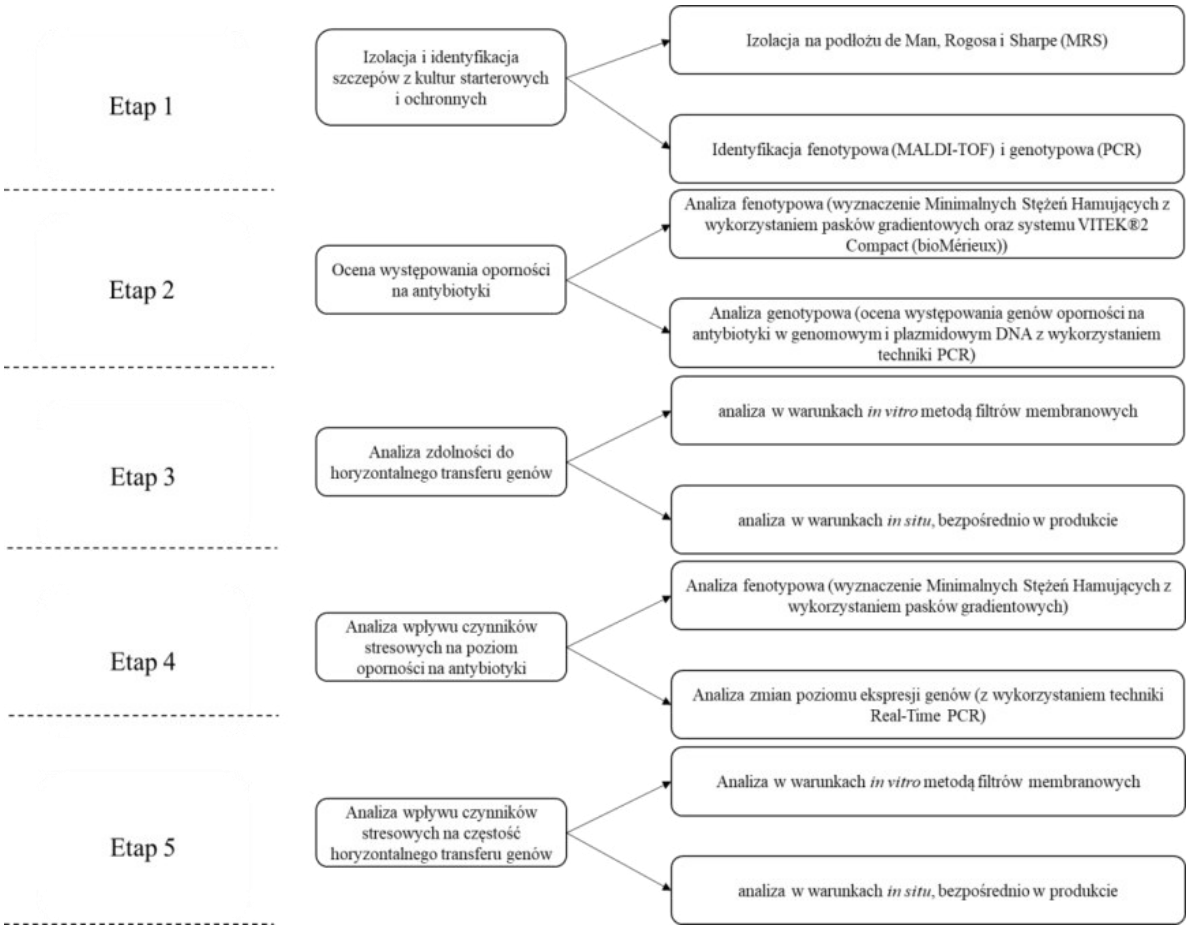
2. CEL I ZAKRES PRACY, HIPOTEZY BADAWCZE

Celem pracy była analiza występowania oporności na antybiotyki wśród szczepów wchodzących w skład komercyjnych kultur starterowych i ochronnych oraz ich zdolność do horyzontalnego transferu genów oporności. Analizie poddano także wpływ czynników stresowych związanych z produkcją i przechowywaniem żywności na zmiany w ekspresji genów kodujących oporność na antybiotyki, jak i częstość transferu genów.

W oparciu o cel badań postawiono następujące hipotezy badawcze:

1. Drobnoustroje wchodzące w skład komercyjnych kultur starterowych i ochronnych mogą być nosicielami genów nabytej oporności na antybiotyki
2. Drobnoustroje te mogą wykazywać zdolność do horyzontalnego transferu genów i przez to brać udział w rozprzestrzenianiu oporności w środowisku
3. Czynniki stresowe związane z produkcją, przetwarzaniem i przechowywaniem żywności mogą prowadzić do zmian w fenotypie oporności na antybiotyki szczepów wchodzących w skład kultur starterowych i ochronnych, ekspresji genów oporności na antybiotyki i/lub częstości transferu genów oporności.

Szczegółowy plan badań przedstawiono poniżej (Ryc. 1).



Ryc. 1 Schemat planu badań

3. MATERIAŁY I METODY

W roku 2020 dokonano zmian w nomenklaturze rodzaju Lactobacillus, w związku z tym w części publikacji stanowiących podstawę niniejszej pracy używane jest stare nazewnictwo, zaś w publikacjach wydanych po zmianie nomenklatury nazewnictwo zostało zaktualizowane. Dla ujednolicenia jednak w tekście pracy używana będzie dawna nomenklatura.

3.1. IZOLACJA I IDENTYFIKACJA SZCZEPÓW

Szczepy do analizy otrzymano z komercyjnych kultur starterowych (n = 30) i ochronnych (n = 10) od kilku producentów oferujących kultury do wykorzystania w przemyśle spożywczym. Liofilizowane kultury ożywiano na bulionie MRS (Merck, Niemcy) w warunkach beztlenowych przez 24 – 48 h w temperaturze 30^{o}C, a następnie izolowano na podłożu agarowym MRS (Merck, Niemcy) do uzyskania czystych szczepów. Następnie przeprowadzono ekstrakcję genomowego i plazmidowego DNA za pomocą zestawów Genomic Mini DNA oraz Plasmid Mini (A&A Biotechnology, Polska), zgodnie z instrukcjami producenta. Otrzymane próbki DNA przechowywano

w temperaturze -20°C. Szczepy przechowywano w temperaturze -80°C w systemie Microbank (Biocorp, Polska), aby zminimalizować ryzyko utraty plazmidów podczas pasażowania.

Identyfikację szczepów przeprowadzono w celu odróżnienia szczepów zawartych w każdej kultur. Szczepy zidentyfikowano fenotypowo za pomocą techniki MALDI-TOF MS w trzech powtórzeniach, zgodnie z zaleceniami producenta. Do walidacji metody użyto szczepu referencyjnego *Escherichia coli* ATCC® 8739.

Ponieważ analiza MALDI-TOF nie umożliwia rozróżnienia gatunków *Lactobacillus pentosus*, *L. plantarum* i *L. paraplantarum* oraz podgatunków *Lactococcus lactis*, w tym celu zastosowano różnicowanie metodą PCR z wykorzystaniem specyficznych starterów i warunków reakcji. Jako kontrole pozytywne w reakcjach PCR wykorzystano szczepy referencyjne *L. pentosus* (ATCC® 8041) i *L. plantarum* (ATCC® 14917™) oraz *L. lactis* ssp. *lactis* (ATCC® 87139) i *L. lactis* ssp. *cremoris* (ATCC® 9596).

Produkty PCR rozdzielano za pomocą elektroforezy na 1,5% żelach agarozowych w buforze 1xTBE wybarwionych roztworem Midori green (0,5 μg/ml) (ABO, Polska) przy 100 V przez 6 min. a następnie przy 80 V przez 90 min. i wizualizowano za pomocą systemu do dokumentacji i analizy barwionych fluorescencyjnie żeli G-BOX F3 (Syngene, UK).

Po identyfikacji zduplikowane szczepy odrzucono i do dalszych analiz wybrano po jednym szczepie danego gatunku od każdego producenta.

3.2. GENOTYPOWANIE SZCZEPÓW

Wszystkie szczepy genotypowano przy użyciu techniki ERIC-PCR. Produkty PCR rozdzielano za pomocą elektroforezy na 1,5% żelach agarozowych w buforze 1xTBE wybarwionych roztworem Midori green (0,5 μg/ml) (ABO, Polska) przy 150 V przez 150 min. i wizualizowano za pomocą systemu do dokumentacji i analizy barwionych fluorescencyjnie żeli G-BOX F3 (Syngene, UK).

Profile ERIC-PCR analizowano za pomocą analizy skupień z wykorzystaniem oprogramowania BioNumerics ver. 7.6.3. (Applied Maths/bioMérieux, Sint-Martens-Latem, Belgia). Odległości podobieństw między profilami ERIC-PCR obliczono za pomocą współczynnika Dice, a dendrogram oparto na metodzie grup nieważonych par ze średnią arytmetyczną UPGMA.

3.3. OKREŚLENIE FENOTYPU OPORNOŚCI NA ANTYBIOTYKI

Minimalne stężenia hamujące wyznaczono dla 9 antybiotyków: ampicylina (AMP), wankomycyna (VA), gentamycyna (CN), kanamycyna (KA), streptomycyna (S), erytromycyna (E), klindamycyna (DA), tetracyklina (TE) i chloramfenikolu (C). Zastosowano dwie metody oznaczania: za pomocą pasków gradientowych (Liofilchem, Włochy) na podłożu agarowym Mueller-Hinton (Merck, Niemcy) oraz przy użyciu systemu VITEK®2 Compact do oznaczania wrażliwości drobnoustrojów (Biomerieux, Durham, Karolina Północna, USA) z kartami AST–P643. Wartości MIC odczytywano po 18 h inkubacji w temp. 30°C. Ze względu na brak aktualnych wytycznych dotyczących oznaczania antybiotykooporności wśród szczepów bakterii kwasu mlekowego, wyniku nie interpretowano w kategoriach wrażliwy/oporny. Podane zostały jedynie wartości MIC określone dla poszczególnych antybiotyków.

3.4. ANALIZA WYSTĘPOWANIA GENÓW OPORNOŚCI NA ANTYBIOTYKI

Wszystkie szczepy przebadano na obecność sześciu genów oporności na tetracyklinę, czterech kodujących białka chroniące rybosom – *tet*M, *tet*O, *tet*S i *tet*W oraz dwóch kodujących pompy efflux – *tet*K i *tet*L. Każdy ze szczepów był analizowany także pod kątem obecności genów kodujących oporność na aminoglikozydy - *aac(6')-Ie-aph(2'')-Ia*, *aph(3'')-IIIa*, *aph(2'')-Ic*, *aac(6')-Ii* and *ant(6')-Ia*, chloramfenikol - *cat*, β - laktamy – *bla*Z, bla_{TEM}, bla_{OXA}, bla_{SHV} i bla_{CTX-M}, glikopeptydy – *van*A i *van*B oraz makrolidy – *erm*A, *erm*B, *erm*C i *msr*A/B. Ponadto użyto specyficznej pary starterów do amplifikacji genu związanego z obecnością transpozonów koniugacyjnych Tn*916*/Tn*1545*. Testy obecności genów przeprowadzono z wykorzystaniem reakcji PCR przy użyciu określonych starterów i warunków. Jako kontrole pozytywne zastosowano szczepy referencyjne *E. faecalis* ATCC® 29212 (*tet*M), *E. faecalis* ATCC® 51299 (*erm*B, *aac(6')-Ie-aph(2'')-Ia*), *S. epidermidis* ATCC® 35984 (*aph(3')-IIIa*, *erm*A, *bla*Z, *ant(6')-la*), *S. epidermidis* ATCC® 12228 (*bla*Z, *tet*K) i szczepy z kolekcji Katedry Mikrobiologii Przemysłowej i Żywności dobrze scharakteryzowane pod kątem występowania genów oporności na antybiotyki.

Rozdział i wizualizację produktów reakcji PCR przeprowadzono tak, jak opisano w punkcie 3.1.

3.5. ANALIZA TRANSFERU GENÓW OPORNOŚCI

Pierwszym etapem analizy transferu genów było wyznaczenie puli dawców. W tym celu sprawdzono, czy zidentyfikowane wcześniej geny oporności były zlokalizowane w chromosomach czy na plazmidach. Na dawców można było wybrać szczepy, które posiadały jednocześnie co najmniej jeden gen oporności zlokalizowany na plazmidzie i wykazywały wysoką wartość MIC dla danego antybiotyku.

Standardowym biorcą w eksperymencie był szczep *E. faecalis* JH2–2 (LMG 19456), wolny od plazmidów, oporny na rifampicynę (MIC>256 μg/ml) i kwas fusydowy (MIC>256 μg/ml).

3.5.1. TRANSFER *IN VITRO*

Zdolność do przenoszenia genów oporności na antybiotyki *in vitro* badano z wykorzystaniem metody filtrów membranowych. W tym celu zmieszano po 1 ml kultury dawcy i biorcy w wykładniczej fazie wzrostu i przefiltrowano przez sterylny filtr membranowy z celulozy o średnicy porów 0,45 μm. Następnie filtry przepłukane 2 ml sterylnego roztworu PPS (Merck, Niemcy) umieszczono na podłożu agarowym BHI i inkubowano przez 24 h w temperaturze 37^{o}C. Po inkubacji kolonie wyhodowane na powierzchni filtra zawieszono w 2 ml PPS, wykonano serie 10-krotnych rozcieńczeń i wysiano na 3 rodzaje pożywek: T-BHI dla transkoniugantów (z dodatkiem 100 μg/ml kwasu fusydowego, 50 μg/ml ryfampicyny i 10 μg/ml tetracykliny), TD-BHI dla dawców (z dodatkiem 10 μg/ml tetracykliny) i B-BHI dla biorców (z dodatkiem 100 μg/ml kwasu fusydowego i 50 μg/ml ryfampicyny). Inkubację prowadzono w temperaturze 37^{o}C przez 24-48 godzin. Częstość transferu genów wyrażono jako stosunek liczby transkoniugantów do liczby biorców.

3.5.2. TRANSFER *IN SITU*

W celu oceny możliwości transferu genów *in situ,* tj. bezpośrednio w produkcie, przygotowano model zakwaszonego mleka. Hodowle dawców i biorców przygotowano identycznie jak w analizie *in vitro*, jednak hodowlę prowadzono nie na podłożu BHI, lecz w mleku. Odtłuszczone mleko w proszku (4% wag./obj.) dodano do 10 ml pasteryzowanego mleka i ogrzewano w temperaturze 63^{o}C przez 30 minut, a następnie schłodzono do temperatury 42^{o}C. Następnie do mleka dodawano po 1 ml hodowli dawcy i biorcy i inkubowano w temperaturze 42°C do uzyskania pH 4,3. Otrzymaną mieszaninę skoagulowanego mleka następnie inkubowano w temperaturze 4^{o}C przez 48 godzin. Po inkubacji wykonane 10-krotne rozcieńczenia próbek wysiano na podłoża, jak opisano

powyżej. Płytki inkubowano przez 24-48 godzin w temperaturze 37°C i częstość transferu wyrażano identycznie jak opisano powyżej.

3.5.3. POTWIERDZENIE TRANSFERU GENÓW, GENOTYPOWANIE TRANSKONIUGANTÓW

Otrzymane transkoniuganty sprawdzono pod kątem ich oporności na antybiotyki z wykorzystaniem pasków gradientowych (Liofilchem, Włochy). Wartość MIC odczytywano po 18 h inkubacji w temperaturze 30°C. Następnie wyizolowano genomowe DNA za pomocą zestawu Genomic Mini (A&A Biotechnology, Polska) i przeprowadzono powtórne reakcje PCR w celu potwierdzenia transferu genów. Genotypowanie transkoniugantów, u których stwierdzono, że nabyły geny oporności, uzyskano w reakcji ERIC-PCR, jak opisano powyżej. Uzyskane profile porównywano z profilami szczepów biorców w celu potwierdzenia transferu genów.

3.6. ODDZIAŁYWANIE CZYNNIKÓW STRESOWYCH ZWIĄZANYCH Z PRZEMYSŁEM SPOŻYWCZYM NA DROBNOUSTROJE

3.6.1. ODDZIAŁYWANIE CZYNNIKÓW STRESOWYCH

Na podstawie przeprowadzonych badań wstępnych wybrano po jednej wartości stężenia NaCl, pH i temperatury chłodniczej oraz dwie wartości wysokiego ciśnienia, które znacząco, ale nie całkowicie, zmniejszyły liczbę komórek w stosunku do próbki nie poddanej działaniu ciśnienia. Ekspozycję na stres kwasowy, osmotyczny i związany z chłodniczym przechowywaniem prowadzono w sterylnych probówkach Falcon z 20 ml bulionu MRS (Merck, Niemcy), do którego dodawano hodowle badanych szczepów w wykładniczej fazie wzrostu (OD_{590} ~ 0,6) w ilości 250 µl. Następnie, aby sprawdzić wpływ stresu kwasowego, do probówek dodano kwas mlekowy do uzyskania wartości pH 4,0 i inkubowano w temperaturze 30°C przez 48 h beztlenowo. W celu sprawdzenia wpływu stresu osmotycznego do kolejnych probówek dodano NaCl do uzyskania stężenia 4,0% i inkubowano beztlenowo w temperaturze 30°C przez 48 godzin. Sterylność dodawania kwasu mlekowego i NaCl zapewniono filtrując dodatki na filtrach strzykawkowych i wprowadzając je do sterylnego podłoża pod komorą laminarną. W celu sprawdzenia efektu chłodniczego przechowywania probówki z zaszczepioną pożywką przeniesiono do lodówki i przechowywano w temperaturze 7°C przez 48 godzin. Dodatkowo przygotowano próbki kontrolne – zaszczepiony bulion MRS bez dodatków, inkubowany w optymalnych warunkach. Test przeprowadzono w trzech powtórzeniach w różnych dniach.

Ekspozycji na wysokie ciśnienia poddawano po 10 ml hodowli badanych szczepów w stacjonarnej fazie wzrostu w bulionie MRS (Merck, Darmstadt, Niemcy). Ciśnieniowanie przeprowadzano w roztworze glikol-woda (1:1, obj./obj.) przy użyciu jednokomorowej aparatury wysokociśnieniowej U4040 (IWC PAN, Warszawa, Polska, Unipress Equipment Division) przy temperaturę 20 ± 3°C, szybkości przyrostu ciśnienia 300 MPa/min oraz czasie obniżania ciśnienia mniejszym niż 5 s.

Po zakończeniu obróbki stresowej próbki od razu poddawano kolejnym etapom badań.

Następnie w dalszej części analizy skupiono się jedynie na wpływie obróbki wysokociśnieniowej na drobnoustroje. Poszerzono jednak analizę o różne warianty wartości ciśnienia i czasu trwania procesu, aby sprawdzić, w jaki sposób efekt oddziaływania HPP na drobnoustroje zależy od warunków procesu.

3.6.2. ANALIZA PRZEŻYWALNOŚCI

3.6.2.1. LIVE/DEAD

Analizie poddano próbki 1 ml hodowli każdej z prób badanych oraz prób kontrolnych w logarytmicznej fazie wzrostu w bulionie MRS (Merck, Niemcy). Do analizy wykorzystano zestaw Survey of Live/Dead® BacLight™ Bacterial Viability Kit (ThermoFischer Scientific, Waltham, Massachusetts, USA) zgodnie z instrukcjami producenta.

Analizy przeprowadzono na cytometrze przepływowym BD FACSLyric™ (Becton Dickinson, San Jose, CA, USA) zgodnie z metodyką (Habtewold i in., 2016).

3.6.2.2. METODA PŁYTKOWA

Przeżywalność szczepów sprawdzono również metodą płytkową. W tym celu wykonano serie dziesięciokrotnych rozcieńczeń dla każdego szczepu przed i po ekspozycji na czynnik stresowy oraz oznaczono liczbę CFU/ml na agarze MRS. Liczbę kolonii zliczono po 48 h inkubacji w warunkach beztlenowych w temperaturze 30°C.

3.7. WPŁYW CZYNNIKÓW STRESOWYCH NA ZMIANY W OPORNOŚCI NA ANTYBIOTYKI

Do analizy wpływu czynników stresowych na poziom oporności na antybiotyki wybrano te spośród szczepów, u których stwierdzono obecność genu oporności na antybiotyki przy jednoczesnej niskiej wartości MIC dla danego antybiotyku. Każdy z badanych szczepów został poddany działaniu następujących czynników stresowych:

niskiego pH, podwyższonego stężenia NaCl, niskiej temperatury przechowywania oraz obróbki wysokociśnieniowej. Zastosowane w badaniach wartości czynników stresowych dobrano na podstawie dostępnej literatury, w której wskazano wartości najczęściej występujących w żywności czynników stresowych oraz na podstawie przeprowadzonych badań wstępnych.

3.7.1. ANALIZA ZMIAN W FENOTYPIE OPORNOŚCI NA ANTYBIOTYKI

Po ekspozycji na czynniki stresowe odzyskano komórki bakteryjne i sprawdzono ponownie wartości MIC dla analizowanych antybiotyków. Analizę przeprowadzono identycznie jak opisano w punkcie 3.3. Następnie przywrócono optymalne warunki wzrostu (szczepy hodowano w pożywkach o optymalnym składzie i inkubowano w optymalnych warunkach) i ponownie sprawdzono wartości MIC, aby zobaczyć, czy zmiany się utrzymują.

3.7.2. ANALIZA ZMIAN EKSPRESJI GENÓW OPORNOŚCI

Całkowite RNA izolowano z hodowli bakteryjnych za pomocą zestawu Total RNA Mini Plus (A&A Biotechnology, Polska) w trzech powtórzeniach dla każdej próbki zgodnie z instrukcją producenta. Otrzymane próbki RNA oczyszczono i zatężono przy użyciu zestawu Clean-Up RNA Concentrator (A&A Biotechnology, Polska). Wszystkie próbki sprawdzono pod kątem jakości wyizolowanego RNA poprzez rozdział elektroforetyczny 10 µl próbki na 1,2% żelu agarozowym w 0,5% buforze TBE pod napięciem 90 V przez 1 godzinę, a następnie zweryfikowano przez wizualizację dwóch prążków (16S i 23S RNA) z wykorzystaniem systemu do dokumentacji i analizy barwionych fluorescencyjnie żeli G-BOX F3 (Syngene, Cambridge, UK). Stężenie RNA mierzono za pomocą Spektrofotometru/Fluorometru DeNovix DS-11 FX (DeNovix Inc., Wilmington, USA), w oparciu o absorbancję próbki przy długości fali 260 nm i 280 nm. Wszystkie próbki RNA zostały natychmiast użyte do syntezy pierwszej nici cDNA przy użyciu zestawu Transcriba Kit (A&A Biotechnology, Polska) zawierającego rekombinowaną odwrotną transkryptazę MMLV, a jako startery zastosowano heksamery o losowej sekwencji.

Analizę ekspresji genów przeprowadzono z wykorzystaniem ilościowej rekcji PCR (ang. quantitative PCR – qPRC) w czasie rzeczywistym - Real-Time PCR z trzema niezależnymi reakcjami dla każdego powtórzenia. Reakcje przeprowadzono przy użyciu systemu RotorGene Q (Qiagen, Niemcy). Pomiary były oparte na metodzie fluorometrycznej przy użyciu barwnika SYBR Green. Reakcje qPCR były prowadzone

z wykorzystaniem specyficznych primerów i warunków reakcji. Dla każdego z genów badanych na podstawie literatury i badań wstępnych dobrano gen referencyjny (ang. housekeeping gene – HG), czyli gen metabolizmu podstawowego, którego ekspresja jest niezmienna. W toku badań rolę genów referencyjnych pełniły następujące geny: 16S rDNA, 23S rRNA i *gap*. Analizie poddano ekspresję następujących genów oporności na antybiotyki: *aac(6')Ie-aph(2'')Ia, aph(3')-IIIa* i *tet*M w pierwszej analizie oraz genów: *aac(6')Ie-aph(2'')Ia, aph(3')-IIIa, tet*M, *tet*W, *bla*Z i *cat* w analizie skupionej jedynie na wpływie HPP. W analizie tej badano także zmiany ekspresji genów ogólnej odpowiedzi na stres: *clp*L, *gro*EL i *dna*K. Specyficzność każdego produktu qPCR sprawdzono na podstawie krzywej topnienia sporządzonej przez ogrzewanie z powolną zmianą temperatury między 60 a 95°C co 0,5°C przez 5 sekund.

Do wyliczenia różnic w ekspresji genów wykorzystano metodę porównawczą CT ($2^{-\Delta\Delta CT}$) (Livak i Schmittgen, 2001). W tej metodzie, obliczony wzór $2^{-\Delta\Delta Ct}$ wylicza proporcję ekspresji danego genu w porównaniu z genem metabolizmu podstawowego (HG).

3.8. WPŁYW CZYNNIKÓW STRESOWYCH NA CZĘSTOŚĆ TRANSFERU GENÓW OPORNOŚCI

Wpływ czynników stresowych na częstość transferu genów oporności podobnie jak zmiany w oporności na antybiotyki analizowano w dwóch etapach badania. W pierwszej części analizie poddano wpływ podwyższonego stężenia NaCl, obniżonego pH i niskiej temperatury przechowywania oraz dwóch wartości wysokiego ciśnienia na częstość transferu genów *aph(3')-IIIa*, *aac(6')-Ie-aph(2'')-Ia, tet*M i *tet*O w warunkach *in vitro*. Następnie przeprowadzono analizę skupiającą się na wpływie różnych wariantów wartości ciśnienia i czasu trwania procesu na częstość transferu genów: *tetM*, *aph(3')-IIIa*, *aac(6')-Ie-aph(2'')-Ia*, *bla*Z i *cat* w warunkach *in vitro* jak i *in situ.*

Pulę dawców w obu etapach analizy stanowiły szczepy będące nosicielami przynajmniej jednego zlokalizowanego na plazmidzie genu oporności na antybiotyki przy jednoczesnej wysokiej wartości MIC dla danego antybiotyku, zaś standardowym biorcą był szczep *E. faecalis* JH2–2 (LMG 19456), wolny od plazmidów, oporny na rifampicynę (MIC>256 μg/ml) i kwas fusydowy (MIC>256 μg/ml).

Zdolność do przenoszenia genów oporności na antybiotyki *in vitro* przez filtrację i *in situ* w produkcie prowadzono w sposób opisany w punkcie 3.5.1 i 3.5.2. Analizie poddano szczepy odzyskane po ekspozycji na warunki stresowe. Inkubację prowadzono beztlenowo przez 48 godzin w temperaturze 30°C. Zastosowano trzy rodzaje pożywek

selekcyjnych na bazie podłoża BHI (Merck, Niemcy): T-BHI dla transkoniugantów (z dodatkiem 100 µg/ml kwasu fusydowego, 50 µg/ml rifampicyny i jednego z badanych antybiotyków: 10 µg/ml tetracykliny, 10 µg/ml chloramfenikolu, 1 µg/ml gentamycyny, 1 µg/ml kanamycyny, 1 µg/ml streptomycyny lub 1 µg/ml ampicyliny), TD-BHI dla dawców (z dodatkiem 10 µg/ml tetracykliny, 10 µg/ml chloramfenikolu, 1 µg/ml gentamycyny, 1 µg/ml kanamycyny, 1 µg/ml streptomycyny lub 1 µg/ml ampicyliny) i B-BHI dla biorców (z dodatkiem 100 µg/ml kwasu fusydowego i 50 µg/ml ryfampicyny). Następnie po wyliczeniu częstości transferu genów przeprowadzono badania mające na celu potwierdzenie zajścia transferu genów, jak i genotypowanie transkoniugantów tak, jak opisano w punkcie 3.5.3.

3.9. ANALIZY STATYSTYCZNE

Analizy statystyczne uzyskanych wyników prowadzono z wykorzystaniem dwóch programów: STATISTICA 13.3 StatSoft® software (Tibco, Inc., Tulsa, OK, USA) oraz Prism 9.3.1. (Prism, GraphPad Software, San Diego). Do analizy wyników wykorzystywano testy: t-Studenta, jednoczynnikową analizę ANOVA, test U – Manna-Whitneya oraz korelację Spermana w zależności od specyfiki analizy. Założony poziom istotności wynosił 0,05.

Szczegółowy opis zastosowanej metodyki przedstawiono w publikacjach, będących podstawą niniejszej pracy.

3.1. – 3.5. – w publikacji:

P2. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zadernowska A. 2022. Microorganisms from starter and protective cultures - Occurrence of antibiotic resistance and conjugal transfer of *tet* genes *in vitro* and during food fermentation. LWT–Food Science and Technology, 153, 112490. https://doi.org/10.1016/j.lwt.2021.112490

3.6. – w publikacji:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

3.7. – w publikacjach:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

P4. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. Effect of high pressure processing on changes in antibiotic resistance genes expression among strains from commercial starter cultures. Food Microbiology, 110, 104169. https://doi.org/10.1016/j.fm.2022.104169

3.8. – w publikacjach:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

P5. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. High-pressure processing effect on conjugal antibiotic resistance genes transfer *in vitro* and in the food matrix among strains from starter cultures. International Journal of Food Microbiology, 388, 110104. https://doi.org/10.1016/j.ijfoodmicro.2023.110104

4. WYNIKI

4.1. OMÓWIENIE PULI SZCZEPÓW ZAKWALIFIKOWANYCH DO ANALIZY

Z 30 dostępnych na rynku kultur starterowych i 10 kultur ochronnych uzyskano łącznie 58 szczepów. Największa liczba szczepów (n = 44; 75,9%) pochodziła z kultur starterowych, 14 izolatów (24,1%) odzyskano z kultur ochronnych. Największa liczba szczepów należała do dawnego rodzaju *Lactobacillus* (n = 26; 44,9%) – w tym według obecnej nomenklatury: *Lactobacillus* (n = 10), *Lacticaseibacillus* (n = 7), *Lactiplantibacillus* (n = 5), *Latilactobacillus* (n = 3) oraz *Lentilactobacillus* (n = 1); następnie *Lactococcus* (n = 17; 29,3%), *Pediococcus* (n = 8; 13,8%), *Streptococcus* (n = 4; 6,9%), *Staphylococcus* (n = 2; 3,4%) i *Leuconostoc* (n = 1; 1,7%) (P2, Table 1). Genotypowanie za pomocą ERIC-PCR wykazało niską różnorodność genetyczną wśród szczepów (P2, Fig. 1).

4.2. WYSTĘPOWANIE OPORNOŚCI NA ANTYBIOTYKI WŚRÓD DROBNOUSTROJÓW Z KULTUR STARTEROWYCH I OCHRONNYCH

Wśród wszystkich badanych szczepów najwyższe wartości MIC odnotowano dla tetracykliny (maksymalna wartość MIC - >256 µg/ml), kanamycyny (maksymalna wartość MIC - 48 µg/ml) oraz chloramfenikolu (maksymalna wartość MIC - 32 µg/ml). Najniższe wartości MIC odnotowano dla erytromycyny i streptomycyny (wszystkie wartości MIC były najniższe od 1 µg/ml). Największe spektrum wartości MIC zaobserwowano dla tetracykliny (0,19 - >256 µg/ml). Nie stwierdzono statystycznie istotnych różnic w obserwowanych wartościach MIC dla każdego antybiotyku w zależności od rodzaju bakterii lub źródła szczepu (P2, Table 2).

Niezależnie od prezentowanego fenotypu oporności, u wszystkich szczepów analizowano obecność 15 różnych determinantów oporności. Obecność genów *van*A i *van*B sprawdzano tylko u szczepów *Lactococcus*, *Streptococcus* i *Staphylococcus* ze względu na wrodzoną oporność na wankomycynę u szczepów *Lactobacillus*, *Leuconostoc* i *Pediococcus*.

Niezależnie od rodzaju, najczęstszym genem kodującym oporność na aminoglikozydy był gen *aph(3')-IIIa*, następnie geny *aac(6')-Ie-aph(2')-Ia*, *aac(6')-Ia i aph(2')-Ic*. Żaden ze szczepów nie wykazywał obecności genu *ant(6')-la*.

Częstość występowania różnych genów oporności na tetracykliny zależała od rodzaju. Wśród szczepów *Lactobacillus* oporność na tetracyklinę była najczęściej związana z obecnością genu *tet*M, następnie *tet*W i *tet*O. Śród szczepów *Lactococcus*, *Pediococcus* i *Staphylococcus* geny *tet*M i *tet*W były przenoszone przez ten sam odsetek szczepów, a gen *tet*O dominował wśród szczepów *Streptococcus*.

Ponad połowa szczepów (55,2%) posiadała gen *cat* kodujący oporność na chloramfenikol. Wśród analizowanych genów kodujących oporność na antybiotyki β-laktamowe dwa szczepy były nosicielami genów bla_{OXA} i *bla*Z. Spośród czterech określonych genów oporności na makrolidy, stwierdzono, że tylko jeden szczep *Latobacillus* jest nosicielem genu *msr*A/B. Pozostałych genów nie oznaczono w żadnym ze szczepów. W żadnym ze szczepów nie stwierdzono także genów oporności na wankomycynę (*van*A i *van*B). Transpozon koniugacyjny z rodziny Tn*916*/Tn*1545* był obecny tylko w czterech izolatach (P2, Table 3).

Analiza statystyczna wykazała istotne różnice w częstości występowania genów: *aph(3')-IIIa*, *aac(6')-Ie-aph(2')-Ia*, *aac(6')-la*, *tet*M, *tet*O, *tet*W i *int* w zależności od rodzaju drobnoustrojów. Analiza częstości występowania genów oporności w zależności od pochodzenia szczepu nie wykazała różnic.

4.3. ZDOLNOŚĆ BAKTERII FERMENTACJI MLEKOWEJ DO HORYZONTALNEGO TRANSFERU GENÓW

Do analizy zdolności bakterii fermentacji mlekowej do horyzontalnego transferu genów oporności wybrano pięć szczepów posiadających zlokalizowany na plazmidzie gen oporności na tetracyklinę (trzy szczepy posiadające gen *tet*K i dwa posiadające gen *tet*M), które pełniły rolę dawców (P2, Table 4).

Możliwość zajścia transferu genów zaobserwowano dla każdego ze szczepów. W metodzie *in vitro* częstość transferu wahała się od $2{,}78 \times 10^{-7}$ do $1{,}71 \times 10^{-5}$ transkoniugantów na biorcę. Wartości MIC tetracykliny dla transkoniugantów mieściły się w zakresie od 24 do 64 µg/ml. W metodzie *in situ* przenoszenie genów obserwowano również dla każdego szczepu, ale częstotliwość przenoszenia była niższa niż w przypadku transferu w warunkach *in vitro* i wahała się od $1{,}36 \times 10^{-8}$ do $1{,}07 \times 10^{-7}$. Wartości MIC tetracykliny dla transkoniugantów mieściły się w zakresie od 12 do 48 µg/ml. Wartości MIC dla tetracykliny były niższe lub równe wartościom MIC wyznaczonym dla dawców z wyjątkiem transferu od szczepów *Pediococcus pentosaceus* LAB-17 i *Lactobacillus rhamnosus* LAB-28, gdzie wartości MIC dla transkoniugantów uzyskanych w transferze *in vitro* były wyższe niż u dawców (P2, Table 5).

Genotypowanie metodą ERIC-PCR ujawniły podobne profile biorców i transkoniugantów. Można zatem wykluczyć, że oporność została nabyta przez mutację w genomie dawcy, co potwierdza klonalny charakter transkoniugantów (P2, Fig. 2).

4.4. PRZEŻYWALNOŚĆ W ODPOWIEDZI NA CZYNNIKI STRESOWE ZWIĄZANE Z PRZETWÓRSTWEM ŻYWNOŚCI

Analizę przeżywalności szczepów w odpowiedzi na czynniki stresowe przeprowadzono metodą cytometrii przepływowej i metodą płytkową. Uzyskane wyniki wskazują, że badane szczepy charakteryzowały się najwyższą przeżywalnością w obecności 4% stężenia NaCl (powyżej 90%). Najniższą przeżywalność zaobserwowano w odpowiedzi na ciśnienie 400 MPa (poniżej 50%). Należy również zauważyć, że wyniki uzyskane metodą płytkową różniły się od wyników uzyskanych w analizie cytometrycznej. Analiza liczby metodą płytkową wykazała wyższy procent redukcji liczby CFU/ml w przypadku każdego z czynników stresowych (P3, Table 2).

4.5. WPŁYW CZYNNIKÓW STRESOWYCH NA ZMIANY W FENOTYPIE ANTYBIOTYKOOPORNOŚCI I EKSPRESJI GENÓW OPORNOŚCI NA ANTYBIOTYKI

4.5.1. ZMIANY W FENOTYPIE OPORNOŚCI

Analiza wpływu różnych czynników stresowych na fenotyp i ekspresję genów oporności na antybiotyki wykazała, że żaden z zastosowanych czynników nie wywołał istotnej statystycznie zmiany w oporności na streptomycynę u żadnego ze szczepów. Zaobserwowano znaczny wzrost wartości MIC dla gentamycyny i kanamycyny po ekspozycji na niskie pH i zwiększone stężenie NaCl (tj. od 0,5 µg/ml do 8 µg/ml), podczas gdy ekspozycja na niską temperaturę przechowywania oraz wysokie ciśnienia wywołała istotną redukcję wartości MIC dla tych antybiotyków (tj. od 0,5 µg/ml do 0,125 µg/ml). Oporność na tetracyklinę nie zmieniła się po ekspozycji na czynnik stresowy wśród szczepów, dla których wyjściowo stwierdzono wysoką wartość MIC. Wśród szczepów, które początkowo wykazywały niską wartość MIC dla tetracykliny, wartość MIC znacznie wzrosła po ekspozycji na niskie pH, wysokie stężenie NaCl i wysokie ciśnienie (tj. od 1 µg/ml do 8 µg/ml). Po ekspozycji na niską temperaturę wartość MIC znacznie się obniżyła (tj. z 1 µg/ml do 0,25 µg/ml). Należy również zaznaczyć, że zmiany te utrzymywały się nawet po przywróceniu optymalnych warunków (P3, Table 3).

Po zawężeniu analizy jedynie do wpływu różnych wariantów obróbki wysokociśnieniowej stwierdzono, że po ekspozycji na czynnik stresowy dochodziło

do zmian w fenotypie oporności na antybiotyki. Zaobserwowano, że wartości MIC dla chloramfenikolu i ampicyliny wzrastały w każdym z wariantów ciśnieniowania, jednak najbardziej wzrosły po zastosowaniu ciśnienia 300 MPa przez 3 minuty (np. z 32 µg/ml do 192 µg/ml dla chloramfenikolu i z 3 µg/ml do 8 µg/ml dla ampicyliny). Wartości MIC dla antybiotyków aminoglikozydowych (gentamycyny i kanamycyny) zmniejszyły się po ekspozycji na każdy z parametrów ciśnieniowania (np. od 2 µg/ml do 0,75 µg/ml). Co ważne, w przypadku aminoglikozydów nie zaobserwowano różnic w wartościach MIC pomiędzy próbkami, niezależnie od zastosowanych wartości ciśnienia i czasu trwania procesu. W przypadku oporności na tetracykliny przy wysokiej początkowej wartości MIC (> 30 µg/ml) nie obserwowano zmian wartości MIC niezależnie od ciśnienia i czasu trwania procesu. Przeanalizowano dwa szczepy o niskich wartościach MIC dla tetracykliny (1 µg/ml i 12 µg/ml). Dla tych szczepów zaobserwowano znaczny wzrost wartości MIC po zastosowaniu obróbki wysokociśnieniowej (np. z 12 µg/ml do 64 µg/ml). Warto dodać, że wartości MIC dla tetracykliny po obróbce wysokociśnieniowej nie różniły się istotnie między próbkami niezależnie od zastosowanej wartości ciśnienia i czasu trwania procesu (P4, Table 4).

4.5.2. ZMIANY W EKSPRESJI GENÓW OPORNOŚCI

Analiza wyników ilościowych reakcji PCR wykazała, że wyniki poziomu ekspresji genów korelowały z wynikami uzyskanymi w analizie fenotypowej. Ekspresja genów oporności na aminoglikozydy (*aac(6')-Ie-aph(2'')-Ia* i aph(3')-*IIIa*) istotnie wzrosła w odpowiedzi na niskie pH i zwiększone stężenie NaCl (względny poziom ekspresji genów > 2,00). Po ekspozycji na niską temperaturę przechowywania i wysokie ciśnienia odnotowano obniżenie poziomu ekspresji genów, ale w większości zmiany te nie były istotne statystycznie (względny poziom ekspresji genów > 0,5). Wyjątkiem były szczepy LAB-39 po ekspozycji na stres związany z chłodniczym przechowywaniem i HPP, LAB-43 w odpowiedzi na ciśnienie 200 MPa oraz LAB-3 i LAB-46b w odpowiedzi na ciśnienie 400 MPa. W tych przypadkach obniżenie poziomu ekspresji genów było znaczące (względny poziom ekspresji genów <0,5). Poziom ekspresji genu *tet*M kodującego oporność na tetracyklinę u szczepów LAB-46b i LAB-8 wzrastał w odpowiedzi na każdy z czynników stresowych poza niską temperaturą. Po ekspozycji na stres zimna zanotowano nieznaczne obniżenie poziomu ekspresji (P3, Fig. 2).

Analiza ekspresji genów skupiająca się jedynie na wpływie różnych wariantów ciśnieniowania wykazała, zgodność między wynikami uzyskanymi w analizie fenotypowej a wynikami ekspresji genów. Uzyskane wyniki wskazują, że ekspresja

genów oporności na aminoglikozydy znacznie zmniejszyła się po ekspozycji na wysokie ciśnienia (względny poziom ekspresji genów < 0,5). Dla żadnego z genów nie zaobserwowano istotnych różnic między poziomami ekspresji w zależności od warunków ciśnieniowania. Jednocześnie zaobserwowano znaczny wzrost ekspresji genów oporności na tetracykliny, z czego największy wzrost poziomu ekspresji odnotowano dla genu *tet*W. W przypadku genu *tet*M nie zaobserwowano różnic między poziomami ekspresji w zależności od wariantu ciśnieniowania. Natomiast dla genu *tet*W poziom ekspresji po zastosowaniu wariantu 400 MPa/3 min. był znacznie niższy niż poziomy ekspresji w pozostałych wariantach procesu. Ekspresja genów kodujących oporność na ampicylinę (*bla*Z) i chloramfenikol (*cat*) również wzrosła po zastosowaniu wysokiego ciśnienia. Co ważne, wzrost ekspresji był największy po zastosowaniu wariantu 300 MPa/3 min, a wraz ze wzrostem wartości ciśnienia i czasu trwania procesu poziom ekspresji nieznacznie obniżał się.

Przeprowadzona dodatkowo analiza ekspresji genów odpowiedzi na stres: *dna*K, *gro*EL i *clp*L wykazała, że ekspresja każdego z trzech analizowanych genów znacznie wzrosła w odpowiedzi na zastosowane wysokie ciśnienia. Poziomy ekspresji genu *clp*L po zastosowaniu każdego z wariantów ciśnieniowania nie różniły się istotnie od siebie. Zaobserwowano jednak różnice między poziomami ekspresji genów *dna*K i *gro*EL w zależności od zastosowanego wariantu obróbki (P4, Fig. 2).

4.6. WPŁYW CZYNNIKÓW STRESOWYCH NA ZMIANY W CZĘSTOŚCI TRANSFERU GENÓW OPORNOŚCI NA ANTYBIOTYKI

Analiza częstości transferu genów oporności przed i po ekspozycji na czynniki stresowe wykazała, że częstość transferu genu oporności na kanamycynę znacznie wzrosła po ekspozycji na niskie pH i zwiększone stężenie NaCl w porównaniu z próbą kontrolną. Częstotliwość przenoszenia genów w próbkach kontrolnych wahała się od $2{,}32 \times 10^{-6}$ do $7{,}23 \times 10^{-6}$. Po ekspozycji na stres osmotyczny częstość genów wahała się od $5{,}87 \times 10^{-6}$ do $1{,}58 \times 10^{-5}$, a po ekspozycji na niskie pH od $5{,}45 \times 10^{-6}$ do $9{,}32 \times 10^{-6}$. Po zastosowaniu niskiej temperatury i wysokiego ciśnienia transfer genów w ogóle nie zachodził. Po ekspozycji na niskie pH i podwyższone stężenie NaCl dochodziło do transferu genów oporności na gentamycynę, czego nie obserwowano w próbce kontrolnej ani po ekspozycji na niską temperaturę i wysokie ciśnienia. W odniesieniu do transferu genów oporności na tetracyklinę, odnotowano zwiększoną częstość transferu po ekspozycji na niskie pH i zwiększone stężenie NaCl. Częstotliwość przenoszenia

genów w próbkach kontrolnych wahała się od $3{,}96x10^{-5}$ do $7{,}75x10^{-5}$. Po ekspozycji na stres osmotyczny częstość transferu genów wahała się od $7{,}23x10^{-5}$ do $9{,}78x10^{-5}$, a po ekspozycji na niskie pH wynosiła od $7{,}04x10^{-5}$ do $8{,}89x10^{-5}$. Po zastosowaniu wysokich ciśnień częstotliwość przenoszenia była porównywalna z częstotliwością w próbkach kontrolnych. Ponadto nie odnotowano transferu genów po ekspozycji na niską temperaturę. Wartości MIC dla transkoniugantów mieściły się w zakresie 1 - 8 µg/ml dla gentamycyny, 1 - 12 µg/ml dla kanamycyny i 1,5 - 32 µg/ml dla tetracykliny. Te wartości MIC były niższe lub równe wartościom MIC określonym dla dawców. Genotypowanie wykazało podobne profile biorców i transkoniugantów, co może sugerować, że oporność nie została nabyta przez mutację w genomie dawcy, co potwierdza klonalny charakter transkoniugantów (P3, Table 4).

Analiza skupiająca się jedynie na wpływie obróbki wysokociśnieniowej na częstość transferu genów oporności wykazała, że transfer genów *aph(3')-IIIa* i *aac(6')-Ie-aph(2'')-la* kodujących oporność na aminoglikozydy nie zachodził w żadnym z wariantów ciśnieniowania, zarówno w warunkach *in vitro*, jak i *in situ* w przeciwieństwie do próbek kontrolnych, niepoddawanych ciśnieniowaniu. Częstość przenoszenia *in vitro* genów: *tet*M kodującego oporność na tetracyklinę, *cat* kodującego oporność na chloramfenikol i *bla*Z odpowiedzialnego za oporność na ampicylinę po ekspozycji na działanie wysokich ciśnień zmieniła się istotnie w porównaniu z próbkami kontrolnymi, zarówno *in vitro*, jak i *in situ*. Częstość transferu genów *bla*Z i *cat* znacznie wzrosły po ekspozycji na wysokie ciśnienia, zarówno w warunkach *in vitro*, jak również *in situ*. Z kolei częstość przenoszenia genu *tet*M zmniejszyła się po ekspozycji na analizowane warianty ciśnieniowania w warunkach *in vitro* i wzrosła w warunkach *in situ*. Stwierdzone wartości MIC dla transkoniugantów wahały się następująco: 0,38 - 0,75 µg/ml dla gentamycyny, 4 - 6 µg/ml dla kanamycyny, 24 - 32 µg/ml dla tetracykliny, 12 - 32 µg/ml dla chloramfenikolu i 0,125 - 0,25 µg/ml dla ampicyliny. Te wartości MIC były niższe lub równe wartościom MIC określonym dla dawców. Co warte odnotowania, wartości MIC dla ampicyliny, tetracykliny i chloramfenikolu wśród transkoniugantów były wyższe niż u biorców, podczas gdy wartości MIC dla gentamycyny i kanamycyny nie różniły się istotnie od szczepów biorców. Nie zaobserwowano istotnych różnic między wartościami MIC wyznaczonymi dla transkoniugantów uzyskanych *in vitro* i *in situ*. Występowanie genów oporności na antybiotyki, których transfer analizowano, stwierdzono zarówno w genomowym, jak i plazmidowym DNA transkoniugantów (P5, Table 3).

Szczegółowe omówienie uzyskanych wyników przedstawiono w publikacjach będących podstawą niniejszej pracy

4.1. – 4.3. – w publikacji:

P1. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424

4.4. – w publikacji:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

4.5. – w publikacjach:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

P4. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. Effect of high pressure processing on changes in antibiotic resistance genes expression among strains from commercial starter cultures. Food Microbiology, 110, 104169. https://doi.org/10.1016/j.fm.2022.104169

4.6. – w publikacjach:

P3. **Zarzecka U.**, Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090

P5. **Zarzecka U.**, Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. High-pressure processing effect on conjugal antibiotic resistance genes transfer *in vitro*

and in the food matrix among strains from starter cultures. International Journal of Food Microbiology, 388, 110104. https://doi.org/10.1016/j.ijfoodmicro.2023.110104

5. DYSKUSJA

Chociaż dostępnych jest wiele badań dotyczących oporności na antybiotyki wśród bakterii fermentacji mlekowej i innych mikroorganizmów związanych z żywnością, wciąż niewiele jest aktualnych danych na temat występowania oporności na antybiotyki wśród szczepów wchodzących w skład kultur starterowych i ochronnych. Z tego wynika potrzeba analizy stanu obecnego.

Z punktu widzenia bezpieczeństwa żywności, szczepy pochodzące z kultur starterowych lub ochronnych nie powinny być nosicielami genów oporności. Wyniki uzyskane w badaniach będących podstawą niniejszej pracy wskazują, że szczepy oporne na antybiotyki mogą być obecne w kulturach drobnoustrojów stosowanych przemysłowo. Analiza fenotypowa wykazała wysokie wartości MIC dla pięciu z dziewięciu analizowanych antybiotyków. Najwyższe wartości MIC odnotowano dla trzech antybiotyków: tetracykliny, kanamycyny i chloramfenikolu, najniższe dla erytromycyny i streptomycyny. Ponadto ponad 10% szczepów charakteryzowało się jednoczesną wysoką wartością MIC dla antybiotyków z trzech lub więcej różnych klas. Wyniki przedstawione przez innych badaczy wskazują na zbliżone bądź większe spektrum oporności na antybiotyki (Cordeiro i in., 2010; Coton i in., 2018; Georgieva i in., 2015; Kastner i in., 2006; Marty i in., 2012; Resch i in., 2008). Wyniki takie w odniesieniu do szczepów stosowanych na szeroką skalę w przemyśle spożywczym wzbudzają niepokój. Wyniki uzyskane dla szczepów należących do dawnego rodzaju *Lactobacillus* są zgodne z wcześniej opublikowanymi badaniami, które wskazują potencjał szczepów do nabywania oporności na tetracyklinę czy chloramfenikol (Devirgiliis i in., 2013; Dušková i in., 2020). Jednak wyniki fenotypu oporności na antybiotyki uzyskane dla innych rodzajów drobnoustrojów nie są zgodne z wynikami innych badaczy, które wykazały najwyższy odsetek szczepów o wysokim MIC dla erytromycyny, podczas gdy jedynie kilka szczepów wykazywało wysokie MIC dla tetracykliny (Federici i in., 2014; Zycka-Krzesinska i in., 2015). Może to wynikać z różnic między poszczególnymi szczepami tego samego rodzaju lub gatunku lub z fluktuacji ekspresji genów odpowiedzialnych za ujawnienie cechy fenotypowej. Tłumaczyłoby to również brak różnic w występowaniu wysokich wartości MIC a rodzajem bakterii lub źródłem szczepu.

Wszystkie szczepy sprawdzono także pod kątem obecności 24 różnych genetycznych determinantów oporności. Łącznie w materiale genetycznym badanych

szczepów stwierdzono obecność 15 z nich, co stanowi wysoki odsetek (62,5%). Największy odsetek szczepów wykazywał obecność genów kodujących oporność na aminoglikozydy. Co ciekawe, pomimo częstego występowania tych genów, nie każdy szczep wykazywał wysoką wartość MIC dla tych antybiotyków. Sugeruje to potrzebę pogłębionych badań w tym zakresie w celu weryfikacji czynników wpływających na ekspresję tych genów. Spośród wszystkich zidentyfikowanych genów oporności największą różnorodność zaobserwowano w przypadku genów oporności na tetracykliny, ponieważ wśród badanych szczepów stwierdzono obecność sześciu różnych z grupy *tet*. Najczęściej występowały geny *tet*M i *tet*W. Podobne wyniki uzyskali inni autorzy (Anisimova i Yarullina, 2018; Cordeiro i in., 2010; Resch i in., 2008; Singla i in., 2018).

Koniugacja jest uważana za podstawowy mechanizm horyzontalnego przenoszenia genów oporności na antybiotyki w środowisku naturalnym. Ponieważ szczepy wykorzystywane przemysłowo są spożywane w dużej liczbie, niepokojącym zjawiskiem jest zdolność drobnoustrojów wchodzących w skład kultur przemysłowych do horyzontalnego przekazywania nabytej oporności na antybiotyki. Dlatego obecność nabytych genów oporności u szczepów przemysłowych powinna być ściśle monitorowana. Anisimova i Yarullina (2020) oraz Campedelli i in. (2019) wskazują, że najściślej monitorowana powinna być oporność na erytromycynę, tetracyklinę i chloramfenikol, ponieważ geny oporności na te antybiotyki są najczęściej zlokalizowane na ruchomych elementach genetycznych, co wiąże się z ryzykiem poziomego transferu genów oporności. Przeprowadzona analiza zdolności przenoszenia genów oporności wykazała, że analizowane szczepy posiadają zdolność poziomego przenoszenia genów oporności na tetracykliny – *tet*M i *tet*K. Potwierdził to zarówno wzrost wartości MIC dla tetracykliny, jak i obecność przeniesionych genów w materiale genetycznym transkoniugantów. Wykazano, że do transferu genów dochodziło zarówno w warunkach laboratoryjnych, jak i bezpośrednio w produkcie. Warto jednak zauważyć, że dla tego samego szczepu częstość transferu w żywności była o około jedną jednostkę logarytmiczną niższa niż częstotliwość transferu w warunkach laboratoryjnych. Feld i in. (2009) udokumentowali przenoszenie genu oporności na makrolidy z *Lactobacillus plantarum* do szerokiego zakresu gospodarzy, w tym *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Enterococcus faecalis*, ale także *Listeria monocytogenes*. Transpozon koniugacyjny Tn*916* zawierający gen oporności na tetracyklinę *tet*(M), który obecny był u szczepu *Lactobacillus paracasei* izolowanego z kultur starterowych wykorzystywanych do produkcji włoskiego tradycyjnego sera Mozzarella di Bufala

Campana, został z powodzeniem przekazany szczepom z gatunku *Enterococcus faecalis*, będącego oportunistycznym patogenem człowieka (Devirgiliis i in., 2009). Nawaz i in. (2011) wykazali przeniesienie genu oporności na makrolidy - *erm*(B) z *Lactobacillus plantarum* i *Lactobacillus brevis* do *Enterococcus faecalis*. Ponadto w plazmidzie *Campylobacter jejuni* znaleziono gen oporności na aminoglikozydy *aad*E, całkowicie identyczny z genem kodowanym przez plazmid *Pediococcus acidilactici*. Postulowano przeniesienie tego genu do patogenu w przewodzie pokarmowym (O'Connor i in., 2007). Co ciekawe, w większości dostępnych badań autorzy wykazali brak transferu genów oporności w produkcie (Flórez i in., 2016; Thumu i Halami, 2018). Ponadto Anisimova i Yarullina (2020) oraz Li i in. (2019) nie wykazali możliwości transferu genów nawet w warunkach *in vitro*. Wyniki te mogą sugerować, że zdolność do przenoszenia genów jest cechą zależną od szczepu. Yang i Yu (2019) sugerują, że niepowodzenie w transferze genów może również wynikać z faktu, że u niektórych szczepów geny oporności mogą znajdować się na plazmidach niekoniugacyjnych, które nie są kompetentne do przenoszenia przez koniugację. W związku z tym można przypuszczać, że ujawnienie zdolności przenoszenia genów przez szczepy LAB może zależeć od wielu czynników środowiskowych. Pozostawia to pole do dalszych badań nad tym zagadnieniem. Warto jednak zauważyć, że w przypadku szczepów celowo dodawanych do żywności, nawet pojedyncze przypadki przeniesienia genu oporności na antybiotyki są zjawiskiem niepożądanym, gdyż przyczyniają się do rozprzestrzeniania się oporności w środowisku.

Procesy związane z produkcją i przechowywaniem żywności wiążą się ze zmianami warunków środowiskowych, które mogą być czynnikiem stresowym dla mikroorganizmów związanych z żywnością, takich jak bakterie fermentacji mlekowej zawarte w kulturach starterowych i ochronnych. Najczęstszymi czynnikami stresowymi, z którymi mają do czynienia mikroorganizmy w łańcuchu przetwórczym, są chłodnicze przechowywanie, niskie pH, podwyższone stężenie NaCl. Wśród nowych procesów związanych z przemysłem spożywczym, które mogą stanowić czynnik stresowy dla drobnoustrojów wymienia się między innymi obróbkę wysokociśnieniową. Zdolność drobnoustrojów do przetrwania w niesprzyjających warunkach zależy od indywidualnych cech. Bakterie fermentacji mlekowej obecne w żywności w dużej liczbie charakteryzują się wysoką przeżywalnością po ekspozycji na czynniki stresowe, co oznacza, że duża liczba komórek pozostaje w żywności. Wykazano, że w warunkach stresowych drobnoustroje te uruchamiają mechanizmy przystosowawcze, co może wpłynąć na ich żywotność i właściwości technologiczne. Pod wpływem czynników stresowych

aktywowane są różne geny kodujące białka odpowiedzi na stres. W rezultacie komórki są zdolne do ekspresji określonego zestawu białek, w tym molekularnych białek opiekuńczych i proteaz. Wśród nich najczęściej wymieniane są białka Clp, które są szeroko rozpowszechnione u bakterii Gram-dodatnich i odgrywają kluczową rolę w systemie kontroli jakości białek (Ferreira i in., 2013). Ponadto, zgodnie z danymi literaturowymi, często aktywowane są również geny kodujące białko opiekuńcze DnaK (gen *dna*K) oraz geny zmieniające translację lub białka opiekuńcze (*gro*EL) (Bucka-Kolendo i in., 2021). Zdolność do przystosowania się do różnych stresujących warunków może również pochodzić z systemów ochrony krzyżowej. Bakterie fermentacji mlekowej są bardzo cenne dla przemysłu spożywczego, dlatego ważne jest dokładne poznanie ich mechanizmów reakcji na stres (Bucka-Kolendo i Sokołowska, 2017; Song i in., 2014).

Badane szczepy charakteryzowały się wysoką przeżywalnością wobec czynników stresowych zastosowanych w badaniach w ramach niniejszej pracy. Uzyskane wyniki znajdują potwierdzenie w wynikach uzyskanych przez innych badaczy (Gandhi i Shah 2016; Hernández i in. 2019). Żywotność bakterii w stresującym środowisku ma kluczowe znaczenie dla funkcjonowania bakterii w produktach mleczarskich, zwłaszcza zawierających duże ilości soli, wysoką kwasowość czy niską wilgotność. Zmniejszona liczba żywych bakterii przy zwiększonej ekspozycji na stres jest prawdopodobnie spowodowana uszkodzeniem integralności błony bakteryjnej (Gandhi i Shah, 2015). Porównanie wyników przeżywalności uzyskanych metodą cytometrii przepływowej i w metodzie płytkowej wykazało różnice w oznaczonym poziomie przeżywalności. W przemyśle spożywczym standardowa metoda płytkowa jest nadal szeroko stosowana do szybkich analiz mikrobiologicznych. Jednak liczba jednostek tworzących kolonie zależy od wielu różnych parametrów, takich jak warunki hodowli, stan fizjologiczny bakterii i uszkodzenia subletalne. Podczas gdy obie metody służą jedynie do oszacowania rzeczywistej liczby bakterii, mniej czynników wpływa na wyniki uzyskane w cytometrii przepływowej (Bonomo i in., 2013).

Obecnie nie ma dostępnych badań dotyczących wpływu stresu związanego z żywnością na oporność na antybiotyki wśród bakterii fermentacji mlekowej. Z tego powodu trudno jest porównywać wyniki uzyskane w tym badaniu z innymi badaniami. Niewiele jest też badań analizujących ten problem wśród innych bakterii Gram-dodatnich i dotyczą one tylko patogenów, takich jak *Listeria monocytogenes* czy *Staphylococcus aureus*. Więcej badań dotyczy ogólnej reakcji na stres, wskazującej na zwiększoną ekspresję białek związanych ze stresem (Al-Nabulsi i in., 2015; Komora i in., 2017).

Stres kwasowy wywołany przez kwas mlekowy jest rodzajem stresu narzuconego przez LAB ze względu na ich właściwości metaboliczne. Kwas mlekowy, a także inne kwasy organiczne wytwarzane przez LAB, m.in. kwas mrówkowy i kwas octowy gromadzą się podczas fermentacji cukru i powodują stres kwasowy (Guan i Liu, 2019). Źródła literaturowe podają, że wśród bakterii fermentacji mlekowej kwasy organiczne dysocjują w cytoplazmie, chociaż LAB są wyposażone w mechanizmy, które pozwalają im przetrwać w fermentowanej żywności, której końcowe pH wynosi około 4,0 (Ferreira i Mendes-Faia, 2020). Mikroorganizmy mogą przystosować się do stresu kwasowego poprzez syntezę białek błony zewnętrznej, takich jak ATPaza przemieszczająca protony, która pomaga stabilizować wewnętrzne pH komórki. Zmianie może ulec również przepuszczalność i płynność błony komórkowej (Chen i in., 2017). Zmiany te jednocześnie mogą mieć bezpośredni wpływ na dopływ i odpływ antybiotyków przez błonę komórkową, a tym samym na wrażliwość drobnoustroju na antybiotyki (Giacometti i in., 2021). W badaniu tym wykazano, że przy niskim pH wzrasta ekspresja genów kodujących oporność na gentamycynę, kanamycynę i tetracyklinę. W kilku publikacjach zbadano możliwy związek między opornością na antybiotyki a tolerancją na kwasowość środowiska. Al-Nabulsi i in. (2015) podali, że szczepy wyizolowane z fermentowanych produktów mleczarskich i mięsnych wykazywały większą wrażliwość na streptomycynę, gentamycynę i kanamycynę w porównaniu ze szczepami niestresowanymi, jednak zmniejszała się ich wrażliwość na tetracyklinę, amoksycylinę, ampicylinę, wankomycynę i ciprofloksacynę, co pozwala sugerować, że efekt ochrony krzyżowej łączący stres kwasowy z opornością na antybiotyki nie dotyczy wszystkich antybiotyków (Wu i in., 2021). Podobne wyniki uzyskali Komora i in. (2017), którzy stwierdzili korelację między poziomem tolerancji na kwasowość środowiska a poziomem wrażliwości na antybiotyki wśród drobnoustrojów Gram-dodatnich. Co ciekawe Lopez i in. (2020) podali, że dojrzewanie fermentowanych produktów mięsnych skutkowało zmniejszeniem liczby opornych na antybiotyki gronkowców koagulazo-ujemnych wchodzących w skład kultury starterowej – *S. xylosus* i *S. carnosus*.

Dodatek NaCl jest jedną z najstarszych metod utrwalania żywności i nadawania pożądanych cech smakowych. W produkcji niektórych rodzajów żywności fermentowanej dodatek soli sprzyja procesowi fermentacji, ponieważ LAB mają wyższą tolerancję na sól w porównaniu z innymi bakteriami, takimi jak mikroorganizmy chorobotwórcze i powodujące psucie się żywności (Fessard i Remize, 2019). To działanie sprawia, że sól jest powszechnym środkiem konserwującym w różnych produktach

spożywczych, w tym fermentowanej żywności, takiej jak salami i ser. Podczas przetwarzania niektórych serów sól może występować w stężeniu nawet 4,0%, co może nagle zwiększyć osmolarność i zmniejszyć żywotność komórek (Ho i in., 2016). Dodatek NaCl zaburza równowagę osmotyczną między środowiskiem pozakomórkowym a cytoplazmą, wywierając ciśnienie osmotyczne na komórki bakteryjne. Aby tolerować takie zmiany, LAB dostosowują swoją osmolarność wewnątrzkomórkową, aby utrzymać turgor i aktywują mechanizmy zapobiegające śmierci komórki. Ponadto mogą wytwarzać cząsteczki ochronne, takie jak białka operonu DnaK i HtrS, które pełnią funkcje ochronne (Mbye i in., 2020). W prezentowanym badaniu zaobserwowano wzrost ekspresji genów kodujących oporność na kanamycynę, gentamycynę i tetracyklinę po ekspozycji na zwiększone stężenie NaCl. Kilka badań wykazało związek między stresem osmotycznym a opornością na antybiotyki, chociaż koncentrowały się one tylko na mikroorganizmach chorobotwórczych. Stwierdzone zmiany oporności na antybiotyki dotyczyły głównie ampicyliny, tetracykliny, wankomycyny i chloramfenikolu (Komora i in., 2017; Ma i in., 2019).

Bakterie doświadczają stresu związanego z chłodniczym przechowywaniem podczas przechowywania żywności w lodówce oraz w trakcie dojrzewania (np. sery) (Liao i in., 2020). Fermentacja żywności jest często przeprowadzana we względnie niskiej temperaturze (0-10^{o}C), w której obecne są odporne na zimno szczepy LAB. W ciągu ostatnich kilkudziesięciu lat przeprowadzono wiele badań nad mechanizmami oporności na zimno u bakterii chorobotwórczych, podczas gdy tylko kilka badań koncentrowało się na bakteriach fermentacji mlekowej. Wiadomo, że odporne na zimno szczepy LAB przystosowują się do niskich temperatur wytwarzając więcej kwasu mlekowego, co poprawia smak produktu i jego właściwości (Liu i in., 2020). Obecnie nie ma dostępnych badań analizujących wpływ stresu zimna na oporność bakterii na antybiotyki. W obecnym badaniu zaobserwowano obniżoną ekspresję genów i wartości MIC dla gentamycyny, kanamycyny i tetracykliny.

Obróbka wysokociśnieniowa jest uważana za jedną z najbardziej obiecujących nowych metod nietermicznego utrwalania żywności. Dzięki wysokiemu ciśnieniu możliwa jest eliminacja patogenów i mikroorganizmów powodujących psucie przy zachowaniu właściwości sensorycznych i użytkowych produktu (Li i in., 2020). Dlatego coraz częściej stosuje się je do utrwalania żywności uzyskanej przy użyciu kultur starterowych lub ochronnych. Przetwarzanie pod wysokim ciśnieniem może zatrzymać lub zahamować wzrost LAB poprzez inaktywację określonych enzymów (Modugno

i in., 2018). LAB reagują na stres HPP z różną intensywnością, chociaż w większości z nich umiarkowana wartość ciśnienia nie powoduje zerwania wiązań kowalencyjnych. Odpowiedź LAB na HPP jest trudniejsza do zidentyfikowania, ponieważ systemy odpowiedzi HPP są bardzo podobne do odpowiedzi na inne czynniki stresowe, potencjalnie ze względu na obecność systemów ochrony krzyżowej (Liu i in., 2018). Obecne badania nad odpowiedzią LAB na stres wywołany wysokim ciśnieniem koncentrują się na zmianach w strukturze, metabolizmie, wzroście i żywotności komórek. Wysokie ciśnienie może wpływać na proteom bakteryjny i indukować zmiany konformacyjne białek (Gayán i in., 2017). W odpowiedzi na wysokie ciśnienie LAB mogą wytwarzać więcej nienasyconych kwasów tłuszczowych, zwiększać płynność błony komórkowej i wytwarzać więcej polisacharydów (Bucka-Kolendo i in., 2020; Yang i in., 2021). Zgodnie z literaturą, obróbka wysokociśnieniowa może być w stanie skutecznie inaktywować *L. plantarum* w różnych matrycach, takich jak piwo, mleko, bufor fosforanowy i bufor cytrynianowy, osiągając 5-logarytmiczne redukcje przy wartościach 400–500 MPa stosowanych przez 30 s do kilka minut (Putnik i in., 2020). W obecnym badaniu zaobserwowano zmiany w ekspresji genu oporności na tetracykliny. Obecnie istnieje niewiele badań dotyczących wpływu HPP na zmiany oporności na antybiotyki. Badania pilotażowe autorów wykazały, że ekspozycja na wysokie ciśnienie zwiększyła ekspresję genu *tet*M w enterokokach przenoszonych przez żywność, ale w badaniu przeprowadzonym przez Tabanelli i in. (2013) zastosowanie HPP nie zmieniło oporności bakterii fermentacji mlekowej na antybiotyki. Wyniki poszerzonej analizy wykazały, że ekspozycja na wysokie ciśnienie wywołała wzrost minimalnego stężenia hamującego dla trzech antybiotyków: chloramfenikolu, tetracykliny i ampicyliny i nieznaczne obniżenie wartości MIC antybiotyków aminoglikozydowych (kanamycyny i gentamycyny). Co ważne, w przypadku chloramfenikolu i ampicyliny największy wzrost wartości MIC zaobserwowano po zastosowaniu najłagodniejszego wariantu ciśnienia (300 MPa/3 min.). Może to sugerować, że łagodniejsze warunki z większym prawdopodobieństwem stymulują zmiany oporności na te antybiotyki. Sugeruje się, że inne czynniki stresowe, głównie stres temperaturowy, mogą powodować podobne zmiany w poziomie antybiotykooporności. Woode i in. (2020) odnotowali spadek oporności na kilka antybiotyków w odpowiedzi na stres cieplny. Jednak Poole (2012) wskazał, że stres cieplny jest powiązany z opornością na β-laktamy, która jest związana z chaperonem ClpL, występującym u mikroorganizmów Gram-dodatnich. To mogłoby prawdopodobnie tłumaczyć obserwowany w tym badaniu wzrost wartości MIC

dla ampicyliny, która jest również antybiotykiem β-laktamowym. W tych samych badaniach Poole (2012) wskazał również na zmniejszenie oporności na aminoglikozydy wśród drobnoustrojów Gram-dodatnich jako efekt stresu cieplnego, co jest zgodne z wynikami uzyskanymi w tym badaniu. Ponadto Dorrian i in. (2011) podali, że wywołanie stresu cieplnego spowodowało wzrost oporności na makrolidy u szczepów *Lactococcus lactis*, chociaż nie zaproponowano żadnego mechanizmu.

W toku badań prowadzonych w ramach niniejszej pracy zaobserwowano wzrost szybkości transferu genów kodujących oporność na gentamycynę, kanamycynę i tetracyklinę po ekspozycji na niskie pH i wysokie stężenie NaCl. Uzyskane wyniki znajdują odzwierciedlenie w danych literaturowych, zgodnie z którymi stres środowiskowy podczas produkcji i przechowywania żywności może wpływać na szybkość transferu genów i liczbę kopii plazmidu w komórkach bakteryjnych. Poziomy transfer plazmidów oporności na antybiotyki może być promowany przez stres związany z utrwalaniem żywności, taki jak wysoka lub niska temperatura, wysokie stężenie NaCl i niskie pH, chociaż mechanizmy są jeszcze nieznane (McMahon i in., 2007a; McMahon i in., 2007b; Obolski i Hadany, 2012; Polit i in., 2018). Na efektywność i częstotliwość transferu genów wpływa wiele czynników, w szczególności odpowiednie zagęszczenie komórek i ich stan fizjologiczny, na który może wpływać wiele czynników fizykochemicznych (James i in., 2021; Lerner i in., 2017). Li i Zhang (2022) wykazali, że na częstotliwość transferu genów mogą mieć również wpływ warunki środowiskowe. Obecnie dostępne są ograniczone dane na temat tego, jak warunki związane z żywnością wpływają na przenoszenie oporności na antybiotyki podczas procesu fermentacji (Gazzola i in., 2012). Obecnie wiadomo, że transfer genów może odbywać się w różnych macierzach. Chociaż wykazano transfer genów w stabilnym środowisku laboratoryjnym, wciąż niewiele wiadomo na temat transferu genów w naturalnych niszach, takich jak żywność (Fletcher, 2015; Thumu i Halami, 2018). Dlatego tak ważne jest poznanie mechanizmów warunkujących rozprzestrzenianie się genów w innych warunkach, aby zidentyfikować wszelkie modyfikowalne interakcje ograniczające lub przerywające rozprzestrzenianie się oporności w środowisku. Wiedza na temat zdolności LAB do przenoszenia oporności na antybiotyki w matrycach żywnościowych jest wciąż ograniczona. Jednym z przykładów może być badanie przeprowadzone przez Leroy i in. (2019), którzy opisali transfer plazmidów przenoszących oporność na antybiotyki podczas fermentacji kiełbas między szczepami *Lactobacillus*.

Według mojej najlepszej wiedzy obecnie nie ma badań dotyczących wpływu HPP, na możliwość horyzontalnego transferu genów oporności na antybiotyki z udziałem szczepów niepatogennych celowo wprowadzanych do żywności. Jednak dane literaturowe wskazują, że w łańcuchu pokarmowym geny oporności na antybiotyki mogą być nabywane przez bakterie narażone na wiele różnych stresów związanych z żywnością (Lopez i in., 2020; Obolski i Hadany, 2012; Oniciuc i in., 2019). Skandalis i in. (2021) donoszą również, że komórki bakteryjne dotknięte czynnikami stresowymi mogą nadal uczestniczyć w poziomym transferze genów. Badania przeprowadzone w ramach niniejszej pracy wykazały, że przeniesienie genów oporności na aminoglikozydy nie nastąpiło po obróbce wysokociśnieniowej, ani *in vitro*, ani *in situ*. Po ekspozycji na HPP częstości przenoszenia genów oporności na tetracyklinę, ampicylinę i chloramfenikol znacznie wzrosły w porównaniu z próbką kontrolną, zarówno *in vitro*, jak i *in situ*. Częstość transferu genów oporności w matrycy pokarmowej w próbkach ciśnieniowych nie różniła się istotnie od szybkości transferu *in vitro*.

Podobne badania przeprowadzono w odniesieniu do szczepów środowiskowych i wykazano, że presja selekcyjna związana ze stresem środowiskowym skutkuje zwiększoną częstością transferu genów u szczepów, które przeżyły ekspozycję na czynniki stresowe. Nabycie genów oporności na antybiotyki wydaje się być odpowiedzią na presję selekcyjną. Narażenie na czynniki stresowe w środowisku wywiera presję, wywołując reakcję stresową i promując poziomy transfer genów, co indukuje rozprzestrzenianie się genów odporności (Bombaywala i in., 2021; Chen i in., 2019; Lin i in., 2019).

Wyniki uzyskane w badaniach będących podstawą niniejszej pracy rzucają nowe światło na problem rozprzestrzeniania się oporności na antybiotyki w środowisku przy udziale drobnoustrojów niepatogennych, a zwłaszcza wpływu warunków przetwarzania i przechowywania żywności na antybiotykooporność. Obecnie w literaturze brak jest obszernej dyskusji na ten temat, dlatego ważne jest dogłębne zrozumienie mechanizmów łączących reakcję stresową ze zmianami oporności na antybiotyki co jest istotne w kontekście przetwórstwa żywności i antybiotykooporności.

6. STWIERDZENIA KOŃCOWE I PODSUMOWANIE

Badania przeprowadzone w ramach realizacji pracy doktorskiej, pozwoliły na sformułowanie następujących spostrzeżeń:

1. Szczepy wchodzące w skład kultur starterowych i ochronnych mogą stanowić środowiskowy wektor genów oporności na antybiotyki.
2. Warunki panujące podczas przetwarzania i przechowywania żywności mogą stanowić dla drobnoustrojów czynniki stresowe indukujące zmiany w fenotypie oporności na antybiotyki jak również ekspresji genów oporności na antybiotyki.
3. Na skutek oddziaływania czynników stresowych związanych z przetwarzaniem żywności może także dochodzić do zmian w częstości transferu genów oporności na antybiotyki zarówno w warunkach laboratoryjnych jak i bezpośrednio w produkcie. Różnice w częstości transferu genów w warunkach *in vitro* i *in situ* wskazują na potrzebę badania możliwości przenoszenia genów nie tylko w warunkach laboratoryjnych ale także bezpośrednio w żywności.

Uzyskane wyniki wskazują, że problem antybiotykooporności dotyczy także szczepów niepatogennych, celowo dodawanych do żywności i od lat uznawanych za bezpieczne. Chociaż zjawisko to nie jest zbyt dobrze poznane, może budzić niepokój. Na szczególną uwagę zasługuje wykazana zdolność przenoszenia genów oporności, co może w istotny sposób przyczynić się do rozprzestrzeniania się oporności w środowisku. Zatem szczepy pochodzące z kultur starterowych i ochronnych, choć nie stanowią bezpośredniego zagrożenia dla konsumentów, mogą im zagrażać pośrednio. Ponadto istnieje duża luka w wiedzy na temat wpływu warunków panujących podczas przetwarzania i przechowywania żywności na drobnoustroje bytujące w produkcie i przeżywające proces, a zwłaszcza ich cechy związane z opornością na antybiotyki. Uzyskane wyniki wskazują, że może dochodzić do zwiększania poziomu ekspresji genów oporności na antybiotyki i/lub częstości transferu genów, a tym samym szybszego rozprzestrzeniania oporności w środowisku. Z tego wynika potrzeba dalszych badań w tym zakresie, aby szczepy wprowadzane do żywności nie stawały się środowiskowym rezerwuarem genów oporności na antybiotyki.

7. PIŚMIENNICTWO

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Starter cultures as a reservoir of antibiotic resistant microorganisms

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ABSTRACT

Starter cultures are selected microbial strains intentionally added to food to obtain a product with desired organoleptic characteristics. The most important group of microorganisms used in starter cultures are bacteria, mostly lactic acid bacteria (LAB). For many years the safety criteria for the use of starter cultures in food production were based mainly on their long history of safe use. However, recent research indicates that starter culture microorganisms are able to acquire antibiotic resistance genes. Starter culture microorganisms can carry variously sized plasmids with resistance determinants. These strains have a high potential for transmitting resistance genes, which raises concerns regarding the antibiotic resistance of food-related bacteria. It should be noted that the antibiotic resistance of starter culture bacteria does not pose a direct risk to consumers because they are not pathogenic. However, they can act as environmental reservoirs of antibiotic resistance determinants. Therefore, bacteria that harbor antibiotic resistance genes on mobile genetic elements should not be used in food production. In addition, it is reported that stress factors associated with the production and storage of food may affect the changes in the antibiotic resistance profile of these microorganisms. Research indicates that the acquisition of antibiotic resistance in starter cultures is not a critical problem, but it poses a concern that cannot be disregarded.

1. Introduction

Starter cultures are pure cultures of carefully selected strains of microorganisms - bacteria, yeast and mold, used on a large scale in the food industry. They allow controlled production process in order to obtain products with the desired organoleptic characteristics. The starter culture production was initiated about 1900 by Emil Christian Hansen and Christian D.E. Hansen. The first patented starter cultures were presented in 1940's, they were liquid, prepared by cultivation of bacteria in sterilized milk. However, these cultures lost their viability during storage due to overacidification. To overcome this problem, drying technologies were developed, leading to improve stability of the cultures. Starter cultures are chilled, frozen or lyophilized to preserve their stability, performance and utility (Blandino, Al-Aseeri, Pandiella, Canterob, & Webb, 2003; Surono & Hosono, 2011).

Antibiotic resistance poses one of the most challenging problems in human and veterinary medicine as well as in food production around the globe. The food chain is considered to be one of the key pathways for the transmission of drug resistance across bacterial populations (Founou, Founou, & Essack, 2016). Research on the prevalence of antibiotic resistance is mainly focused on clinically relevant bacterial species. Regarding to antibiotic resistance among food-borne microorganisms, researchers' interest is centered around pathogens and opportunistic pathogens. However, it is speculated that non-pathogenic microorganisms intentionally added to food, as starter cultures can act as reservoirs of antibiotic resistance genes because the bacteria included in starter cultures can show acquired, transferable resistance to many antibiotics. Literature data indicate that starter culture bacteria can carry genes that encode resistance, mainly to tetracycline: *tet*(M), *tet*(K) and *tet*(W) and macrolides: *erm*(B), *erm*(C) and *erm*(G). If genes are localized on mobile genetic elements they can be transferred to other microorganisms, both commensal and pathogenic (Cordeiro, Du, Mulvey, Krause, & Hooley, 2010; Kastner et al., 2006; Resch, Nagel, & Hertel, 2008).

It should be also noted that starter cultures are added to foods in large quantities that approximate 10^7–10^8 CFU/g. Numerous bacterial cells are introduced into the gastrointestinal tract, where they are in contact with many microorganisms cells. Authors indicate that the human gastrointestinal tract is also an ideal site for genetic exchanges among bacteria (Lerner, Matthias, & Aminov, 2017). The European Food Safety Authority (EFSA) has committed to developing criteria for assessing the safety of the microorganisms used in food production. These criteria are based on the concept of qualified presumption of safety (QPS) (Herman et al., 2019; Leuschner et al., 2010). In the United

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States a GRAS system (generally recognized as safe) developed by the American Food and Drug Administration (FDA) was introduced (FDA, 2010).

There are only a few reports in the literature of antibiotic resistance in starter cultures. although the results of the available studies indicate a problem, this is not a topic often raised by researchers. In addition, some researchers indicate that stress factors associated with the conditions of food production and storage, such as high and low temperature, low pH or high concentration of NaCl may affect the changes in the antibiotic resistance profile of these microorganisms, most often manifested by an increase in the MIC value. The proposed reason is the combination of molecular mechanisms of stress response and antibiotic resistance, which entails changes in the expression of the genes responsible for antibiotic resistance in response to stressors.

Therefore the aim of this review is to summarize and critically evaluate the current state of knowledge about antibiotic resistance in starter cultures microorganisms. Due to the huge number of species of microorganisms used in starter cultures, attention will be focused only on bacteria of the genera *Lactobacillus, Lactococcus, Pediococcus, Streptococcus* and *Staphylococcus*, since only these genera have been the subject of research on antibiotic resistance in starter cultures and literature data on this topic is available.

2. Starter cultures in food production

Starter cultures are preparations of selected microbial strains which contain live or dormant cells whose metabolic activity allows obtaining the final product with the desired organoleptic properties and characteristics. They are added to enhance the appearance, flavor and aroma of food products, and to prolong their shelf life. Functional microbiota and additional microbiota can be distinguished among the microorganisms included in the starter culture Functional microbiota is responsible for the fermentation process, the production of flavor and aroma compounds, as well as proteolytic and lipolytic processes. Additional microbiota exerts a positive influence on the consumers' health, prolongs the product's shelf life and prevents the development of pathogens. Starter cultures may be categorized as mesophilic or thermophilic, according to the manufacturing temperatures under which they are used. Mesophilic cultures grow and produce lactic acid at optimal levels, at a temperature about 30 °C, whereas thermophilic cultures optimally function at a temperature about 42 °C (Surono & Hosono, 2011). Starter cultures have been also classified based on the diversity of microbial strains. Single-strain cultures have been identified which contain only one strain of a given microbial species, single-species cultures which contain at least two strains of a given microbial species that are selected based on their optimal growth temperature and mutual growth stimulation, and multi-species cultures which contain at least two strains belonging to different microbial species.

Species composition is the basic criterion for classifying starter cultures. Starter cultures can be composed of bacterial, yeast and fungal cells. The majority of starter culture are composed of bacteria, and LAB are most frequently used. Lactic acid bacteria are Gram-positive, optionally anaerobic non-sporulating cocci or bacilli with low Guanine + Cytosine (G + C) content. The most important feature of their metabolism is the ability to ferment carbohydrates to produce lactic acid. Lactic acid bacteria are an important group of microorganisms in food production. They are responsible for the taste and texture of numerous food products and for preventing the development of pathogenic microorganisms. (Wright & Axelsson, 2012). Lactic acid bacteria can be classified as homofermentative and heterofermentative based on their by-products of carbohydrate fermentation. Homofermentative LAB convert hexoses to lactic acid according to the Embden-Meyerhof-Parnass (EMP) pathway. In homofermentation, 1 mol of glucose is converted into 2 mol of pyruvate. Then there is a simultaneous reduction of pyruvate and NADH oxidation, resulting in 2 mol of lactic acid and 2 mol of ATP. Small amounts of by-products, mainly carbon dioxide, are also produced. Homofermentative bacteria include most genera *Lactococcus*, *Pediococcus* and *Streptococcus* and some of *Lactobacillus* species, i.e. *Lactobacillus delbrueckii, Lactobacillus plantarum* and *Lactobacillus acidophilus*. Heterofermentation follows the pentose phosphate pathway. As a result of heterofermentation, 1 mol of glucose is converted to 1 mol of lactic acid and 1 mol of by-products, such as ethanol, carbon dioxide or acetic acid. Heterofermentative bacteria include bacteria of the genera *Leuconostoc* and such *Lactobacillus* species as *Lactobacillus brevis* and *Lactobacillus fermentum*. Heterofermentation is the effect of the lack of some key enzymes for the homofermentation process, including aldolase and triphosphate isomerase (Wright & Axelsson, 2012). Thus, lactic acid bacteria are applicable in starter cultures used in the food industry with an enormous variety of fermented dairy products, meat, fish, fruit, vegetable and cereal products. Besides, LAB contribute to the flavour, texture and nutritional value of the foods, through production of aroma components, and used as adjunct cultures (Ammor & Mayo, 2007). Other microbial species used in starter cultures also play an important role, but they are used only in the production of selected foods. They include, for example, coagulase-negative species of the genus *Staphylococcus – S. xylosus* and *S. carnosus* (meat starter cultures), species of the genus *Pediococcus – P. pentosaceus* and *P. acidilactici* (meat starter cultures), propionic bacteria of the genus *Propionibacterium* (hard ripened cheese starter cultures) and *Streptococcus thermophilus* (yogurt starter cultures). According to EFSA guidelines, strains of the genus *Enterococcus* cannot be used in food production although they are classified as lactic acid bacteria. Enterococci can cause opportunistic infections in immunocompromised people and contribute to the spread of antibiotic resistance; therefore their use in the food industry poses an excessive risk (Leuschner et al., 2010).

3. Transfer of antibiotic resistance genes

As mentioned earlier foods produced with starter cultures are important vehicles of enormous amounts of living bacterial cells into the human body. As literature data indicate these microorganisms may carry antibiotic resistance genes (Kastner et al., 2006; Resch et al., 2008; Cordeiro, Du, Mulvey, Krause, & Holley, 2010; Federici et al., 2014). Natural resistance as well as resistance induced by spontaneous mutations did not raise concern due to the low risk of horizontal transfer and a minimal threat to consumer health. However, researchers suggest that microorganisms included in the food cultures can easily adapt to new environments and be able to acquire antibiotic resistance genes. Toomey, Bolton, and Fanning (2009) showed that the starter strain *Lactococcus lactis* is capable of obtaining erythromycin (*erm*(B)) and tetracycline (*tet*(M)) resistance genes from another strain belonging to the same species. It should be also noted that the antibiotic resistance of starter culture bacteria does not pose a direct risk to consumers because these microorganisms are not pathogenic. However, non-pathogenic microorganisms in, such as LAB, can act as reservoirs of determinants of antibiotic resistance. These strains have a high potential for transmitting resistance genes, which raises concerns regarding the antibiotic resistance of food-related. Mobile genetic elements play a key role in the horizontal transfer of resistance genes between bacterial cells. Presence of highly organized mobile elements such as plasmids and transposons in bacterial genomes indicates potential transfer of genetic material, including genes encoding antibiotic resistance (Fig. 1, Fig. 2). Simple mobile genetic elements such as insertion sequences (IS), capable of autonomous transposition, have been also identified in association with antibiotic resistance genes (Fraqueza, 2015).

Most of the mobile genetic elements can be transferred by host strains to the other bacterial cells. Gene transfer can occur both between microorganisms belonging to one species and interspecifically. Transfer between species involves the risk of spreading antibiotic resistance genes to human pathogens such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Listeria monocytogenes*. The

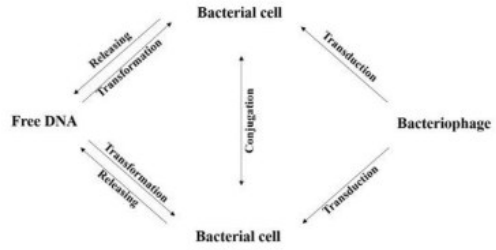


Fig. 1. Horizontal gene transfer mechanisms (Verraes et al., 2013; von Wintersdorff et al., 2016).

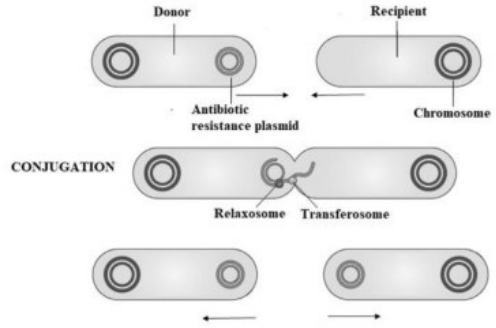


Fig. 2. Antibiotic resistance transfer by conjugation (Marston, Dixon, Knisely, Palmore, & Fauci, 2016; Wang, Zheng, Ma, & Song, 2018).

conjugative transposon Tn*916* containing tetracycline resistance gene *tet*(M) occurred in *Lactobacillus paracasei* isolated from natural whey starter cultures employed in the manufacture of the Italian traditional cheese Mozzarella di Bufala Campana, was horizontally transferable to the opportunistic pathogen *Enterococcus faecalis* (Devirgiliis, Caravelli, Coppola, Barile, & Perozzi, 2009). Nawaz et al. (2011) showed transfer of the erythromycin resistance gene *erm*(B) from *Lactobacillus plantarum* and *Lactobacillus brevis* to *Enterococcus faecalis*.

Foods produced with starter cultures contain large quantities of microorganisms, live cells come into the large intestine where contact with commensal microorganisms. The human digestive tract offers a highly supportive environment for the transfer of conjugative plasmids and transposons that carry genes encoding antimicrobial resistance (Nawaz et al., 2011). The presence of resistance genes in strains isolated from food products and in human gut-derived commensal bacteria provides the first evidence that commercial strains used for food production can contribute to the transmission of resistance genes (Devirgiliis, Zinno, & Perozzi, 2013). What is significant, the interspecies transmission of plasmid-carried resistance genes between commercial strains and commensal gut bacteria has been confirmed *in vitro* and *in vivo* - in food and in model organisms (Flórez et al., 2016; Thumu & Halami, 2019; Toomey et al., 2009). According to the literature, genes encoding resistance to tetracyclines and erythromycin are most frequently transmitted (Toomey et al., 2009).

4. Guidelines relating to the safe application of starter cultures

Producers who use starter cultures in the food production process have to guarantee that the added microorganisms do not pose a direct or indirect threat to consumer health. Therefore, it was necessary to create legal regulations regarding the admission of bacterial strains to contact with food. In the United States Generally Recognized as Safe (GRAS) designation has been introduced by Food and Drug Administration (FDA) in the process of assessing the safety of starter cultures used in the food production (Mattia & Merker, 2008). In the year 2007, European Food Safety Authority (EFSA) launched the Qualified Presumption of Safety (QPS) initiative. Although QPS status is usually considered as the European correspondent of a GRAS determination for microbial food cultures, some differences should be highlighted between these two approaches. These differences arise from the different regulatory frameworks in Europe and the USA.

4.1. US regulatory framework: GRAS system

According to US regulations, microbial starter cultures added to a food may be considered as safe food additives. A GRAS determination refers to substances generally recognized as safe under the conditions of its intended use. In the case of cultures used prior to year 1958, GRAS status can be established through experience based on a substantial history of use in food by a significant number of consumers. Also, a consensus among experts about GRAS status must be achieved. To simplify the achievement of a GRAS determination, in 1997 the procedure where any person or company may notify the FDA that a specific usage of a microorganism is GRAS was proposed. The FDA resolves to accept the basis for the notifier's GRAS determination or concludes that it does not provide an enough basis for a GRAS determination (FDA, 2010). Due to their long safe history in food production, cultures of lactic acid bacteria (LAB) are the most representative microorganisms included in the GRAS list (FDA, 2015). A starter culture should be considered as GRAS not only on the basis of its food application. There are also some technological features important, such as the form in which the microorganisms are added to the food or the microbial cells concentration achieved in the food.

4.2. The European regulatory framework: The qualified presumption of safety (QPS) system

Qualified presumption of safety (QPS) is an approach developed by the European Food Safety Authority (EFSA) to evaluate the safety of biological agents intentionally added to food. Its objective is to harmonize the risk assessment of microbial cultures for feed and food production across the EFSA Scientific Panels and Units. QPS is a preassessment at a species level, which has to be complemented by a safety assessment of a specific culture. The role of EFSA's Panel on Biological Hazards is to assess the QPS status of a strain and declare that the microorganism displays no safety concerns. Microorganisms on the QPS list need no more safety assessment. QPS never establishes the safety of strains. It's the responsibility of a specific Scientific Panel. The assessment for QPS suitability relies on the following criteria: (a) Taxonomic unit; (b) Current state of knowledge; (c) Possible pathogenicity and (d) Description of end use (EFSA, 2007). The most of microorganisms recommended for the QPS list are Gram-positive non-sporulating bacteria. This group includes many commensals of the gastrointestinal tract with a previous history of safe use. For this group, the EFSA's Scientific Committee applies requiring that all strains not carry any transferable antimicrobial resistance (Leuschner et al., 2010). Microorganisms which safety properties are not fully understood are subjected to safety assessments. They have to include a taxonomic classification and a strain characterization, including whole-genome sequence analysis to identify potential virulence and antibiotic resistance genes and their horizontal transfer abilities. However, in the case of recently discovered microorganisms the availability of genome sequences might not be enough to identify virulence or antibiotic resistance genes. Often comparison with available data is required, so functional studies on these microorganisms are needed (Fig. 3).

The QPS system is an important milestone in the process of evaluating the safety of starter cultures in the food industry. However, strains

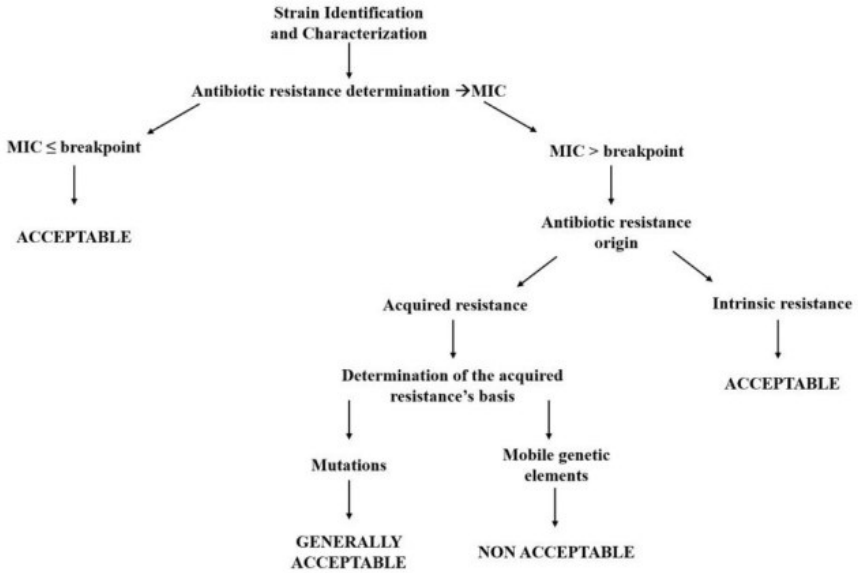


Fig. 3. Scheme of strains evaluation for their antibiotic resistance (European Commision 2003; EFSA, 2012).

Table 1
Role of the entities involved in the food cultures safety assessment in the European Union (Laulund et al., 2017).

Name of the entity	The role of entity in the safety evaluation of food cultures
EU Commission	creates the General Food Law and risk management of safety systems together with the Member States
The European Food Safety Authority (EFSA)	responsible for the risk assessment
The EFSA Panel on Additives and Products or Substances Used in Animal Feed	provides scientific advice on the safety and/or efficacy of microorganism-based additives and products or substances used in animal feed
The EFSA panel on Biological Hazards (BIOHAZ)	provides scientific advice on biological hazards in relation to food safety and microbiological criteria
The International Dairy Federation (IDF)	represents the global dairy sector and ensures that the best scientific expertise is used to support high quality milk and nutritious, safe, and sustainable dairy products
The European Food and Feed Cultures Association (EFFCA)	cooperates, both within the EU and globally, with a wide range of stakeholders in the area of microbial food cultures

which status of safe microorganisms based on their long history of safe use are regarded as traditional food ingredients and are admitted for use in the EU without additional tests. The concept of antibiotic resistance determinants is relatively new. Therefore, there is no information about resistance genes and mobile genetic elements in strains that have been used in food production for many years. These strains can be potential reservoirs of resistance genes and contribute to their spread. Strains with QPS status can also acquire resistance genes through horizontal gene transfer and become their carriers (EFSA, 2007; Laulund, Wind, Derkx, & Zuliani, 2017). In the safety assessment of food cultures, including starter cultures, several entities participate, whose role in the assessment process is precisely defined (Table 1).

5. Antibiotic resistance of starter culture microorganisms

The first documented study of antibiotic resistance in starter culture bacteria was conducted in 1980 (Sozzi & Smiley, 1980). However, this study was not a large-scale research project. Researches into the antibiotic resistance of commercial starter culture strains have attracted considerable interest in recent years but there is still little data on this topic. The studies investigating the antibiotic resistance of starter culture bacteria are reviewed in Table 2.

5.1. *Lactobacillus*

Antibiotic resistance in *Lactobacillus* strains has been extensively documented. Many species belonging to this genus are used in the production of starter cultures, including *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus curvatus*. According to the literature, all species of the genus *Lactobacillus*, including those used in the production of starter cultures are intrinsically resistant to several classes of antibiotics. Resistance to glycopeptides, as vancomycin, is the result of the terminal D-alanine residue's replacement by D-lactate or D-serine in the muramylpentapeptide. Lactobacilli are also naturally resistant to quinolones, such us ciprofloxacin, norfloxacin and nalidixic acid and to antibiotics being nucleic acid inhibitors such as trimethoprim, but mechanism responsible for this is not fully understood yet. The intrinsic quinolones resistance is not considered to be associated with mutations in the *gyr*A and *par*C genes as occurred in other Gram-positive bacteria, thus this could have resulted from intrinsic characteristics such as cell wall structure, its permeability, or an efflux mechanism (Fraqueza, 2015; Hummel, Hertel, & Holzapfel, 2007). Resistance to aminoglycosides, such as neomycin, kanamycin and streptomycin is being attributed to the absence of cytochrome-mediated electron transport, which

Table 2
Antibiotic resistance and occurrence of resistance genes in microorganisms included in starter cultures - literature review.

Isolation source	Genus/species	Phenotype	Antibiotic resistance genes	Mechanism of resistance	Source
Cheese starter culture	*Lactobacillus helveticus*	FD, K, F, OFX, S, TE, TOB	n. d.	–	Kastner et al. (2006)
	Leuconostoc mesenteroides	CIP, S, C	*cat*	Enzymatic inactivation	Hummel et al. (2007)
	Lactococcus lactis ssp. *lactis*	V, RD, S, B, E, W	*dfr*A	Enzymatic inactivation	Liu et al. (2009)
Yogurt starter culture	*L. delbrueckii* subsp. *bulgaricus*	AMP, CN, K, TE, MY, N, P, ROX, S	*tet*(M),	Ribosomal protection protein	Zhou et al. (2012)
			aph (3′)-III, *ant* (6)	Enzymatic inactivation	
			erm(B)	Enzymatic inactivation	
		N	n. d.	–	Sozzi and Smiley (1980)
	Lactococcus sp.	K, NA, F, S, TOB	n. d.	–	Kastner et al. (2006)
	Streptococcus thermophilus	E	*erm*(B)	Enzymatic inactivation	Nawaz et al. (2011)
		N	n. d.	–	Sozzi and Smiley (1980)
Meat starter culture	*Staphylococcus carnosus*	CTX, DA, E, NA, P, TE, V	n. d.		Kastner et al. (2006)
	Lactobacillus curvatus	FD, K, OFX, OX, S, TE, V, NA	n. d.	–	Kastner et al. (2006)
		FD, K, OFX, OX, S, TE, TOB, VA	n. d.	–	
		OXT	*tet*(M)	Ribosomal protection protein	Klare et al. (2007)
	Lactobacillus plantarum	E	*erm*(B)	Enzymatic inactivation	Devirgiliis et al. (2010)
	Lactobacillus pentosus	CC, K, MY, OFX, OX, P, S, TE, NN, VA	n. d.	–	Kastner et al. (2006)
	S. xylosus	CTX, NA, P, TE	*tet*(K)	Efflux protein	Kastner et al. (2006)
		NA, P, TE	*tet*(K)	Efflux protein	
		MY, AMP, TE, FD, P	*blaZ*, *tet*(K)	Enzymatic inactivation Efflux protein	Resch et al. (2008)
	Pediococcus pentosaceus	CTX, DA, F, OFX, OX, RD, TE, TOB, VA	*tet*(K)	Efflux protein	Resch et al. (2008)
		S, CN, AMP, TE, C, E, DA	*tet*(M)	Ribosomal protection protein	Federici et al. (2014)
			tet(W)	Ribosomal protection protein	
			tet(K)	Efflux protein	
			tet(L)	Efflux protein	
			tet(S)	Ribosomal protection protein	
			erm(A) *erm*(B)	Enzymatic inactivation	
		CTX, DA, F, OX, RD, TE, TOB, V	*tet*(K)	Efflux protein	Kastner et al. (2006)

Abbreviations: AMP - ampicillin, CIP - ciprofloxacin, C - chloramphenicol, CN - gentamicin, K - kanamycin, N - neomycin, S - streptomycin, RL - sulphametoxazole, W - trimethoprim, V – vancomycin, FD – fusidic acid, F - nitrofurantoin, OFX - ofloxacin, TE - tetracycline, TOB – tobramycin, RD - rifampicin, B - bacitracin, E – erythromycin, P - penicillin, ROX - roxithromycin, NA – nalidixic acid, CTX - cefotaxime, DA - clindamycin, OX - oxacillin, MY – lincomycin; OXT – oxytetracycline; n.d. – no data.

mediates drug uptake (Ammor, Flórez, & Mayo, 2007). Aminoglycoside resistance in lactobacilli is encoded most often by following genes: *aadA, aadE, aphA3, aph* (3′)-III and *ant(6)*. Lactobacilli are also intrinsic resistant to other antibiotics such as bacitracin, cefoxitin, metronidazole and nitrofurantoin (Bernardeau, Vernoux, Henri-Dubernet, & Guégen, 2008). However, resistant genotypes also arise by mutation. The mechanisms how the resistome evolves and changes are presently not well understood (Berkner, Konradi, & Schönfeld, 2014). The presence of intrinsic antibiotic resistance is not a major safety concern, because these genes have a low risk of conjugative transmission to other bacteria.

Apart from the intrinsic resistance *Lactobacillus* has also the potential to acquire resistance to all classes of antibiotics. The most common is acquired resistance to antibiotics that inhibit protein synthesis, including chloramphenicol, macrolides (erythromycin) and lincosamides (clindamycin) (Federici et al., 2014). Chloramphenicol resistance is most often due to the presence of the *cat* gene (Hummel, Hertel, Holzapfel, & Franz, 2007). Resistance to erythromycin is often associated with the presence of the *erm*(A), *erm*(B) and *erm*(C) genes. Resistance to lincosamides can be encoded by the *lnu*(A) gene (Ammor et al., 2007). Acquired tetracycline resistance in *Lactobacillus* strains is usually encoded by the *tet*(M), *tet*(S) and *tet*(W) genes (Korhonen et al., 2008). These microorganisms also often acquire resistance to antibiotics inhibiting cell wall synthesis, such as β-lactams (penicillin and ampicillin), β-lactamase inhibitors and cephalosporins (cephalotin, cefuroxime, ceftriaxone and cefoxitin). Many *Lactobacillus* species also demonstrate acquired resistance to tetracycline (Korhonen et al., 2008; Thumu & Halami, 2019). Many studies described the presence of antimicrobial resistance in *Lactobacillus* and mechanisms of resistance acquisition. Resistance could be the result of point genes mutations under antibiotic pressure and vertical spreading but it can be also caused by horizontally acquired determinants located on mobile genetic elements (Ammor et al., 2007; Hummel et al., 2007). What's worrying, the problem of acquiring resistance determinants by *Lactobacillus* species also applies to strains used in starter cultures. According to Cordeiro et al. (2010) *Lactobacillus curvatus* and *Lactobacillus plantarum* strains from meat starter cultures were resistant mostly to tetracyclines (tetracycline, chlortetracycline and oxytetracycline) but resistance to lincomycin, sulfamethazine, carbadox, monensin and ampicillin was also observed. The genes encoding the ribosomal protective proteins, *tet* (M), *tet*(O), *tet*(S) and *tet*(Q), responsible for tetracycline resistance were genes the most commonly detected by these authors. Klare et al. (2007) analyzed antibiotic resistance among *Lactobacillus* strains, including 26 strains used in starter cultures. These authors showed that 2 out of 26 strains, both belonging to the *L. curvatus* species, were resistant to oxytetracycline. They also found that this resistance was encoded by the *tet*(M) gene. Kastner et al. (2006) analyzed antibiotic resistance among starter cultures and probiotic microorganisms, included two *Lactobacillus helveticus* strains from cheese starter culture and *Lactobacillus pentosus* from meat starter. These strains were resistant mostly

to tetracycline, nalidixic acid and ofloxacin. The authors did not detect in these strains any of the resistance genes they analyzed. Morover, a widespread resistance to penicillins, especially penicillin G, was observed in lactobacilli used as starter cultures (Federici et al., 2014). Some studies report chloramphenicol resistance encoded by acetyltransferase *cat* gene. That gene was not expressed in chloramphenicol-resistant *Lactobacillus* strains from meat starter-cultures (Hummel et al., 2007). Furthermore, Thumu and Halami (2012) reported that erythromycin-resistant *Lactobacillus* species isolated from meat starter cultures harbored only the *erm*(B) gene, and that the MLS (Macrolide, Lincosamide, and Streptogramin) phenotype displayed by these strains was a result of a mutation in the leader peptide of *erm* genes. In this sense, the acquired resistance to MLS antibiotics is frequently associated with the presence of methylase genes, mainly *erm*(B) gene, but also *erm*(C), *erm*(G) and *erm*(T) genes. Rozman, Mohar Lorbeg, Acetto, & Bogovič Matijašić (2020) provided *in silico* analysis and identified genes responsible for antibiotic resistance in *Lactobacillus* strains being a component of starter cultures. Genes encoding macrolide and lincosamide resistance – *lmrC, lsaA, mreA* and *lmrB* were the most frequent, but they identified also glycopeptide resistance genes – *vanRI* and *vanRF*.

5.2. Lactococcus

In comparison with *Lactobacillus* strains, the antibiotic resistance of genus *Lactococcus* has been scantily researched. According to the literature, all of *Lactococcus* species are intrinsically resistance to metronidazole, trimethoprim and cefoxitin. *Lactococcus* species are also able to acquire resistance to Gram-positive spectrum antibiotics, such as macrolides (erythromycin and lincomycin), glycopeptides (vancomycin and teicoplanin), β-lactams and to some broad-spectrum antibiotics, for example rifampicin and chloramphenicol. A slightly lowered susceptibility is also observed towards quinolones, such as ciprofloxacin, dicloxacillin and norfloxacin (Devirgiliis et al., 2013). A significant increase in erythromycin and tetracycline resistance is observed among *Lactococcus* species the most often. Among the various identified resistance genes *erm*(B) is the most frequently found gene among lactococci and is known to be expressed constitutively or inducibly (Erginkaya, Turhan, & Tatlı, 2018). Tetracycline resistance in *Lactococcus* is encoded mainly by the *tet*(M) gene (Devirgiliis, Barile, Caravelli, Coppola, & Perozzi, 2010).

Knowledge about the antibiotic resistance of *Lactococcus* strains derived from starter cultures is limited. This knowledge gap can be attributed to the fact that *L. lactis* subsp. *lactis, Lactococcus lactis* ssp. *diacetylactis* and *L. lactis* subsp. *cremoris* are the only *Lactococcus* species to be used in the food processing industry. Based on the published data, the antibiotic resistance of *Lactococcus* strains does not pose a widespread problem. Hummel et al. (2007) obtained results that showed that strains of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *diacetylactis* from yoghurt and cheese starter cultures were most often resistant to aminoglycosides (streptomycin and gentamycin) and ciprofloxacin, and the resistance gene that was most often found in these strains was the resistance gene chloramphenicol - *cat*. The results of these studies indicate a discrepancy between the phenotype and the genotype of resistance, since the strains showed resistance, although the corresponding resistance genes were not found. This contradiction may be due to the fact that various genetic mechanisms are responsible for phenotypic resistance to a particular antimicrobial, some of which remain unknown. In turn, sensitivity to chloramphenicol, despite the presence of an appropriate resistance gene, can be explained by disruption of gene expression, the presence of pseudogens, or mutations within it that prevent transcription or translation (Resch et al., 2008). Klare et al. (2007) found no resistance to any of the antibiotics among the analyzed *Lactococcus* strains derived from starter cultures.

5.3. Staphylococcus

Bacteria from genera *Staphylococcus*, like all Gram-positive bacteria, are intrinsically resistant to aztreonam, temocillin, polymyxin B/colistin, nalidixic acid and ceftazidime. Literature indicates that staphylococci are able to acquire resistance to broad spectrum of antibiotics (Leclercq et al., 2013). There are two species of coagulase-negative staphylococci used in meat starter cultures – *Staphylococcus xylosus* and *Staphylococcus carnosus*. There are only a few studies on their antibiotic resistance. In study by Resch et al. (2008) including staphylococcal strains from meat starter cultures antibiotic resistance was more frequent in *S. xylosus* than in *S. carnosus* strains. Analyzed strains the most often demonstrate phenotypic resistance to tetracyclines, ampicillin, fusidic acid, lincomycin, penicillin and oxacillin. The more frequent incidence of antibiotic resistance in *S. xylosus* strains is explained by the close relatedness of this species to the opportunistic pathogen *S. saprophyticus*, while *S. carnosus* is member of the phylogenetic *S. simulans* group, in which only nonpathogenic staphylococci are included. It has been speculated that *S. carnosus* is less likely related to the incidence of antimicrobial resistances in starter cultures because of the close phylogenetic relationship with nonpathogenic species (Cordeiro et al., 2010). This hypothesis is supported by these authors' finding that *S. xylosus* strains were antibiotic resistant more frequently than the *S. carnosus* strains, especially to resistances against penicillin, oxacillin and tetracycline. In turn, aminoglycoside resistance is rarely observed (Landeta, Curiel, Carrascosa, Munoz, & de las Rivas, 2013). According to literature antibiotic resistance in staphylococci from meat starter cultures is encoded by genes the most frequently observed in other staphylococcal strains, mainly by *tet*(M) and *tet*(K) for tetracycline resistance, *erm*(B) and *erm*(C) for erythromycin resistance and *blaZ* and *mec*A for β-lactam antibiotics. It is also important that methicillin-resistance is rarely observed in those strains (Kastner et al., 2006).

5.4. Streptococcus

Microorganisms from genus *Streptococcus* are intrinsically resistant only to fusidic acid and aminoglycosides (Cattoir, 2016). However, very little is known about the antibiotic resistance of commercial *Streptococcus* strains. The only *Streptococcus* species that is used in starter cultures is *S. thermophiles* included in yoghurt starter culture. Despite the ubiquitous occurrence of *S. thermophilus* strains in yoghurt starter cultures, the prevalence of antibiotic-resistant strains has not been widely researched to date. Individual reports indicate that *S. thermophilus* strains can be resistant to trimethoprim, macrolides (especially erythromycin) and tetracyclines. The genetic factors implicated in the resistance to the above antibiotics have also been insufficiently investigated in *S. thermophilus*. Resistance to tetracyclines is encoded by *tet*(S) and *tet*(M) genes, whereas resistance to erythromycin – by the *erm* (B) gene. High percentages of *S. thermophilus* strains from yoghurt starter cultures were resistant to gentamicin and penicillin (Gad, Abdel-Hamid, & Farag, 2014).

5.5. Pediococcus

According to current knowledge, species of the genus *Pediococcus* are intrinsically resistant to glycopeptides, aminoglycosides, quinolones and trimethoprim/sulfamethoxazole. These microorganisms are also able to acquire resistance to penicillin G, imipenem, clindamycin, rifampin, chloramphenicol, daptomycin, ramoplanin tetracyclines (tetracycline, oxytetracycline and chlortetracycline), macrolides (erythromycin and lincomycin) and bacitracin (Ammor et al., 2007). Little data about antibiotic resistant of starter pediococci is available. It may be due to the fact that only two species: *P. pentosaceus* and *P. acidilactici* are part of commercial meat starter cultures. According to present state of knowledge starter pediococci are resistant to tetracyclines, macrolides and lincosamides the most frequently (Kastner et al., 2006). *P.*

pentosaceus can also exhibite cross-resistance to antibiotics belonging to the lincosamide and macrolide classes. It may be due to the modification of the target site by the *erm* genes (Cordeiro et al., 2010). Tetracycline resistance in pediococci is encoded by *tet*(B), *tet*(H), *tet*(J), and *tet*(W) genes (Kobashi, Hasebe, Nishio, & Uchiyama, 2007).

6. Starter culture stress response under food processing factors

During food manufacturing and storage as well as in the gastrointestinal tract starter cultures microorganisms are exposed to various stress factors, such as heat, cold, high acidity, presence of bile salts, osmotic and oxidative stress. Their capacity to survive to these challenges is essential for their efficacy. Growth performance and robustness of starter cultures are properties essential for the final product. They should survive all the steps of production process. In addition, the most of these strains survive gastrointestinal conditions - low pH and presence of bile salts. These are also stress factors that can cause changes in microbial cells. Stress response rely on the coordinated expression of genes altering different cellular processes, e.g. cell division, DNA metabolism, membrane composition, transport etc. to improve the stress tolerance. In stress response mechanisms, some aspects of cell physiology are changed (Papadimitriou et al., 2016). The main change is induction of changes in the gene expression by intracellular signaling networks. Stress responses imply several genetic switches controlling metabolic changes. The stress response is accomplished by changes in the patterns of gene expression for those genes whose products are required to protect bacterial cells. Microorganisms respond to stress in a specific way which depend on many factors, mainly the species, and on the type of stress (Rossi, Zotta, Iacumin, & Reale, 2016).

Still little is known about the stress response in starter culture microorganisms, but the topic is well received in relation to, among others, all lactic acid bacteria. Since these are microorganisms that are used in starter cultures, it can be concluded that the same reactions can occur during the exposure of starter cultures to stress factors associated with the production and storage of food. There are studies indicating that as a result of the stress factor acting on the cell, there may be changes in the level of antibiotic resistance of microorganisms. Few studies have examined the effects of exposure to stress factors on the antibiotic susceptibility of lactobacilli. Acid-stressed and bile-stressed lactobacilli have shown modified susceptibility. Increased or reduced susceptibilities have been observed, varying with type of stress, species or strain, and type of antibiotic (Amund, 2016). Natt and Garcha (2011) analyzed the antibiotic susceptibility of *Lactobacillus acidophilus* strain exposed to acid stress in the pH range from 3.5 to 7.5. The strain selected for analysis was sensitive to all antibiotics used except erythromycin, i.e. ampicillin, streptomycin, vancomycin, penicillin, chloramphenicol and tetracycline. The authors showed that after exposure to the stress factor, the test strain showed acquired resistance to all other antibiotics, except for tetracycline and chloramphenicol. In case of chloramphenicol and tetracycline, results indicate that the microorganism became less sensitive towards these antibiotics since the zones of growth inhibition decreased, however, the strain could still be considered as sensitive. Casado Muñoz et al. (2016) observed changes in minimal inhibitory concentrations (MICs) in lactic acid bacteria (*Leuconosctoc pseudomesenteroides* and *Lactobacillus pentosus*) exposed on physiochemical stresses. The increase in MIC was observed the most frequently for ampicillin, chloramphenicol, ciprofloxacin and tetracycline. Dorrian, Briggs, Ridley, Layfield, and Kerr (2011) also showed that stress response in *Lactococcus lactis* is associated with increasing resistance to ribosomally active antibiotics.

It is reported that observed changes in the antibiotic resistance level in response to a stress factor may result from the induction of a stress response, which implies changes in the expression of antibiotic resistance genes. It occurs when the antibiotic resistance genes are located on the same operon as the genes induced at the time of the stress factor and are induced simultaneously. Rossi et al. (2016) looked insight into the heat shock response (HSR) regulation in *Lactobacillus* strains. They found that all the strains in their genomes share 25 various HSR related genes. Among them there were alternatives factors σ^{32} and σ^{24}. They also showed the inclusion in the σ^{32} and σ^{24} regulons of genes with relevant functions such as substrate utilization, tolerance to high acidity and hydrogen peroxide, DNA repair, regulation, cation transport, carbon metabolism, translation and membrane function. In the context of this review the most important is inclusion in these regulons of antimicrobial resistance genes, such as *lmr*B encoding lincomycin resistance efflux protein *van*Z – glycopeptide resistance gene, *ytm*P encoding aminoglycoside phosphotransferase family protein. The multiple antibiotic resistance (mar) operon is also associated with the σ^{32} regulon, responsible for antibiotic resistance genes expression.

This phenomenon still requires intensified research and knowledge. Both physiological and gene expression studies can play a key role in obtaining new information on the impact of stress factors associated with food production and storage on bacterial cells and their resistance to antibiotics.

7. Conclusions

Research indicates that the acquisition of antibiotic resistance in starter cultures is not a critical problem, but it poses a serious concern that cannot be disregarded. Starter culture strains are highly capable of acquiring new genes and transferring resistance genes to other strains. Interspecies transfer of antibiotic resistance is also possible. It is also possible to widen the profile of antibiotic resistance due to the stress factor associated with the conditions of food production and storage. Therefore, bacteria that harbor antibiotic resistance genes on mobile genetic elements should not be distributed or used in food production.

Declaration of competing interest

None.

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028X-73.5.916 In this study authors analysed the antibiotic resistance of microorganisms from meat starter cultures. Their results indicate that incidence of antimicrobial resistance was higher among Pediococcus pentosaceus and lower for Staphylococcus carnosus strains but genetic determinants were not found using PCR. Phenotypic resistance in the absence of known resistance genes found here suggests that other mechanisms or genes might have contributed to the negative results.

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Microorganisms from starter and protective cultures - Occurrence of antibiotic resistance and conjugal transfer of *tet* genes *in vitro* and during food fermentation

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ABSTRACT

This study analyzed antibiotic resistance and the occurrence of resistance genes in strains from starter and protective cultures. The largest number of obtained strains belonged to the genus *Lactobacillus* (n = 26; 44.9%), followed by *Lactococcus* (n = 17; 29.3%), *Pediococcus* (n = 8; 13.8%), *Streptococcus* (n = 4; 6.9%), *Staphylococcus* (n = 2; 3.4%) and *Leuconostoc* (n = 1; 1.7%). Among all strains, the highest Minimum Inhibitory Concentrations (MICs) were noted for tetracycline (max. >256 μg/ml), kanamycin (max. 48 μg/ml) and chloramphenicol (max. 32 μg/ml). The most common resistance genes were *aph(3')-IIIa* (n = 35; 60.3%) and *cat* (n = 32; 55.2%). Strains derived from starter and protective cultures have the ability to horizontally transfer resistance genes *in vitro* and *in situ*. It should be noted that antibiotic resistance of bacteria from starter and protective cultures does not present an immediate risk to consumers. However, these microorganisms could become an environmental reservoir of antibiotic resistance. The obtained results indicate that antibiotic resistance in starter and protective cultures is not a critical issue, however it creates a concern that cannot be ignored.

1. Introduction

Starter cultures, i.e. pure cultures of selected microbial strains, mainly lactic acid bacteria (LAB), are introduced into food at the production stage to obtain a product with desired characteristics. Without them, it would not be possible to obtain a proper product, as these microorganisms are involved in major biochemical transformations (Bintsis, 2018). Protective cultures, i.e. viable microorganisms added intentionally to food in order to control its microbiological status, are distinguished among industrial cultures as well. They can inhibit the growth of many pathogenic microorganisms due to the production of metabolites with antibacterial and antifungal properties (Ng, Zarin, Lee, & Tan, 2020). Although the use of starter cultures has been practiced in the production of food for years, the use of protective cultures has become popular in recent years. The reason were efforts to obtain a so-called "clean label" and the abandonment of the use of preservatives. At this point, the introduction of bacterial strains that are safe for consumers yet act antagonistically towards other microorganisms was proposed (Quinto et al., 2019).

According to the World Health Organization (WHO), the increasing prevalence of microbial antibiotic resistance is associated with serious risks and is becoming one of the most serious public health problems. The WHO has warned of the lack of new antibiotics to combat growing drug resistance. Most of the available drugs are modifications to existing antibiotics which, in this case, are only short-term solutions (Talebi Bezmin Abadi, Rizvanov, Haertlé, & Blatt, 2019).

Since the industrially used microorganisms enter food in a quantity of the order of 10^7–10^8 CFU/g, it is necessary to make sure that they are not a source of antibiotic resistance genes. In the United States, a system for the assessment of microorganisms added intentionally to food (GRAS – Generally Recognized As Safe) has been in place for many years. In 2007 the European Food Safety Authority (EFSA) outlined a scheme of the concept of qualified presumption of safety (QPS). Many strains, mainly LAB, due to their long history of safe use, have been recognized as traditional food ingredients. They were legally approved for use in food production within the European Union with no need to carry out studies confirming their safety. Strains which were granted "safe" status in the past and are approved for industrial use in food production do not need to be regularly checked for the occurrence of acquired antibiotic resistance. Since the analysis of the genetic resistance determinants

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occurrence has been applied for a relatively short time, there is no information on the occurrence of resistance genes and mobile genetic elements in the strains used in the production of food for many years (Laulund, Wind, Derkx, & Zuliani, 2017). However, according to literature data, antibiotic resistance may also occur in the strains intentionally added to food in the form of industrial cultures. Intrinsic resistance and resistance resulting from spontaneous mutations do not raise concern as it exhibits a low risk of horizontal transfer. However, it is more and more frequently reported that the strains generally recognized as safe may acquire resistance genes and transfer them horizontally to other strains (Rozman, Mohar Lorbeg, Accetto, & Bogovič Matijašić, 2020).

Antibiotic resistance in strains from starter and protective cultures has been documented most accurately for the genus *Lactobacillus*. According to the literature, lactobacilli are intrinsically resistant to glycopeptides and quinolones. They also exhibit the potential for acquiring resistance to chloramphenicol (encoded by the *cat* gene), tetracycline (encoded mainly by the *tet*M, *tet*S and *tet*W genes) and macrolides (encoded by the *erm*A, *erm*B and *erm*C genes) (Fraqueza, 2015; Georgieva et al., 2015). The genus *Lactococcus* has intrinsic resistance to metronidazole, trimethoprim and cefoxitin (Devirgiliis, Zinno, & Perozzi, 2013). According to the literature, lactococci may acquire resistance to macrolides, tetracyclines, rifampicin and chloramphenicol. The resistance genes most frequently found in *Lactococcus* are *erm*B and *tet*M (Erginkaya, Turkan, & Tatli, 2018). *Pediococcus* is naturally resistant to glycopeptides, ciprofloxacin, trimethoprim and sulfamethoxazole and might also exhibit acquired resistance to tetracyclines, encoded by the genes *tet*K, *tet*O, *tet*S and *tet*W, and to macrolides, encoded by the *erm*B gene (Shani, Oberhaensli, & Arias-Roth, 2020; Singla, Mandal, Sharma, Anand, & Tomar, 2018).

Given that starter and protective cultures strains are not pathogenic organisms, the main hazard linked to the occurrence of antibiotic resistance in them is the risk of horizontal transfer of resistance genes. The possibility of resistance genes transfer from these strains was confirmed under both *in vitro* and *in situ* conditions. According to the literature, the most frequently occurring transfer is that of tetracycline and macrolide resistance genes (Thumu & Halami, 2018). Some authors point to the possibility of transferring resistance genes from starter cultures microorganisms to pathogens in food, especially during fermentation (Flórez et al., 2016; Toomey, Monanghan, Fanning, & Bolton, 2009). The issues concerning resistance gene transfer by microorganisms from starter and protective cultures are already quite well known. There are few reports on the conjugative transfer of antibiotic resistance determinants from the bacteria used in industrial cultures to other microorganisms. This implies the need to undertake research in this area.

The aim of this study was to determine the antibiotic resistance pattern of the strains included in the commercially available starter and protective cultures and an analysis of the occurrence of antibiotic resistance genes. The ability of these strains to horizontally transfer resistance genes was also investigated.

2. Materials and methods

2.1. Bacterial strains

The strains for analysis were obtained from commercial starter (n = 30) and protective (n = 10) cultures. They were sourced from several producers offering cultures for use in the food industry. For this purpose, freeze-dried cultures were cultured on de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany), incubated for 24–48 h at 30 °C and then plated on MRS agar plates (Merck, Germany). The plates were then incubated under anaerobic conditions at 30 °C for 48 h. After incubation, individual colonies were selected and isolated on MRS agar plates. When pure strains were obtained, they were passaged onto the base medium and stored at −80 °C in Microbank system (Biocorp, Poland) prior to further analysis to minimize the risk of plasmid loss during passage.

2.2. Genomic and plasmid DNA isolation

For genomic and plasmid DNA extraction, bacterial strains were streaked on MRS agar (Merck, Germany) and incubated at 37 °C for 24 h. Genomic DNA extraction were provided using the Genomic Mini DNA isolation kit and plasmid DNA was isolated using Plasmid Mini kit (A&A Biotechnology, Poland), according to the manufacturer's instructions. The obtained genomic DNA samples was suspended in 200 μl Tris-HCl buffer (10 mM, pH 8.5) and stored at −20 °C until further analysis. Plasmid DNA samples were suspended in 60 μl Tris-HCl buffer (10 mM, pH 8.5) and also stored at −20 °C.

2.3. Strains identification

The identification of the strains was performed in order to distinguish the strains included in each culture. Strains were identified using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). Before the analysis strains were cultured for 48 h at 30 °C on Tryptic Soy Agar - TSA (Merck, Germany). Then single colonies were transferred to the target plate, covered with 1 μl of CHCA (α-cyano-4-hydroxy-cinnamic acid) solution and air-dried completely. Measurements were taken using a VITEK MS (bioMérieux, Durham, NC) with acceleration voltage 200 kV, mass range 2–20 kDa, laser frequency 50 Hz, and extraction delay time 200 ns. All mass fingerprints were analyzed by the VITEK MS V2.0 research use only database (RUO; SARAMIS version 4.13) (bioMérieux, Durham, NC). Strains were tested in triplicate using the direct transfer protocol according to the manufacturers' recommendations. *Escherichia coli* ATCC® 8739 was used to validate the method.

As the MALDI-TOF MS analysis does not distinguish between *Lactobacillus pentosus, L. plantarum* and *L. paraplantarum* species and *Lactococcus lactis* subspecies, for this purpose, differentiation by PCR using primers and reaction conditions contained in Table S1 was used. PCR reactions were conducted in a thermal cycler Mastercycler nexus GX2 (Eppendorf, Germany). Primers were synthesized by the commercial enterprise – Genomed (Gdańsk, Poland).

The differentiation of *L. pentosus L. plantarum* and *L. paraplantarum* species was carried out in accordance with Torriani, Felis, and Dellaglio (2001). The reaction was set up using 1x PCR buffer without Mg^{2+} (ThermoFisher Scientific, Waltham, Massachusetts, USA), 1.5 mM $MgCl_2$, the primers paraF, pentF, and pREV (0.25 mM each), and primer planF (0.12 mM), 12 mM deoxynucleotide triphosphates, 0.025 U/μl of Taq DNA Polymerase (ThermoFisher Scientific, Waltham, Massachusetts, USA), 5 ng/ml of DNA template and ddH_2O to a final volume of 25 μl. The cycling conditions were as follows: an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 10 s and elongation at 72 °C for 30 s, and final extension at 72 °C for 5 min. Reference strains of *L. pentosus* (ATCC® 8041) and *L. plantarum* (ATCC® 14917™) were used as positive controls.

The differentiation of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* was carried out according to Pu, Dobos, Limsowtin, and Powell (2002). The reaction was set up using 1x DreamTaq PCR buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA), 0.2 mM dNTPs, 50 pmol of LaCreR, LacF and CreF primers, 1 U DreamTaq DNA polymerase (ThermoFisher Scientific, Waltham, Massachusetts, USA) and 2 μl of DNA template. The cycling conditions were as follows: an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s and elongation at 72 °C for 2 min, and final extension at 72 °C for 5 min. Reference strains of *L. lactis* ssp. *lactis* (ATCC® 87139) and *L. lactis* ssp. *cremoris* (ATCC® 9596) were used as positive controls.

The PCR products were visualized by electrophoresis on 1.5% agarose gels in 1 × TBE (Tris-borate-EDTA) buffer stained by 0.5 μg/ml

of Midori green (ABO, Poland) at 100 V for 6 min and then at 80V for 90 min and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, UK).

After identification, duplicated strains were discarded and one strain of a given species from each culture manufacturer was selected for further analysis.

2.4. Molecular typing by enterobacterial repetitive intergenic consensus - polymerase chain reaction (ERIC-PCR)

All strains were genotyped using ERIC-PCR assay according to Pusparini, Waturangi, Usia, and Nikastri (2018). The PCR reactions were performed in a volume 25 μl containing 12.5 μl DreamTaq Green PCR Master Mix (2x) (ThermoFisher Scientific, Waltham, Massachusetts, USA), 1.25 μl of 10 pmol ERIC1 (5′- ATG TAA GCT CCT GGG GAT TCA-3′) and ERIC2 (5′- AAG TAA GTG ACT GGG GTG AGC G-3′) primers synthesized by the commercial enterprise – Genomed (Gdańsk, Poland), 1.5 μl of DNA template and 8.5 μl of ddH_2O. PCR reactions were conducted in a thermal cycler Mastercycler nexus GX2 (Eppendorf, Germany) using the following cycling conditions: initial denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 65 °C for 8 min and a final elongation at 65 °C for 15 min before cooling at 4 °C. The PCR products were visualized by electrophoresis on 1.5% agarose gels in 1 × TBE (Tris-borate-EDTA) buffer stained by 0.5 μg/ml of Midori green (ABO, Poland) at 150 V for 150 min and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, UK).

The ERIC-PCR profiles were examined by cluster analysis with BioNumerics ver. 7.6.3. software (Applied Maths/bioMérieux, Sint-Martens-Latem, Belgium). The similarity distances between ERIC-PCR profiles were calculated using the Dice coefficient, and the dendrogram was based on the unweighted pair group method with arithmetic mean (UPGMA).

2.5. Phenotypic antibiotic resistance - minimal inhibitory concentrations (MICs) determination

The minimum inhibitory concentrations (MICs) of 9 antibiotics: ampicillin (AMP), vancomycin (VA), gentamicin (CN), kanamycin (KA), streptomycin (S), erythromycin (E), clindamycin (DA), tetracycline (TE) and chloramphenicol (C) were determined. Two methods of determination were used. The MIC values were determined using gradient strips (Liofilchem, Italy) on Mueller-Hinton agar (Merck, Germany) as well as using VITEK® 2 Compact system for determining the susceptibility of microorganisms (Biomerieux, Durham, NC) with AST–P643 cards. The MIC value was read following after 18 h of incubation at 30 °C. Due to the lack of current guidelines for the determination of antibiotic resistance among the strains of lactic acid bacteria, the result was not interpreted in terms of susceptible/resistant. Only the MIC values determined for individual antibiotics were reported.

2.6. Molecular detection of antibiotic-resistant genes

All the strains were tested for the presence of four tetracycline resistance genes encoding ribosomal protection proteins - *tet*M (Roberts, 1994), *tet*O, *tet*S and *tet*W (Flórez et al., 2014) and two genes encoding tetracycline efflux pumps - *tet*K (Gevers, Danielsen, Huys, & Swings, 2003) and *tet*L (Roberts, 1994). Moreover, each of the strains was analyzed for the presence of genes encoding resistance to aminoglycosides - *aac(6′)-Ie-aph(2″)-Ia*, *aph(3″)-IIIa* (Padmasini, Padmaraj, & Ramesh, 2014), *aph(2″)-Ic*, *aac(6′)-Ii* and *ant(6′)-Ia* (Kobayashi et al., 2001), chloramphenicol - *cat* (Goksel et al., 2016), β-lactams – *blaZ* (Kaase et al., 2008), bla_{TEM}, bla_{OXA}, bla_{SHV} and bla_{CTX-M} (Kim et al., 2009), glycopeptides – *van*A and *van*B (Dutka-Malen, Evers, & Courvalin, 1995) and macrolides – *erm*A, *erm*B, *erm*C and *msr*A/B (Młynarczyk, Młynarczyk, Szymanek, & Luczak, 2006). In addition, a specific primer pair was used to amplify gene involved the presence of Tn*916*/Tn*1545* conjugative transposons (Macovei & Zurek, 2006). Gene presence assays were performed using the Simple and Multiplex PCR methods using specific primers and conditions (Table S1). As positive controls, *E. faecalis* ATCC® 29212 (*tet*M), *E. faecalis* ATCC® 51299 (*erm*B, *aac (6′)-Ie-aph(2″)-Ia*), *S. epidermidis* ATCC® 35984 (*aph(3′)-IIIa*, *erm*A, *bla*Z, *ant(6′)-Ia*), *S. epidermidis* ATCC® 12228 (*blaZ*, *tet*K) and strains of Department's collection carrying well characterized antibiotic resistance genes was used in the amplification reactions.

The PCR products were visualized by electrophoresis on 1.5% agarose gels in 1 × TBE (Tris-borate-EDTA) buffer stained by 0.5 μg/ml of Midori green (ABO, Poland) at 100 V for 6 min and then at 80 V for 90 min and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, UK).

2.7. Antibiotic resistance genes transfer

2.7.1. Recipient and donor strains

In order to select the donor strains, it was checked whether the previously identified resistance genes were chromosomally- or plasmid-located in individual strains. For this purpose, strains in which at least one of the resistance genes had been determined were selected and PCR reactions were repeated for these genes, using the previously isolated plasmid DNA as a template instead of genomic DNA. The presence of the band at the appropriate height indicated the plasmid localization of the gene, while the absence of the band indicated the chromosomal gene localization. All strains which had simultaneously at least one plasmid-encoded resistance gene and showed high MIC value for given antibiotic were selected as donors. Ultimately, 5 donor strains that had plasmid encoded tetracycline resistance genes and a high tetracycline MIC were selected for analysis.

E. faecalis JH2–2 (LMG 19456), free from plasmids, resistant to rifampicin (MIC >256 μg/ml) and fusidic acid (MIC >256 μg/ml) was the standard recipient in the experiment.

2.7.2. In vitro filter mating

Transferability of antibiotic resistance genes was examined by filter mating. For this purpose, overnight cultures of donor and recipient strains have been prepared on Brain Heart Infusion (BHI) broth medium (Merck, Germany). Then 1 ml of the each culture was added to 5 ml of fresh BHI medium and incubated for 4 h at 37 °C until the mid-exponential phase of growth was obtained. Then the 1 ml of donor culture was added to the 1 ml recipient culture and the mixture was filtered through a sterile cellulose membrane filter with a pore diameter of 0.45 μm. After donor and recipient cells were filtered, the filters were rinsed with 2 ml sterilized peptone physiological saline solution (PPS) (Merck, Germany). The filters were placed on BHI-agar medium and were incubated overnight at 37 °C. After incubation, colonies grown on the filter surface were resuspended in 2 ml PPS. Series of 10-fold dilutions were made from the obtained suspension and plated on 3 types of media: T-BHI (with the addition of 100 μg/ml of fusidic acid, 50 μg/ml of rifampicin and 10 μg/ml of tetracycline), TD-BHI (with the addition of 10 μg/ml of tetracycline) and B–BHI (with the addition of 100 μg/ml fusidic acid and 50 μg/ml rifampicin). Transconjugant colonies were to grow on T-BHI medium, donor colonies on TD-BHI medium, and recipient colonies on B–BHI medium. Incubation was carried out at 37 °C for 24–48 h. The transfer frequency was expressed as the ratio of the number of transconjugants to the number of recipients.

2.7.3. In situ mating in the food matrix

In order to assess the possibility of *in situ* conjugal transfer from LAB strains in food matrix, a model of milk acidification was prepared. Donor and recipient cultures were prepared as described above, however the 4-h incubation was conducted not in BHI broth but in milk. Skim-milk powder (4% w/v) was added to 10 ml of pasteurized milk. This mix

was heated at 63 °C for 30 min in a preheated water bath, and then cooled to 42 °C. Then 1 ml of donor and recipient culture were added to milk and incubated at 42 °C until the pH 4.3 was obtained. The resulting coagulated milk mixture was then incubated at 4 °C for 48 h. After incubation, serial 10-fold dilutions of fermented milk samples were plated onto selective media as described above. Plates were incubated for 24–48 h at 37 °C and the transfer frequency was expressed identically as described above.

2.7.4. Confirming the presence of resistance genes in transconjugants

Obtained transconjugants were screened for their tetracycline resistance. The MIC value of tetracycline was determined by applying a gradient strip (Liofilchem, Italy) on an inoculated Mueller-Hinton agar plate (Merck, Germany). The MIC value was read following after 18 h of incubation at 30 °C. Then, genomic DNA was isolated from the transconjugants using the Genomic Mini kit (A&A Biotechnology, Poland) and repeated PCR reactions were performed to confirm gene transfer.

2.7.5. Genotyping of transconjugants

The fingerprints of transconjugants found to have acquired resistance genes were obtained by ERIC-PCR reaction as described above. Then, fingerprints were compared to the fingerprints of recipient strains for confirmation purposes.

2.8. Statistical analysis

Statistical analyses were performed using STATISTICA 13.3 StatSoft® software (StatSoft, Inc., Tulsa, OK, USA) using the Spearman correlation test or the Mann–Whitney *U* test. For the analyses, the significance level $\alpha = 0.05$ was assumed.

3. Results

3.1. Strain identification and genotyping

After removing duplicate strains, i.e. strains of a given species from cultures of the same producer (there was any mis-identity), a total number of 58 strains from 40 commercially available starter and protective cultures were obtained. The largest number of strains (n = 44; 75.9%) came from starter cultures and 14 isolates (24.1%) were obtained from protective cultures. The strains belonged to 16 various species from six genera: *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Pediococcus*, *Streptococcus* and *Leuconostoc*. The largest number of obtained strains belonged to the genus *Lactobacillus* (n = 26; 44.9%), followed by *Lactococcus* (n = 17; 29.3%), *Pediococcus* (n = 8; 13.8%), *Streptococcus* (n = 4; 6.9%), *Staphylococcus* (n = 2; 3.4%) and *Leuconostoc* (n = 1; 1.7%) (Table 1). The confidence level of identification for each isolate was in the range of 91–99%.

Genotyping by ERIC-PCR showed low genetic diversity among the strains from the protective and starter cultures (Fig. 1). The ERIC1 and ERIC2 primers generated in the analyzed strains 2–10 amplicons with a molecular weight ranging from 250 to 11,000 bp, and based on UPGMA analysis, 53 different ERIC-PCR profiles were recognized in all 58 strains. The most dominant profiles (n = 5) consisted of two strains, while each of the other profiles was detected in only one isolate.

3.2. Phenotypic antibiotic resistance

Among all the tested strains, the highest MIC values were noted for tetracycline (maximum MIC value - >256 μg/ml), kanamycin (maximum MIC value - 48 μg/ml) and chloramphenicol (maximum MIC value - 32 μg/ml). The lowest MIC values were noted for erythromycin and streptomycin (all MICs were lowest than 1 μg/ml). The largest spectrum of MIC values was observed for tetracycline (0.19 - >256 μg/ml) (Table 2, Table S2). There were no statistically significant differences in the observed MIC values for each antibiotic and the bacterial genus or source of the strain.

Table 1
Strains chosen to the analysis - results of identification.

Species/genus (No. of strains)	Number (%) of strains	
	Starter cultures	Protective cultures
Lactobacillus		
L. paracasei (5)	5 (100.0)%	–
L. paraplantarum (4)	4 (100.0%)	–
L. delbrueckii (2)	2 (100.0%)	–
L. plantarum (1)	1 (100.0%)	–
L. kefiri (1)	1 (100.0%)	–
L. sakei (2)	–	2 (100.0%)
L. rhamnosus (2)	–	2 (100.0%)
L. curvatus (1)	1 (100.0%)	–
L. helveticus (1)	1 (100.0%)	–
Lactobacillus sp. (7)	6 (85.7%)	1 (14.3%)
Total (26)	21 (80.7%)	5 (19.3%)
Lactococcus		
L. lactis ssp. *lactis* (15)	14 (93.3%)	1 (6.7%)
L. lactis ssp. *cremoris* (2)	2 (100.0%)	–
Total (17)	16 (94.1%)	1 (5.95)
Staphylococcus		
S. xylosus (1)	–	1 (100.0%)
S. carnosus (1)	1 (100.0%)	–
Total (2)	1 (50.0%)	1 (50.0%)
Pediococcus		
P. pentosaceus (4)	1 (25.0%)	3 (75.0%)
P. acidilactici (4)	–	4 (100%)
Total (8)	1 (12.5%)	7 (87.5%)
Streptococcus		
S. thermophilus (4)	4 (100.0%)	–
Leuconostoc		
L. mesenteroides ssp. *cremoris* (1)	1 (100.0%)	–
Total (58)	44 (75.9%)	14 (24.1%)

Although the majority of the strains (46.5%) presented a high MIC value to two antibiotics, 10.3% had high a MIC for three antibiotics (Table 2). No statistically significant differences were found in the number of antibiotics to which strains presented high MIC value depending on the genus.

3.3. Presence of antibiotic resistance genes

Regardless of the resistance phenotype presented, 15 different resistance determinants were established in all strains. The presence of the *van*A and *van*B genes was determined only in *Lactococcus*, *Streptococcus* and *Staphylococcus* strains due to intrinsic vancomycin resistance in *Lactobacillus*, *Leuconostoc* and *Pediococcus* strains.

Regardless of the genus, the most common gene encoding aminoglycoside resistance was *aph(3′)-IIIa*, followed by *aac(6′)-Ie-aph(2′)-Ia*, *aac(6′)-Ia* and *aph(2′)-Ic*. None of the strains showed the presence of the *ant(6′)-Ia* gene.

The frequency of the various tetracycline resistance genes was dependent on the genus. Among the *Lactobacillus* strains, tetracycline resistance was most often associated with the presence of the *tet*M gene, followed by *tet*W and *tet*O. Furthermore, among *Lactococcus*, *Pediococcus* and *Staphylococcus* strains, the *tet*M and *tet*W genes were carried by the same percentage of strains, and the *tet*O gene was dominant among *Streptococcus* strains.

More than half of the strains (55.2%) carried the *cat* gene encoding chloramphenicol acetyltransferase. Among the tested genes encoding resistance to β-lactam antibiotics, two strains were carriers of the bla_{OXA} and *blaZ* genes. Of the four macrolide resistance genes determined, only one *Latobacillus* strain was found to carry the *msr*A/B gene. The remaining genes were not marked in any of the strains. No vancomycin resistance genes (*van*A and *van*B) were found in any of the strains. The conjugative transposon of the Tn*916*/Tn*1545* family was present in only four isolates (Table 3, Table S2).

An analysis of significant differences in the frequency of resistance

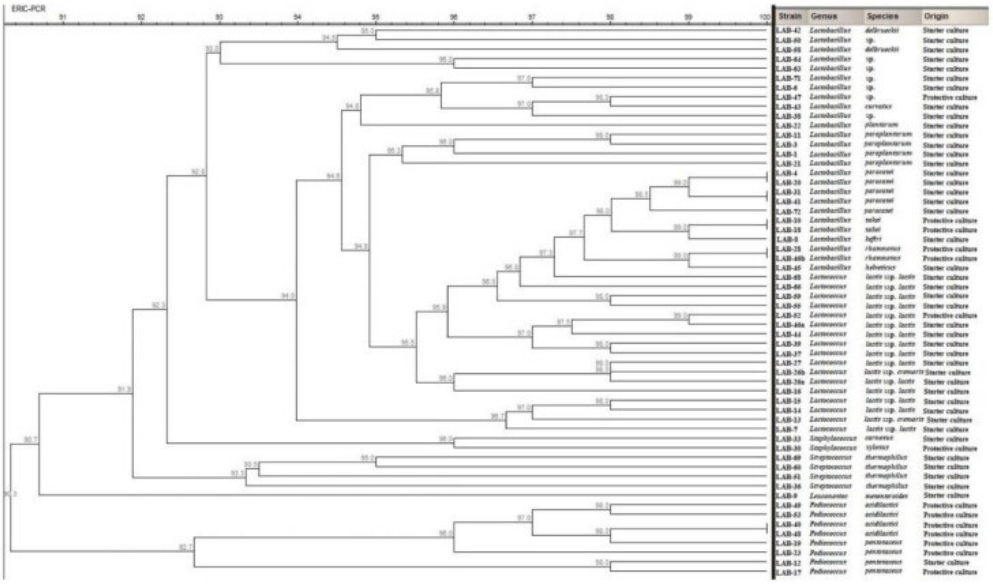


Fig. 1. ERIC-PCR-based dendrogram showing genetic relatedness among strains from starter and protective cultures.

Table 2
Phenotypic antibiotic resistance patterns.

Genus	No. of strains	MIC value (μg/mL)								
		AMP	CN	C	TE	E	DA	K	S	VA
L. curvatus	1	0.125	0.5	16	64	0.125	0.38	6	0.125	–
L. delbrueckii	2	1.5–2	0.75	6–12	24	0.19–0.25	0.023–0.38	12–16	0.19–0.25	–
L. helveticus	1	0.75	0.25	0.12	32	0.125	0.032	6	0.125	–
L. kefiri	1	0.125	0.19	0.25	0.19	0.125	0.75	0.19	0.125	–
L. paracasei	5	0.19–1.5	0.38–8	4–32	1–32	0.023–0.25	0.023–0.5	4–48	0.023–0.25	–
L. paraplantarum	4	0.25–1.5	0.38–0.75	6–32	12 - >256	0.125–0.25	0.25–0.75	6–16	0.125–0.25	–
L. plantarum	1	3	1	6	32	0.19	0.047	16	0.19	–
L. rhamnosus	2	0.125–0.38	0.75–2	4–8	1–32	0.047–0.32	0.125–0.19	3–8	0.047–0.32	–
L. sakei	2	0.5–2	0.25–1	12–24	1,5 - 48	0.094–0.125	0.023–0.032	8–16	0.094–0.125	–
***Lactobacillus* sp.**	7	0.125–3	0.5–1	6–24	12–48	0.094–0.25	0.016–0.75	4–16	0.094–0.25	–
L. lactis* ssp. *lactis	15	0.094–3	0.19–1.5	4–24	0.75–48	0.023–0.19	0.023–1.5	6–32	0.094–0.19	0.094–32
L. lactis* ssp. *cremoris	2	0.25	0.38–4	12–16	32	0.19–0.25	0.25–0.75	8–12	0.19–0.25	0.19
P. pentosaceus	4	0.19–3	0.5–8	4–12	1.5–24	0.094–0.125	0.023–0.094	16–48	0.094–0.125	–
P. acidilactici	4	0.094–2	0.25–1	6–32	16–64	0.094–0.25	0.016–0.75	12–32	0.094–0.25	–
S. thermophilus	4	0.25–3	0.5–1	2–24	16–32	0.094–0.38	0.016–0.75	1–24	0.094–0.38	0.125–0.25
S. xylosus	1	4	0.75	6	12	0.19	0.38	24	0.19	0.25
S. carnosus	1	1	0.38	8	24	0.125	0.016	6	0.125	32
L. mesenteroides	1	0.38	0.5	24	16	0.125	0.5	8	0.125	–

Abbreviations: AMP – ampicillin, CN – gentamicin, C – chloramphenicol, TE – tetracycline, E – erythromycin, DA – clindamycin, K – kanamycin, S – streptomycin, VA – vancomycin, „-" – MIC was not determined due to the intrinsic resistance.

genes, depending on the type, found differences for the following genes: *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2′)-Ia*, *aac(6′)-Ia*, *tet*M, *tet*O, *tet*W and *int*. However, when comparing the frequency of resistance genes with the source of the strain, no differences were found.

3.4. Tetracycline resistance genes transfer

Plasmid location was found for five tetracycline-resistance genes. They were carried by five different strains which were therefore selected as donors in the study of the ability of the strains to transfer resistance genes. The characteristics of donor strains are presented in Table 4.

This ability was analyzed using two methods: *in vitro* filter mating and *in situ* in food matrix. Three mating pairs examined *tet*K gene transfer and two - *tet*M gene. Of these, gene transfer was observed in each pair.

In *in vitro* filter mating, the transfer frequency from donors to *E. faecalis* JH2-2 ranged from 2.78×10^{-7} up to 1.71×10^{-5} trans-conjugants per recipient. Tetracycline MIC values for the trans-conjugants ranged from 24 to 64 μg/ml. The same five mating pairs were inoculated into the fermented milk to investigate the transfer possibility *in situ* in the food matrix. Gene transfer was also observed in each pair, but the transfer frequency was lower than *in vitro* filter mating and

Table 3
Occurrence of antibiotic resistance genes.

Resistance gene	Number (%) of strains with antibiotic resistance genes								Total n = 58
	Lactobacillus n = 26	*Lactococcus* n = 17	*Pediococcus* n = 8	*Streptococcus* n = 4	*Staphylococcus* n = 2	*Leuconostoc* n = 1	Starter cultures (n = 44)	Protective cultures (n = 14)	
Aminoglycosides									
aph(3′) IIIa	16 (61.5%)	10 (58.8%)	5 (62.5%)	1 (25.0%)	2 (100.0%)	1 (100.0%)	25 (56.8%)	10 (71.4%)	35 (60.3%)
aac(6′) Ie-aph (2′)-Ia	16 (61.5%)	8 (47.1%)	3 (37.5%)	1 (25.0%)	1 (100.0%)	1 (100.0%)	24 (41.4%)	7 (50.0%)	31 (53.4%)
aac(6′) Ia	7 (26.9%)	3 (17.6%)	1 (12.5%)	–	–	1 (100.0%)	10 (22.7%)	2 (14.3%)	12 (20.7%)
aph(2′)-Ic	3 (11.5%)	–	–	–	–	–	2 (4.5%)	1 (7.1%)	3 (5.2%)
ant(6′) Ia	–	–	–	–	–	–	–	–	–
Tetracyclines									
tetM	12 (46.2%)	5 (29.4%)	2 (25.0%)	1 (25.0%)	2 (100.0%)	1 (100.0%)	17 (38.6%)	6 (42.9%)	23 (39.6%)
tetL	2 (7.7%)	–	–	–	–	–	1 (2.3%)	1 (7.1%)	2 (3.4%)
tetK	1 (3.8%)	1 (5.9%)	1 (12.5%)	–	–	–	2 (4.6%)	1 (7.1%)	3 (5.2%)
tetO	4 (15.4%)	2 (11.7%)	1 (12.5%)	3 (75.0%)	1 (50.0%)	–	10 (22.7%)	1 (7.1%)	11 (18.9%)
tetS	1 (3.8%)	–	–	–	–	–	1 (2.3%)	–	1 (1.7%)
tetW	6 (23.1%)	5 (29.4%)	3 (37.5%)	–	2 (100.0%)	–	10 (22.7%)	6 (42.9%)	16 (27.6%)
Macrolides									
ermA	–	–	–	–	–	–	–	–	–
ermB	–	–	–	–	–	–	–	–	–
ermC	–	–	–	–	–	–	–	–	–
msrA/B	1 (3.8%)	–	–	–	–	–	1 (2.3%)	–	1 (1.7%)
Glycopeptides									
vanA	n.d.	–	n.d.	–	-	n.d.	-	-	-
vanB	n.d.	–	n.d.	–	-	n.d.	-	-	-
β-lactams									
bla_{TEM}	–	–	–	–	-	-	-	-	-
bla_{SHV}	–	–	–	–	-	-	-	-	-
bla_{CTX-M}	–	–	–	–	-	-	-	-	-
bla_{OXA}	1 (3.8%)	1 (5.9%)	–	–	-	-	2 (4.6%)	–	2 (3.4%)
blaZ	1 (3.8%)	1 (5.9%)	–	–	-	-	2 (4.6%)	–	2 (3.4%)
Chloramphenicol									
cat	15 (57.7%)	9 (52.9%)	5 (62.5%)	3 (75.0%)	-	-	23 (52.3%)	9 (64.3%)	32 (55.2%)
Conjugative transposon									
int (Tn916-Tn1545)	1 (3.8%)	3 (17.6%)	–	–	-	-	3 (6.8%)	1 (7.1%)	4 (6.9%)

Abbreviations: „-" – trait has not been present, „n.d." – no data; has not been analyzed due to the intrinsic resistance.

Table 4
Characteristic of donor strains.

Strain	Identification	Source	Antibiotic resistance	
			Tetracycline MIC	Genotype
LAB-14	*Lactococcus lactis* ssp. *lactis*	Starter culture	48 μg/ml	***tetK***, *aph(3′)-IIIa, aac(6′) Ie-aph (2′) Ia*
LAB-16	*Lactococcus lactis* ssp. *lactis*	Starter culture	32 μg/ml	***tetM***, *aph(3′)-IIIa, aac(6′) Ie-aph(2′) Ia, aac (6′)-Ia*
LAB-17	*Pediococcus pentosaceus*	Protective culture	24 μg/ml	***tetK***, *aph(3′)-IIIa, aac(6′) Ie-aph (2′) Ia*
LAB-22	*Lactobacillus plantarum*	Starter culture	32 μg/ml	***tetK***, *aph(3′)-IIIa, aac(6′) Ie-aph (2′) Ia*
LAB-28	*Lactobacillus rhamnosus*	Protective culture	32 μg/ml	***tetM***, *aph(3′)-IIIa, aac(6′) Ie-aph(2′) Ia, aph (2′) Ic*
16680 EK	*Enterococcus faecalis*	Clinical (positive control)	128 μg/ml	*ermB*, ***tetM***, *int, aac(6′) Ie-aph (2″) Ia, ant(6′)-Ia*

ranged from 1.36×10^{-8} up to 1.07×10^{-7}. Tetracycline MIC values for the transconjugants ranged from 12 to 48 μg/ml. The MIC values for tetracycline were lower or equal to MICs determined for the donors. There were two exceptions: *Pediococcus pentosaceus* LAB-17 and *Lactobacillus rhamnosus* LAB-28, where MIC values for the transconjugants obtained in the *in vitro* filter mating were higher than in the donors (Table 5).

ERIC-PCR reactions revealed similar recipient and transconjugant profiles. It might therefore be excluded that the resistance was acquired by a mutation in the donor genome, which confirms the clonal nature of the transconjugants (Fig. 2).

4. Discussion

While there are many studies on antibiotic resistance among lactic acid bacteria and other food-related microorganisms, there is still little information on the prevalence of antibiotic resistance in starter and protective cultures and, in particular, a lack of current data is apparent. This created a need to analyze the current state.

From a safety point of view, strains from starter or protective cultures cannot harbor transferable resistance genes, in order to be used as food additives and to exert an effect on the human host. The results obtained in this study indicate that antibiotic-resistant strains may be present in microbial cultures used industrially. The phenotypic method did not indicate any strains with high MIC value to four out of nine analyzed

Table 5
Results of conjugal transfer experiment.

Mating strains	Donor (D)	Recipient (R)	Transconjugants (TC)	Transfer Rate TC/R	Tetracycline MIC of transconjugants [μg/mL]
In vitro filter mating					
Lactococcus lactis ssp. *lactis* LAB-14	5.40×10^{8}	9.40×10^{7}	1.00×10^{2}	1.06×10^{-6}	16
Lactococcus lactis ssp. *lactis* LAB-16	3.10×10^{8}	1.30×10^{8}	2.00×10^{2}	1.54×10^{-7}	24
Pediococcus pentosaceus LAB-17	6.20×10^{8}	3.60×10^{8}	1.00×10^{2}	2.78×10^{-7}	48
Lactobacillus plantarum LAB-22	9.80×10^{7}	8.60×10^{7}	3.00×10^{2}	3.49×10^{-6}	24
Lactobacillus rhamnosus LAB-28	2.60×10^{8}	9.80×10^{7}	2.00×10^{2}	2.04×10^{-6}	48
E. faecalis 16680 EK (positive control)	7.00×10^{8}	3.10×10^{8}	5.30×10^{3}	1.71×10^{-5}	64
***In situ* mating in food matrix**					
Lactococcus lactis ssp. *lactis* LAB-14	3.20×10^{7}	6.30×10^{7}	2.00×10^{1}	3.17×10^{-7}	16
Lactococcus lactis ssp. *lactis* LAB-16	1.00×10^{7}	9.30×10^{7}	1.00×10^{1}	1.07×10^{-7}	24
Pediococcus pentosaceus LAB-17	3.40×10^{7}	8.90×10^{7}	1.00×10^{1}	1.12×10^{-7}	24
Lactobacillus plantarum LAB-22	3.50×10^{6}	7.30×10^{8}	1.00×10^{1}	1.36×10^{-8}	12
Lactobacillus rhamnosus LAB-28	2.20×10^{7}	6.20×10^{7}	2.00×10^{1}	3.22×10^{-7}	24
E. faecalis 16680 EK (positive control)	5.50×10^{7}	8.10×10^{7}	2.00×10^{1}	$2,46 \times 10^{-7}$	24

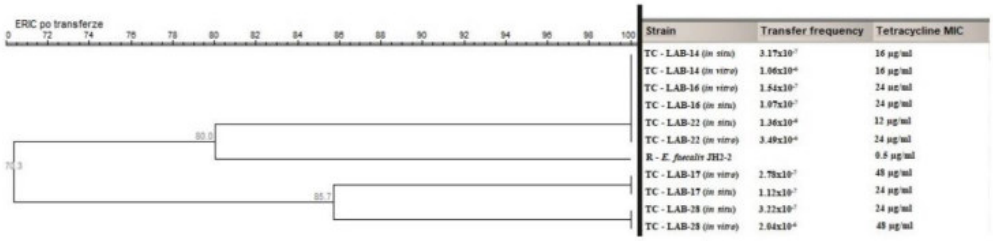


Fig. 2. Unweighted pair group method with the arithmetic mean (UPGMA) dendrogram derived from ERIC-PCR profiles showing restriction pattern similarity among *E. faecalis* JH2–2 and transconjugants.
Abbreviations: TC – tranconjugant, R – recipient.

antibiotics. However, the strains analyzed by Coton et al. (2018) showed larger spectrum of antibiotic resistance. The highest MIC values were noted for three antibiotics: tetracycline, kanamycin and chloramphenicol. The lowest MIC values were noted for erythromycin and streptomycin. In addition, more than 10% of the strains showed high MIC value to antibiotics of three or more classes simultaneously. This result for strains used on an industrial scale is worrying. The occurrence of antibiotic resistance in *Lactobacillus* strains has been the most extensively documented, probably due to the fact that many species belonging to this genus are used in the production of starter cultures (Zarzecka, Zadernowska, & Chajęcka-Wierzchowska, 2020). The current results are in agreement with previously published studies showing that the genus *Lactobacillus* generally has low MIC values to ampicillin, erythromycin and clindamycin but might acquire resistance to such antibiotics as tetracycline or chloramphenicol (Devirgiliis et al., 2013). On the other hand, study by Dušková et al. (2020) showed that among the *Lactobacillus* strains from starter cultures, the highest MIC values were for aminoglycosides. However, the results of phenotypic antibiotic resistance patterns of other genera are not in agreement with other studies, which showed the highest percentage of strains with high MIC for erythromycin, while few strains showed high MIC for tetracycline (Federici et al., 2014; Zycka-Krzesinska, Boguslawska, Aleksandrzak-Piekarczyk, Jopek, & Bardowski, 2015). This may be due to differences between individual strains of the same genus or species, or to fluctuations in the expression of the genes responsible for revealing the phenotypic trait. This would also explain the lack of differences in the occurrence of high MIC values and the bacterial genus or source of the strain.

All strains were checked for the presence of 24 different genetic resistance determinants. A total number of 15 were found, which is also quite a high result. The highest percentage of strains showed the presence of genes encoding resistance to aminoglycosides. Interestingly, despite the frequent occurrence of these genes, not each strain presented a high MIC value for these antibiotics. This suggests the need for in-depth research in this area in order to verify factors influencing the expression of these genes. Of all the resistance genes identified, the greatest diversity was observed for the tetracycline resistance genes as the strains tested had six different genes. The *tet*M and *tet*W genes were the most common. Similar results were obtained by other authors (Anisimova & Yarullina, 2018; Cordeiro, Du, Mulvey, Krause, & Holley, 2010; Kastner et al., 2006; Resch, Nagel, & Hertel, 2008; Singla et al., 2018). Interestingly, Lopez, Callegari, Patrone, and Rebecchi (2020) reported that the ripening of fermented meat resulted in a reduction in the number of antibiotic-resistant coagulase-negative staphylococci included in the starter culture - *S. xylosus* and *S. carnosus*. These results open up the field for further research in this area to determine if, and how, antibiotic resistance changes during the manufacturing process.

An alarming phenomenon is if the presence of these genes is not considered intrinsic, because these strains are used industrially and are consumed at high dosages. Therefore, such acquired resistances should be monitored most closely. Anisimova and Yarullina (2020) indicate that the resistance to erythromycin, tetracycline and chloramphenicol should be the most closely monitored because the genes of resistance to these antibiotics are the most often located on mobile genetic elements. Campedelli et al. (2019) indicate that a large percentage of lactic acid bacteria strains show resistance to tetracycline, chloramphenicol, clindamycin and erythromycin. Moreover, the vast majority of the genes responsible for resistance to these antibiotics were located on mobile genetic elements, which is associated with the risk of horizontal transfer of resistance genes.

Conjugation is considered to be the basic mechanism of horizontal transfer of AR genes under natural environments. The analysis of the resistance genes transferability showed that the analyzed strains have the ability to horizontally transfer the tetracycline resistance genes -

*tet*M and *tet*K. This was confirmed both by the increase in MIC values for tetracycline and the presence of the transferred genes in the genetic material from transconjugants. The transfer took place both *in vitro* and *in situ*. It is worth noting, however, that the transfer frequency in the food model was about one log unit lower than the filter mating transfer frequency for the same strain. Interestingly, most of the authors showed that their research did not transfer resistance genes in the food model (Flórez et al., 2016; Thumu & Halami, 2018; Toomey, Monaghan, Fanning, & Bolton, 2009). The newest studies point to the possibility of gene transfer by lactic acid bacteria (Ashwini, Ray, Halami, & Sumana, 2021; Ojha, Shah, & Mishra, 2021). Moreover, Anisimova and Yarullina (2020) and Li et al. (2019) did not show the possibility of gene transfer even *in vitro*. These results may suggest that the ability to gene transfer is a strain-dependent feature (Rossi, Rizzotti, Felis, & Torriani, 2014). Yang and Yu (2019) also state that failure in gene transfer may also result from the fact that in some strains, the resistance genes may be located on non-conjugative plasmids that are not competent to transfer via conjugation. The observed differences may suggest that the disclosure of the ability to transfer genes by LAB strains may depend on many environmental factors. This leaves room for further research on this issue. However, it is worth noting that in the case of strains intentionally added to food, even single cases of antibiotic resistance gene transfer are an undesirable phenomenon, as they contribute to the spread of resistance in the environment.

5. Conclusions

The obtained results indicate that antibiotic resistance may also occur among the microorganisms included in the starter and protective cultures. While this phenomenon is not very widespread or well understood, it may be of concern for microorganisms intentionally added to food. Of particular note is the demonstrated ability to transfer resistance genes, which may significantly contribute to the dissemination of resistance in the environment. Thus, strains from starter and protective cultures, although they do not pose a threat to consumers due to their non-pathogenicity, may threaten them indirectly. These results indicate the need for further research in this area, to ensure that the strains introduced into food do not constitute an environmental reservoir of antibiotic resistance genes.

CRediT authorship contribution statement

Urszula Zarzecka: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Wioleta Chajęcka-Wierzchowska:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Anna Zadernowska:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

Authors declared no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112490.

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Table S1 Primers used in the study

Species/Gene	Primer	Primer sequence 5'-3'	Amplicon size (bp)	Annealing temperature	Reference
Identification					
L. paraplantarum	paraF	GTCACAGGCATTACGAAAAC	107	56ºC	Torriani et al., 2001
L. pentosus	pentF	CAGTGGCGCGGTTGATATC	218		
L. plantarum	planF	CCGTTTATGCGGAACACCTA	318		
	pREV	TCGGGATTACCAAACATCAC			
L. lactis ssp. lactis	LacF	GTACTTGTACCGACTGGAT	217	45ºC	Pu et al., 2002
L. lactis ssp. cremoris	CreF	GTGCTTGCACCGATTTGAA	217		
	LaCreR	GGGATCATCTTTGAGTGAT			
Tetracyclines resistance genes					
***tet*M**	tetM-F	GTGGACAAAGGTACAACGAG	406	62ºC	Roberts, 1994
	tetM-R	CGGTAAAGTTCGTCACACAC			
***tet*L**	tetL-F	TGGTGGAATGATAGCCCATT	229		
	tetL-R	CAGGAATGACAGCACGCTAA			
***tet*K**	tetK-F	TTATGGTGGTTGTAGCTAGAAA	348	55ºC	Gevers et al., 2003
	tetK-R	AAAGGGTTAGAAACTCTTGAAA			
***tet*O**	tetO-F	AATGTCAGAACTGGAACAGGAAGAA	84	60ºC	Flórez et al., 2014
	tetO-R	CGTGATAAACGGGAAATAACGTT			
tetS	tetS-F	CGAGGTCATTCTCATTGGTGAA	60		
	tetS-R	CAGACACTGCGTCCATTTGTAAA			
***tet*W**	tetW-F	ACGGCAGCGCAAAGAGAA	69		
	tetW-R	CGGGTCAGTATCCGCAAGTT			
Macrolides resistance genes					
***erm*A**	ermA-F	TCTAAAAAGCATGTAAAAGAA	645	58ºC	Młynarczyk et al., 2006
	ermA-R	TGATTATAATTATTTGATAGCTTC			
***erm*B**	ermB-F	GAAAAGGTACTCAACCAAATA	639		
	ermB-R	CATTTGTTAAATTCATGGCAATGA			
***erm*C**	ermC-F	TCAAAACATAATATAGATAAA	642		
	ermC-R	TAACTGCTAAATTTGTTATAATCG			
***msr*A/B**	msrA/B-F	GCAAATGGTGTAGGTAAGACAACT	399		
	msrA/B-R	TAAAACAAATGTAGTGTACTA			
β-lactams resistance genes					

***bla*TEM**	bla_{TEM}-F	AGTGCTGCCATAACCATGAGTG	431	61ºC	Kim et al., 2009
	bla_{TEM}-R	CTGACTCCCCGTCGTGTAGATA			
***bla*OXA**	bla_{OXA}-F	ATTATCTACAGCAGCGCCAGTG	296		
	bla_{OXA}-R	TGCATCCACGTCTTTGGTG			
***bla*SHV**	bla_{SHV}-F	GATGAACGCTTTCCCATGATG	214		
	bla_{SHV}-R	CGCTGTTATCGCTCATGGTAA			
***bla*CTX-M**	bla_{CTX-M}-F	GACAAAGAGAGTGCAACGGATG	501		
	bla_{CTX-M}-R	TCAGTGCGATCCAGACGAAA			
blaZ	blaZ-F	CAAAGATGATATAGTTGCTTATTCTC	400	50ºC	Kaase et al., 2008
	blaZ-R	TGCTTGACCACTTTTATCAGC			
Aminoglycosides resistance genes					
aac(6')-Ie-aph(2'')-Ia	Gent1-F	CAGGAATTTATCGAAAATGGTAGAAAAG	369	58ºC	Padmasini et al., 2014
	Gent1-R	CACAATCGACTAAAGAGTACCAAT			
aph(3'')-IIIa	Amino1-F	GGCTAAAATGAGAATATCACCGG	523		
	Amino1-R	CTTTAAAAAATCATACAGCTCGCG			
aph(2'')-Ic	Gent2-F	ATACAATCCGTCGAGTCGCT	837	55ºC	Kobayashi et al., 2001
	Gent2-R	GTTGGCCTTATCCTCTTCCA			
aac(6')-Ii	Gent3-F	TGGCCGGAAGAATATGGAGA	410		
	Gent3-R	GCATTTGGTAAGACACCTACG			
ant(6')-Ia	Strepto-F	CGGGAGAATGGGAGACTTTG	563		
	Strepto-R	CTGTGGCTCCACAATCTGAT			
Glycopeptides resistance genes					
***van*A**	A-F	GGGAAAACGACAATTGC	723	54ºC	Dutka-Malen et al., 1995
	A-R	GTACAATGCGGCCGTTA			
***van*B**	B-F	ATGGGAAGCCGATAGTC	635		
	B-R	GATTTCGTTCCTCGACC			
Chloramphenicol resistance genes					
cat	cat-F	CATATCAAATGAACTTTAATA	718	52ºC	Goksel et al., 2016
	cat-R	CGTTTTGTGAAGTAGTACACT			
Conjugative transposon Tn*916*/Tn*1545*					
int	int-F	GCGTGATTGTATCTCACT	1046	50ºC	Macovei & Zurek, 2006
	int-R	GACGCTCCTGTTGCTTCT			

Table S2 Characteristic of all strains used in the study

Strain	Identification	Application of culture	Antibiotic MICs									Resistance genes
			AMP	CN	C	TE	E	DA	K	S	VA	
Strains from starter cultures												
LAB-45	*Lactobacillus helveticus*	Cheese	0.75	0.25	0.12	32	0.125	0.032	6	0.125	-	*tet*O, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-4	*Lactobacillus paracasei*		1.5	8	24	32	0.19	0.5	12	0.19	-	*aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia, cat*
LAB-20	*Lactobacillus paracasei*		0.5	6	6	1.5	0.064	0.032	48	0.064	-	*cat*
LAB-31	*Lactobacillus paracasei*		0.19	2	4	1	0.023	0.032	32	0.023	-	*tet*M, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-41	*Lactobacillus paracasei*		1	0.38	32	32	0.125	0.023	4	0.125	-	*tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia, cat*
LAB-72	*Lactobacillus paracasei*		0.38	0.75	12	32	0.25	0.5	8	0.25	-	*tet*M, *tet*W, *cat*
LAB-22	*Lactobacillus plantarum*		3	1	6	32	0.19	0.047	16	0.19	-	*tet*K, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-6	*Lactobacillus* sp.		0.125	0.75	12	16	0.25	0.75	12	0.25	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia, cat*
LAB-71	*Lactobacillus* sp.		0.38	0.75	6	32	0.19	0.094	4	0.19	-	*aph(2')-Ic*
LAB-13	*Lactococcus lactis* ssp. *cremoris*		0.25	0.38	16	32	0.19	0.25	8	0.19	0.19	*tet*M, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, bla*$_{OXA}$, *cat*
LAB-15	*Lactococcus lactis* ssp. *lactis*		3	1	8	1.5	0.125	0.047	32	0.125	0.25	-
LAB-27	*Lactococcus lactis* ssp. *lactis*		0.094	2	24	32	0.125	0.38	16	0.125	32	*aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-44	*Lactococcus lactis* ssp. *lactis*		1.5	1	6	12	0.19	0.032	24	0.19	0.19	-
LAB-55	*Lactococcus lactis* ssp. *lactis*		0.25	0.38	12	48	0.094	0.25	8	0.094	0.25	*tet*W, *aph(3')-IIIa, cat*
LAB-66	*Lactococcus lactis* ssp. *lactis*		0.5	0.75	12	48	0.125	0.5	6	0.125	0.094	*cat*
LAB-68	*Lactococcus lactis* ssp. *lactis*		3	1.5	16	24	0.125	0.023	24	0.125	0.19	-
LAB-9	*Leuconostoc mesenteroides*		0.38	0.5	24	16	0.125	0.5	8	0.125	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia*
LAB-51	*Streptococcus thermophilus*		1.5	0.75	12	32	0.38	0.75	16	0.38	0.25	*tet*O, *cat*
LAB-60	*Streptococcus thermophilus*		1.5	0.75	2	16	0.094	0.016	8	0.094	0.125	*tet*O
LAB-8	*Lactobacillus kefiri*	Kefir	0.125	0.19	0.25	0.19	0.125	0.75	0.19	0.125	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-1	*Lactobacillus paraplantarum*		0.5	0.75	8	12	0.19	0.5	6	0.19	-	*tet*M, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia,*
LAB-3	*Lactobacillus paraplantarum*		0.25	0.5	8	32	0.25	0.75	6	0.25	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia, cat*
LAB-21	*Lactobacillus paraplantarum*		1.5	0.75	6	16	0.125	0.25	16	0.125	-	*tet*L
LAB-38	*Lactobacillus* sp.		2	1	6	32	0.094	0.016	16	0.094	-	*tet*O, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-26b	*Lactococcus lactis* ssp. *cremoris*		0.25	4	12	32	0.25	0.75	12	0.25	0.19	*tet*M, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, blaZ, cat*
LAB-16	*Lactococcus lactis* ssp. *lactis*		0.19	1	8	32	0.125	0.25	8	0.125	0.094	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia*
LAB-26a	*Lactococcus lactis* ssp. *lactis*		0.19	2	4	0.75	0.023	0.023	32	0.023	32	*aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia*
LAB-37	*Lactococcus lactis* ssp. *lactis*		3	0.75	6	24	0.125	0.023	16	0.125	0.19	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-39	*Lactococcus lactis* ssp. *lactis*		0.25	0.25	8	12	0.19	0.125	6	0.19	0.25	*tet*M, *tet*O, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia*
LAB-59	*Lactococcus lactis* ssp. *lactis*		3	1.5	8	24	0.094	0.023	32	0.094	0.125	-
LAB-43	*Lactobacillus curvatus*	Meat	0.125	0.5	16	64	0.125	0.38	6	0.125	-	*tet*M, *tet*O, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-11	*Lactobacillus paraplantarum*		0.25	0.38	32	>256	0.25	0.25	8	0.25	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aph(2')-Ic, cat*

LAB-12	*Pediococcus pentosaceus*		1.5	0.5	8	24	0.094	0.023	16	0.094	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-33	*Staphylococcus carnosus*		1	0.38	8	24	0.125	0.016	6	0.125	32	*tet*M, *tet*O, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-42	*Lactobacillus delbrueckii*	Yoghurt	1.5	0.75	6	24	0.25	0.023	16	0.25	-	*msr*A/B
LAB-58	*Lactobacillus delbrueckii*		2	0.75	12	24	0.19	0.38	12	0.19	-	*tet*O, *cat*
LAB-50	*Lactobacillus* sp.		1	0.5	6	12	0.25	0.023	16	0.25	-	*tet*S, *aac(6')-Ia*
LAB-63	*Lactobacillus* sp.		3	0.5	8	48	0.19	0.047	16	0.19	-	*bla*Z
LAB-64	*Lactobacillus* sp.		0.25	0.5	24	32	0.094	0.25	8	0.094	-	*cat*
LAB-7	*Lactococcus lactis* ssp. *lactis*		1	1	16	16	0.19	1.5	12	0.19	0.125	*cat*
LAB-14	*Lactococcus lactis* ssp. *lactis*		0.19	0.19	12	48	0.125	1.5	12	0.125	0.19	*tet*K, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-46a	*Lactococcus lactis* ssp. *lactis*		0.5	0.75	12	16	0.19	0.38	12	0.19	0.094	*tet*O, *tet*W, *cat*
LAB-36	*Streptococcus thermophilus*		3	1	12	24	0.19	0.75	24	0.19	0.125	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-69	*Streptococcus thermophilus*		0.25	0.5	24	32	0.19	0.75	1	0.19	0.19	*tet*O, *cat*
Strains from protective cultures												
LAB-28	*Lactobacillus rhamnosus*	Dairy	0.125	0.75	8	32	0.32	0.19	8	0.32	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aph(2')-Ic, cat*
LAB-46b	*Lactobacillus rhamnosus*		0.38	2	4	1	0.047	0.125	3	0.047	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*
LAB-52	*Lactococcus lactis* ssp. *lactis*		0.19	0.75	12	16	0.19	0.5	8	0.19	0.25	*tet*W, *aph(3')-IIIa, cat*
LAB-10	*Lactobacillus sakei*	Meat	0.5	0.25	24	1.5	0.125	0.023	8	0.125	-	*tet*M, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia, cat*
LAB-18	*Lactobacillus sakei*		2	1	12	48	0.094	0.032	16	0.094	-	*tet*M, *tet*L, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-47	*Lactobacillus* sp.		2	0.38	8	16	0.125	0.023	16	0.125	-	*tet*W, *cat*
LAB-17	*Pediococcus pentosaceus*		3	1	12	24	0.125	0.023	24	0.125	-	*tet*K, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, cat*
LAB-19	*Pediococcus pentosaceus*		0.19	8	4	1.5	0.094	0.094	48	0.094	-	*tet*W
LAB-23	*Pediococcus pentosaceus*		3	0.5	4	16	0.125	0.023	16	0.125	-	*aph(3')-IIIa*
LAB-40	*Pediococcus acidilactici*		0.75	0.25	32	48	0.25	0.032	12	0.25	-	*tet*M, *tet*O, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia, aac(6')-Ia,*
LAB-48	*Pediococcus acidilactici*		2	0.75	6	24	0.094	0.016	16	0.094	-	*cat*
LAB-49	*Pediococcus acidilactici*		1	1	16	64	0.19	0.032	32	0.19	-	-
LAB-53	*Pediococcus acidilactici*		0.094	0.38	12	16	0.25	0.75	12	0.25	-	*tet*W, *aph(3')-IIIa, cat*
LAB-30	*Staphylococcus xylosus*		4	0.75	6	12	0.19	0.38	24	0.19	0.25	*tet*M, *tet*W, *aph(3')-IIIa, aac(6')-Ie-aph(2'')-Ia*

Abbreviations: AMP – ampicillin, CN – gentamicin, C – chloramphenicol, TE – tetracycline, E – erythromycin, DA – clindamycin, K – kanamycin, S – streptomycin, VA - vancomycin

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High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures

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ABSTRACT

This study analyzed the effect of food-related stresses on the expression of antibiotic resistance of starter and protective strains and resistance gene transfer frequency. After exposure to high-pressure processing, acidic and osmotic stress, the expression of genes encoding resistance to aminoglycosides (*aac(6′)Ie-aph(2″)Ia* and *aph(3′)-IIIa*) and/or tetracyclines (*tet*M) increased. After cold stress, a decrease in the expression level of all tested genes was observed. The results obtained in the gene expression analysis correlated with the results of the phenotype patterns. After acidic and osmotic stresses, a significant increase in the frequency of each gene transfer was observed. To the best of the authors' knowledge, this is the first study focused on changes in antibiotic resistance associated with a stress response among starter and protective strains. The results suggest that the physico-chemical factors prevailing during food production and storage may affect the phenotype of antibiotic resistance and the level of expression of antibiotic resistance genes among microorganisms. As a result, they can contribute to the spread of antibiotic resistance. This points to the need to verify strains used in the food industry for their antibiotic resistance to prevent them from becoming a reservoir for antibiotic resistance genes.

1. Introduction

Antibiotic resistance is currently one of the most important public health issues. It has been shown that this problem may concern not only pathogenic microorganisms but also strains used on a large scale in the food industry in the form of starter and protective cultures. In addition, it has been shown that strains used in the food industry demonstrate the capability of horizontal gene transfer and thus contribute to the spread of resistance in the environment (Casado Muñoz et al., 2016; Zarzecka et al., 2020, 2022a).

An important aspect, which has not yet been addressed in scientific research, is the influence of the food production and storage conditions on the antibiotic resistance of strains used in starter or protective cultures. These microorganisms face various environmental stresses during food production. They may be associated with changes in the environment during fermentation, e.g. a decrease in pH due to the accumulation of organic acids, or may result from food processing/preservation processes. The most frequently mentioned are: low temperature during refrigeration storage and the addition of NaCl. Currently, high-pressure processing (HPP) is also mentioned. HPP is a promising new method of non-thermal food preservation (Mbye et al., 2020; Ma et al., 2019).

In response to various stressful conditions, these microorganisms have developed comprehensive strategies to prevent damage caused by an unfavorable environment (Ma et al., 2020; Wu et al., 2013; H. Yang et al., 2021). The studies conducted so far on the impact of food-related stresses on microorganisms indicate that the adaptation of microorganisms to unfavorable conditions is usually associated with the induction of a large number of genes, synthesis of stress response proteins, and the development of cross-resistance to other types of stress. The regulation of these responses is complex and closely related to metabolic pathways. Other studies have confirmed that lactic acid bacteria show many physiological and adaptive responses under unfavorable

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environmental conditions (Hussain et al., 2013). Typically, this adaptation takes place as a bacterial cellular response to a stress factor by regulating molecular mechanisms. The activation of these mechanisms may result in the selection of variants with increased tolerance or resistance to other stress factors, including reduced sensitivity to several antibiotics, including antibiotics important in clinical practice (Al-Nabulsi et al., 2015; Giacometti et al., 2021; McMahon et al., 2007; Komora et al., 2017). It has also been reported that exposure to various stress conditions may result in the mobilization of certain genetic elements and thus to an increased frequency of gene transfer (Giacometti et al., 2021; Mahon et al., 2007). All of these studies have only been performed for pathogenic microorganisms. There are no studies on the link between stress response and antibiotic resistance among non-pathogenic microorganisms such as lactic acid bacteria. It is already known that the microorganisms included in starter and protective cultures can also carry a number of mobile genetic elements with antibiotic resistance genes. The role of stress in triggering the mobilization of such elements has been demonstrated, i.a., for the *Lactobacillus crispatus* strain (Strøman et al., 2003).

The aim of this study was to determine the effect of food-related stresses on the expression of antibiotic resistance genes in strains derived from commercial starter and protective cultures. The survival rate and changes in the frequency of resistance gene transfer under the influence of these factors were also assessed.

2. Material and methods

2.1. Strains

The study was carried out using eight strains derived from commercial starters and protective strains selected from the collection described previously (Zarzecka et al., 2022a). One of the strains came from dairy protective culture, and seven from starter cultures used for meat and dairy. Seven of the strains belong to the previous *Lactobacillus* genus and one to the *Lactococcus* genus. The genus names of bacteria have been updated in line with the new nomenclature (Zheng et al., 2020). The characteristics of the strains selected for analysis are summarized in Table 1. The criterion for selecting the strains used in this study was to have at least one plasmid-encoded antibiotic resistance gene particularly for tetracycline and aminoglycosides with a simultaneous low Minimum Inhibitory Concentration (MIC) value for these antibiotics. Each of the strains had low MICs for gentamicin, streptomycin and kanamycin, and the two strains: LAB-8 and LAB-46b, simultaneously had low MICs for tetracycline.

2.2. Stress treatment

Each of the eight tested strains was subjected to the following stress factors: low pH, increased NaCl concentration, cold stress and high-pressure treatment. The values of the stress factors used in the study were selected on the basis of the available literature, which indicated values of the intrinsic and technological factors most common in food as well as on the basis of preliminary studies.

2.2.1. Acidic, osmotic and cold stress

The preliminary studies about acidic, osmotic and cold stresses were determined based on the methods described previously (Zarzecka et al., 2022b). The influence of osmotic stress was analyzed using the culture in 5 mL of de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) with the addition of NaCl in the range of 1–10%. To check tolerance to acidic stress the culture was conducted in 5 mL of MRS broth with the pH value in the range of 2.00–5.00. Incubation was provided for 48 h in 30 °C in anaerobic conditions. Effect of cold stress was tested in 5 mL of MRS broth incubated for 48 h in 4 °C and 7 °C. Then, tenfold dilutions were made for control and tested samples and the number of colony forming units (CFU)/mL on MRS agar (Merck, Germany) was determined. According to the literature data, the average pH of fermented food is 4.0, the concentration of NaCl in food produced with the use of industrial strains reaches 4.0%, the temperature of refrigerated food storage is about 7.0 °C (Cabello-Olmo et al., 2020; Obande and Young, 2020; Oh and Jung, 2015; Sharma et al., 2020). Based on literature and preliminary studies such values were selected for analysis.

Stress treatment was carried out in sterile Falcon tubes with 20 mL MRS broth (Merck, Germany) to which the exponentially growing cell cultures (OD_{590} ~ 0.6) in amount of 250 μl was added. Then, to check the effect of acid stress, lactic acid was added to 8 tubes to obtain a pH value of 4.0 and incubated at 30 °C for 48 h anaerobically. To check the effect of osmotic stress, NaCl was added to the next 8 tubes to obtain a concentration of 4.0% and incubated anaerobically at 30 °C for 48 h. The sterility of adding lactic acid and NaCl was ensured by filtering the additives on syringe filters and adding them to a sterile medium under a laminar chamber. The pH of the substrate after the addition of lactic acid was checked with a litmus paper. In order to check the effect of refrigeration, 8 tubes with inoculated medium were transferred to a refrigerator and stored at 7 °C for 48 h. In addition, a control samples were prepared - inoculated MRS broth without additives, incubated under optimal conditions. The assay was performed in triplicate on different days (Fig. 1).

Table 1
Characteristic of strains analyzed in the study.

Strain	Identification	Origin	Antibiotic MICs [μg/mL]									Antibiotic resistance genes
			AMP	CN	C	TE	E	DA	K	S	VA	
LAB-45	*Lactobacillus helveticus*	Cheese starter culture	0.75	0.25	0.12	32	0.125	0.032	6	0.125	–	*tetO, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, cat*
LAB-43	*Latilactobacillus curvatus*	Meat starter culture	0.125	0.5	16	64	0.125	0.38	6	0.125	–	*tetM, tetO, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, cat*
LAB-41	*Lacticaseibacillus paracasei*	Cheese starter culture	1	0.38	32	32	0.125	0.023	4	0.125	–	*tetW, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, cat*
LAB-3	*Lactiplantibacillus paraplantarum*	Kefir starter culture	0.25	0.5	8	32	0.25	0.75	6	0.25	–	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, cat*
LAB-1	*Lactiplantibacillus paraplantarum*	Kefir starter culture	0.5	0.75	8	12	0.19	0.5	6	0.19	–	*tetM, tetW, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, aac(6′)-Ia, bla_{OXA}*
LAB-8	*Lentilactobacillus kefiri*	Kefir starter culture	0.125	0.19	0.25	0.19	0.125	0.75	0.19	0.125	–	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*
LAB-46b	*Lacticaseibacillus rhamnosus*	Dairy protective culture	0.38	2	4	1	0.047	0.125	3	0.047	–	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*
LAB-39	*Lactococcus lactis ssp. lactis*	Kefir starter culture	0.25	0.25	8	12	0.19	0.125	6	0.19	0.25	*tetM, tetO, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, aac(6′)-Ia*

Abbreviations: AMP – ampicillin, CN – gentamicin, C – chloramphenicol, TE – tetracycline, E – erythromycin, DA – klindamycin, K – kanamycin, S – streptomycin, VA – vancomycin, „-„ – non-analyzed.

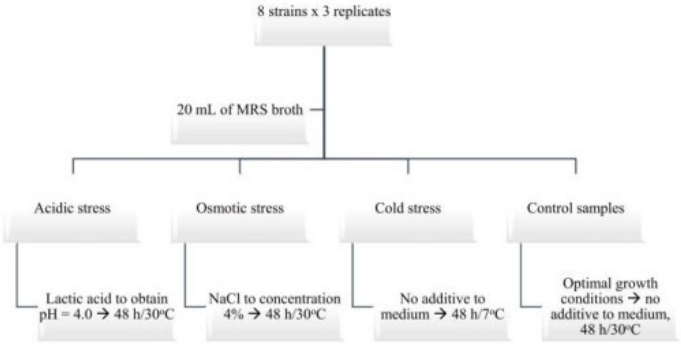


Fig. 1. Flow chart describing the stress treatment procedure for acidic, osmotic and cold stresses.

2.2.2. High pressure processing

For the selection of pressure value, preliminary tests were carried out in which pressures of 100, 200, 300, 400, 500 and 600 MPa were checked. Each of these pressures was applied for 5 min. After pressurization, a series of ten-fold dilutions were made and the number of colony forming units (CFU)/mL was determined on MRS agar for tested and control samples. The analyzes were carried out simultaneously for each of the pressure values, and they were performed in triplicate on different day. Two pressure values were selected for the analysis, which significantly lowered the number of cells in relation to the previous value: 200 and 400 MPa.

The pressurization was carried out for 5 min in glycol-water solution (1: 1, v/v) with the use of the high-pressure single-chamber U4040 (IWC PAN, Warsaw, Poland, Unipress Equipment Division). Temperature was kept at 20 ± 3 °C, the pressure rise rate was 300 MPa/min, and the pressure relief time was less than 5 s. The procedure was subjected to overnight cultures of isolates on MRS broth (Merck, Germany).

After the stress treatment was completed, the samples were immediately subjected to the next stages of the study.

2.3. Analysis of the survival of strains under the influence of food-related stresses

2.3.1. Control sample preparation

Monocultures were inoculated into fresh MRS broth (Merck, Germany) and incubated overnight at 37 °C to reach the mid-log phase (OD~0.8). A 1 mL sample of each monoculture was subsequently centrifuged at 10,000×*g*/10 min to recover bacterial cells. Cell pellets were resuspended in 1 mL of potassium phosphate buffer (50 mM, pH = 7) to get control cells and live cells. The population of dead cells was generated by resuspended pellets in 100% isopropanol for 30 min. Dead cells were recovered by centrifugation at 10,000×*g*/10 min and washed once in potassium phosphate buffer. Equal volumes of live (untreated), and dead cells were mixed with Survey of Live/Dead® BacLight™ Bacterial Viability Kit (ThermoFischer Scientific, Waltham, Massachusetts, USA) according to manufacturer's instructions and were used for set up.

2.3.2. Stress assay preparation

A 1 mL sample of each monoculture obtained after stress treatment was subsequently centrifuged at 10,000×*g* for 10 min to recover bacterial cells. Cell pellets were resuspended in 1 mL of potassium phosphate buffer and stained with accordance to manufacturer's instructions.

2.3.3. Flow cytometer measurements

Analysis were performed on BD FACSLyric™ flow cytometer (Becton Dickinson, San Jose, CA, USA) according to Habtewold et al. (2016). For set up SYBR was excited by a 488 nm (50 mW) laser and PI was excited by a 561 nm (50 mW) laser. The voltages were set on both fluorescent parameters so that the SYBR and PI positive cells were both on scale and above threshold. All parameters were displayed as height measurements on a logarithmic scale. Thresholds were established using unstained cells so that a minimal number of events were present at the threshold limits. The compensation was calculated using single stained fixed cells. Tubes containing stained cells were vortexed for 20 s before acquisition on a low flow rate (approximately 12 μL/min), and at least 20,000 events were collected.

First, the forward and side scatter of cells was evaluated in a bivariate dot plot. The population was gated by the relative value of the forward and side scatter to eliminate debris. The gated population was further divided according to SYBR emission (on the abscissa axis) and the PI emission (on the ordinate axis) in a bivariate dot plot. An additional bivariate plot displaying bead fluorescence against time allowed the stability of the acquisition rate to be monitored so that any perturbations that had the potential to affect the count calculations could be identified and excluded. The counting calculations were performed according to manufacturer's instructions.

2.3.4. Plate count method

The survival of the strains was also checked by the plate method. For this purpose, a series of ten-fold dilutions were made for each strain before and after exposure to the stress factor and the number of colony forming units (CFU)/mL was determined on MRS agar. The number of colonies was counted after 48 h of anaerobic incubation at 30 °C.

2.4. Determination of antibiotic resistance phenotype

After the stress treatment, the MIC values were determined for four antibiotics - gentamicin (CN), kanamycin (KA), streptomycin (S) and tetracycline (TE). The MIC values were determined as described previously (Zarzecka et al., 2022a) using gradient strips (Liofilchem, Italy) on Mueller-Hinton agar (Merck, Germany). The MIC value was read following after 18 h of anaerobic incubation at 30 °C. Then, optimal growth conditions were restored (the strains were cultured into media with optimal composition and incubated under optimal conditions) and the MIC values were rechecked to see if the changes would persist.

2.5. RNA extraction and reverse transcription into cDNA

Total RNA was extracted from bacterial cultures using Total RNA Mini Plus kit (A&A Biotechnology, Poland). RNA extraction was conducted in triplicate for each sample. Obtained RNA samples were purificated and concentrated using Clean-Up RNA Concentrator (A&A Biotechnology, Poland). All of the samples were checked for their integrity by loading 10 μl on a 1.2% agarose gel in 0.5% Tris-borate-EDTA (TBE) buffer and running at 90V for 1 h and verified by visualization of the two bands (16S and 23S RNA) using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, Cambridge, UK). RNA concentration was measured using Spectrophotometer/Fluorometer DeNovix DS-11 FX (DeNovix Inc., Wilmington, USA), based on sample absorbances at wavelengths of 260 nm and 280 nm. All the RNA samples were immediately used for the synthesis of first cDNA strand using Transcriba Kit (A&A Biotechnology, Poland) containing recombinant MMLV reverse transcriptase with low RNAse H activity. Optimal DNA polymerase activity was at temperature of 37–42 °C, template RNA was protected with a recombinant RNAse inhibitor and random sequence hexamers were used as primers.

2.6. Gene expression analysis

Gene expression analysis was performed with three independent qPCR reactions were for each replicate. Real-Time PCR reactions were conducted using the RotorGene Q system (Qiagen, Germany).

To determine the expression level of the aminoglycoside resistance genes the *gap* gene encoding glyceraldehyde-3-phosphate was selected as the housekeeping gene. The qPCR reactions were performed in a volume of 15 μl containing 7.5 μl of PowerUp SYBR Green MasterMix (ThermoFischer Scientific, Waltham, Massachusetts, USA), 1.05 μl of forward and reverse primer (10 pmol/μl) and 1–10 ng of template cDNA, filled up with ddH_2O to a final volume. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 60 s at 72 °C. The melting curve was constructed by heating in a slow ramp between 60 and 95 °C in increments of 0.5 °C for 5 s.

To determine the expression level of the tetracycline resistance genes the gene encoding the 16S rDNA was selected as the housekeeping gene. The qPCR reactions were performed in a volume of 10 μl containing 5 μl of PowerUp SYBR Green MasterMix (ThermoFischer Scientific, Waltham, Massachusetts, USA), 900 nM of each primer and 100 ng of template cDNA, filled up with with ddH_2O to a final volume. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Then the melting curve was constructed as described above.

Primer sequences for the *gap* gene and the aminoglycosides resistance genes *aac(6')Ie-aph(2'')Ia* and *aph(3')-IIIa* were obtained from Jaimee and Halami (2017) and primers for the 16S rDNA and tetracycline resistance gene *tet*M were obtained from Flórez et al. (2014) (Table S1). All the oligonucleotide primers were synthesized by the commercial enterprise – Genomed (Gdansk, Poland).

Differences in gene expression were determined using relative quantification, in which relevant gene expression is normalized to a housekeeping gene. Comparative CT ($2^{-\Delta\Delta CT}$) method was used for the analysis of results (Livak and Schmittgen, 2001). A threshold was determined by the software used for qPCR reactions. Obtained ratios equal to or greater than 2.00 represented a significant increase in gene expression, while ratios equal to or less than 0.50 represented a significant decrease in gene expression.

2.7. Analysis of horizontal gene transfer possibility

In order to check whether and how the frequency of resistance gene transfer changed under the influence of stress factors, a donor pool was prepared, which included the tested strains before the stress treatment and the strains recovered after exposure to the stress factors.

Plasmid-free *Enterococcus faecalis* JH2–2 (LMG, 19456) strain, resistant to rifampicin (MIC >256 μg/mL) and fusidic acid (MIC >256 μg/mL) was the standard recipient in the experiments.

Transferability of antibiotic resistance genes by filter mating as well as confirming the gene transfer and genotyping of transconjugants were examined as described previously (Zarzecka et al., 2022a). Incubation was carried out anaerobically for 48 h at 30 °C. Three types of selective media were used based on Brain Heart Infusion (BHI) broth (Merck, Germany): T-BHI for transconjugants (with the addition of 100 μg/mL of fusidic acid, 50 μg/mL of rifampicin and 10 μg/mL of tetracycline/1 μg/mL of gentamicin/1 μg/mL of kanamycin/1 μg/mL of streptomycin), TD-BHI for donors (with the addition of 10 μg/mL of tetracycline/1 μg/mL of gentamicin/1 μg/mL of kanamycin/1 μg/mL of streptomycin) and B-BHI for recipients (with the addition of 100 μg/mL fusidic acid and 50 μg/mL rifampicin).

2.8. Statistical analysis

Statistical analyses were performed using the Spearman correlation test or/and the one-way ANOVA with Prism 9.3.1. (Prism, GraphPad Software, San Diego). Statistical comparison of means was performed using Student's *t*-test. For all of the analyses, the significance level $\alpha = 0.05$ was assumed.

3. Results

3.1. Survival analysis

An analysis of the survival of the strains in response to stress factors was carried out using the flow cytometry and plate count methods. The obtained results indicate that the tested strains had the highest survival rate in the presence of a 4% NaCl concentration (above 90%). The lowest survival rate was observed in response to a pressure of 400 MPa (less than 50%). It should also be noted that the results obtained with the plate count method differ from the results obtained in the cytometric analysis. Plate count analysis showed a higher percentage of strain reduction in each of the stress factors (Table 2).

3.2. Changes in the antibiotic resistance phenotype

Based on an analysis of the results, it was observed that the treatment with any of the factors did not cause a statistically significant change in the resistance to streptomycin in any of the strains. Regarding resistance to gentamicin and kanamycin, a significant increase in MIC values was observed after exposure to low pH and increased NaCl concentration (i.e. from 0.5 μg/mL to 8 μg/mL), while exposure to low temperature and high pressure caused a significant reduction in MIC values (from 0.5 μg/mL to 0.125 μg/mL). Tetracycline resistance did not change after the exposure to stress factor among the strains with an initial high MIC value. In strains that initially showed a low MIC value for tetracycline, the MIC value increased significantly after exposure to low pH, high NaCl concentration and the high pressure (i.e. from 1 μg/mL to 8 μg/mL). After exposure to low temperature, the MIC value decreased significantly (i.e. from 1 μg/mL to 0.25 μg/mL) (Table 3).

It should also be noted that these changes continued even after the restoration of optimal conditions.

3.3. Changes in antibiotic resistance genes expression

The effect of food-related stresses on the expression level of genes encoding resistance to gentamicin: *aac(6')-Ie-aph(2'')-Ia*, kanamycin: *aph(3')-IIIa* and tetracycline *tet*M was analyzed. Relevant quantification showed that the results of gene expression correlated with the results obtained in the phenotypic analysis. The expression of aminoglycoside resistance genes increased significantly in response to low pH and increased NaCl concentration (relative gene expression level >2.00).

Table 2
Results of survival analysis.

Strain	pH = 4.0			4% NaCl			Temp. 7 °C			200 MPa			400 MPa		
	L [%]	D [%]	CFU reduction - plate count method [%]	L [%]	D [%]	CFU reduction - plate count method [%]	L [%]	D [%]	CFU reduction - plate count method [%]	L [%]	D [%]	CFU reduction - plate count method [%]	L [%]	D [%]	CFU reduction - plate count method [%]
LAB-1	87.12	12.88	15.40	98.46	1.54	10.43	97.73	2.27	10.45	97.49	2.51	11.94	30.21	69.79	72.43
LAB-3	85.77	14.23	19.35	98.16	1.84	9.46	95.42	4.58	12.53	97.46	2.54	12.03	24.71	75.29	80.64
LAB-8	89.37	10.63	15.34	98.03	1.97	10.23	95.90	4.10	13.10	96.50	3.50	13.85	42.14	57.86	68.34
LAB-43	85.21	14.79	18.80	95.67	4.33	12.43	97.38	2.62	10.32	95.66	4.34	13.89	12.53	87.47	90.00
LAB-41	84.84	15.16	20.49	98.53	1.47	9.85	87.58	12.42	20.56	95.61	4.39	14.00	43.87	56.13	64.32
LAB-45	88.06	11.94	18.48	98.66	1.34	10.23	96.77	3.23	11.28	90.56	9.44	17.73	50.99	49.01	58.24
LAB46b	81.25	18.75	25.43	98.05	1.95	10.42	95.96	4.04	12.67	97.79	2.21	9.21	13.40	86.60	93.45
LAB-39	84.75	15.25	20.73	92.55	7.45	15.32	96.54	3.46	13.37	88.52	11.48	23.72	21.06	78.94	87.42

Abbreviations: L – live cells, D – dead cells, CFU – colony forming unit.

Table 3
Changes in MICs values after exposure to food-related stresses.

Strain		LAB-45	LAB43	LAB-41	LAB-3	LAB-1	LAB-8	LAB-46b	LAB-39
MIC for streptomycin [μg/mL]	C	0.125^{a}	0.125^{a}	0.125^{a}	0.25^{a}	0.19^{a}	0.125^{a}	0.047^{a}	0.19^{a}
	pH 4.0	0.19^{a}	0.125^{a}	0.125^{a}	0.38^{a}	0.25^{a}	0.125^{a}	0.032^{a}	0.19^{a}
	4% NaCl	0.125^{a}	0.19^{a}	0.19^{a}	0.25^{a}	0.125^{a}	0.19^{a}	0.032^{a}	0.125^{a}
	7 °C	0.125^{a}	0.19^{a}	0.125^{a}	0.19^{a}	0.19^{a}	0.19^{a}	0.047^{a}	0.125^{a}
	200 MPa	0.19^{a}	0.125^{a}	0.19^{a}	0.25^{a}	0.19^{a}	0.125^{a}	0.064^{a}	0.25^{a}
	400 MPa	0.125^{a}	0.125^{a}	0.125^{a}	0.38^{a}	0.125^{a}	0.125^{a}	0.047^{a}	0.19^{a}
MIC for gentamicin [μg/mL]	C	0.25^{a}	0.5^{a}	0.38^{a}	0.5^{a}	0.75^{a}	0.19^{a}	2^{a}	0.25^{a}
	pH 4.0	1.5^{b}	2^{b}	1.5^{b}	1.5^{b}	2^{b}	1.5^{b}	6^{b}	1^{b}
	4% NaCl	8^{c}	6^{c}	4^{c}	8^{c}	6^{c}	4^{c}	6^{b}	6^{c}
	7 °C	0.094^{a}	0.25^{a}	0.19^{a}	0.125^{a}	0.38^{a}	0.094^{a}	1^{a}	0.094^{a}
	200 MPa	0.125^{a}	0.125^{a}	0.19^{a}	0.19^{a}	0.38^{a}	0.064^{a}	1^{a}	0.125^{a}
	400 MPa	0.125^{a}	0.25^{a}	0.125^{a}	0.19^{a}	0.38^{a}	0.094^{a}	1^{a}	0.094^{a}
MIC for kanamycin [μg/mL]	C	6^{a}	6^{a}	4^{a}	6^{a}	6^{a}	0.19^{a}	3^{a}	6^{a}
	pH 4.0	32^{b}	12^{b}	32^{b}	12^{b}	12^{b}	12^{b}	48^{b}	12^{b}
	4% NaCl	24^{b}	8^{c}	16^{c}	12^{b}	24^{c}	8^{c}	24^{c}	12^{b}
	7 °C	3^{c}	4^{a}	3^{a}	4^{a}	4^{a}	0.125^{a}	3^{a}	1.5^{c}
	200 MPa	1.5^{d}	1^{d}	2^{d}	2^{c}	3^{d}	0.094^{a}	1.5^{d}	2^{c}
	400 MPa	6^{a}	4^{a}	2^{d}	3^{c}	3^{d}	0.094^{a}	1.5^{d}	2^{c}
MIC for tetracycline [μg/mL]	C	32^{a}	64^{a}	32^{a}	32^{a}	12^{a}	0.19^{a}	1^{a}	12^{a}
	pH 4.0	32^{a}	64^{a}	32^{a}	32^{a}	12^{a}	6^{b}	6^{b}	12^{a}
	4% NaCl	32^{a}	64^{a}	32^{a}	32^{a}	12^{a}	6^{b}	8^{c}	12^{a}
	7 °C	8^{b}	24^{a}	12^{b}	12^{b}	3^{b}	0.094^{a}	0.25^{d}	12^{a}
	200 MPa	32^{a}	64^{a}	32^{a}	32^{a}	12^{a}	8^{b}	6^{b}	12^{a}
	400 MPa	32^{a}	64^{a}	32^{a}	32^{a}	12^{a}	6^{b}	8^{c}	12^{a}

Abbreviations: C - control sample – before stress treatment; $^{a-d}$ - mean values in rows with different letters differ significantly at $P < 0.05$.

After cold and high-pressure treatment, a decrease in expression was observed but, in the majority of cases, these changes were not significant (relative gene expression level >0.5). The exceptions were the LAB-39 strain after exposure to cold stress and HPP, LAB-43 in response to a pressure of 200 MPa, and LAB-3 and LAB-46b in response to a pressure of 400 MPa. In these cases, the reduction in gene expression was significant (relative gene expression level <0.5) (Fig. 2, Table S2).

For LAB-46b and LAB-8 strains the expression of *tet*M gene encoding tetracycline resistance increased in response to each of the stress factors except low temperature. After exposure to cold stress, gene expression slightly decreased (Fig. 2, Table S2).

3.4. Changes in horizontal gene transfer frequency

As a result of the analysis of the frequency of resistance gene transfer before and after exposure to stress factors, it was found that the frequency of kanamycin resistance gene transfer increased significantly after exposure to low pH and increased NaCl concentration compared to the control sample. Gene transfer frequency in the control sample ranged from 2.32×10^{-6} to 7.23×10^{-6}. After osmotic stress treatment, gene frequency ranged from 5.87×10^{-6} to 1.58×10^{-5} and after acidic stress treatment, it ranged from 5.45×10^{-6} to 9.32×10^{-6}. After exposure to low temperature and high pressure, gene transfer did not occur at all. After exposure to low pH and increased concentration of NaCl, gene transfer of gentamicin resistance was observed, which was not observed in the control sample, or after exposure to low temperature and high pressure.

Regarding tetracycline resistance gene transfer, an increased frequency of gene transfer was observed after exposure to low pH and increased NaCl concentration. Gene transfer frequency in the control sample ranged from 3.96×10^{-5} to 7.75×10^{-5}. After osmotic stress treatment, the gene transfer frequency ranged from 7.23×10^{-5} to 9.78×10^{-5} and after acidic stress treatment it ranged from 7.04×10^{-5} to 8.89×10^{-5}. After exposure to high pressures, the transfer frequency was comparable to that in the control sample. Moreover, no gene transfer was observed after exposure to low temperatures (Table 4).

MIC values for the transconjugants ranged from 1 to 8 μg/mL for gentamicin, from 1 to 12 μg/mL for kanamycin and from 1.5 to 32 μg/mL for tetracycline. These MIC values were lower or equal to MICs determined for the donors.

Similar recipient and transconjugant profiles were revealed which might suggest that the resistance was not acquired by a mutation in the donor genome, which confirms the clonal nature of the transconjugants (Figs. S1–S3).

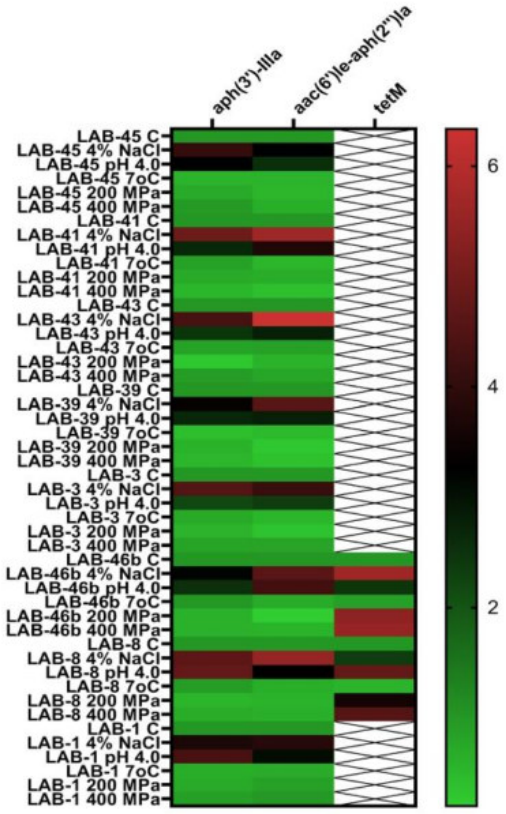


Fig. 2. Changes in the relative expression level of the antibiotic resistance genes in response to food-related stresses Abbreviation: C – control sample (before stress treatment).

4. Discussion

Food production and storage are associated with changes in environmental conditions, which may be a stress factor for food-related microorganisms, such as lactic acid bacteria (LAB) included in starter and protective cultures. The most common stress factors that microorganisms deal with in the food chain include cold, acidity, NaCl and high pressure. Their ability to survive under adverse conditions depends on the individual characteristics of bacteria. In stressful conditions, LAB activates mechanisms that allow them to adapt to new conditions, which may affect their viability and technological properties. The ability to adapt to various stressful conditions can also originate from cross-protection systems. LAB are very valuable for the food industry, so it is important to thoroughly understand their stress response mechanisms (Bucka-Kolendo and Sokołowska, 2017; Song et al., 2014).

The tested strains had high survival ability against the stress factors used in this study. The obtained results have been confirmed by other researchers (Gandhi and Shah 2016; Hernández et al., 2019). The viability of the bacteria in a stressful environment is crucial for the functioning of the bacteria in dairy products, especially those containing high levels of salt, high acidity or low humidity. The reduced number of viable bacteria with increased stress exposure is likely due to damage to the integrity of the bacterial membrane (Gandhi and Shah, 2015). A comparison of the survival results obtained in the plate and flow cytometry methods shows that in a plate count analysis a higher percentage of strain reduction under the influence of stress was obtained. In the food industry, the standard plate method is still widely used for rapid microbiological analyses. However, the number of colony-forming units depends on many different parameters such as culture conditions, the physiological state of the bacteria, and sub-lethal damage. While both methods are only used to estimate the actual number of bacteria, fewer factors influence the results obtained in flow cytometry (Bonomo et al., 2013).

There are currently no studies available on the effect of food-related stresses on antibiotic resistance among LAB. For this reason, it is difficult to compare the results obtained in this study to other studies. There are also only a few studies analyzing this issue among other Gram-positive bacteria (and only pathogens, such as *Listeria monocytogenes* or *Staphylococcus aureus*). More research concerns general stress response, indicating increased expression of stress-related proteins (Al-Nabulsi et al., 2015; Komora et al., 2017; Ma et al., 2019).

Acidic stress caused by lactic acid is a type of stress self-imposed by the LAB due to their metabolic properties. Lactic acid, the major metabolite of LAB, as well as other organic acids produced by LAB, e.g. formic acid and acetic acid accumulate during sugar fermentation and cause acidic stress (Guan and Liu, 2019; Ferreira and Mendes-Faia, 2020). It has been reported that among lactic acid bacteria organic acids dissociates in the cytoplasm, although LAB are equipped with mechanisms that allow them to survive in fermented foods that have a final pH of around 4.0 (Koponen et al., 2012). Microorganisms can adapt to acidic stress by synthesizing outer membrane proteins, like proton translocating ATPase, which helps stabilize the internal pH of the cell. The permeability and fluidity of the cell membrane might also be changed (Pérez Montoro et al., 2018; Chen et al., 2017; Heunis et al., 2014). These changes simultaneously might have a direct impact on the inflow and outflow of antibiotics through the cell membrane and thus on the antibiotic sensitivity of the microorganism (Giacometti et al., 2021). In this study it was shown that at a lower pH, the expression of genes encoding resistance to gentamicin, kanamycin and tetracycline increased. Several publications have investigated the possible relationship between antibiotic resistance and tolerance to the acidity of the environment. Al-Nabulsi et al. (2015) reported that strains isolated from fermented dairy and meat products exhibited higher susceptibility to such antibiotics as streptomycin, gentamicin, kanamycin and doxycycline compared to unstressed strains, however, they were more resistant to tetracycline, amoxicillin, ampicillin, vancomycin and ciprofloxacin. Thus, the cross-protection effect linking acid stress with antibiotic resistance does not apply to all antibiotics (Wu et al., 2021). Similar results were obtained by Ma et al. (2019) and Komora et al. (2017) who found a correlation between the level of tolerance to the acidity of the environment and the level of susceptibility to antibiotics among Gram-positive microorganisms.

The addition of NaCl is one of the oldest methods of food preservation. Salt is also added to provide the food with the desired taste characteristics. In the production of certain types of fermented foods, an addition of salt promotes the fermentation process, as LAB have a higher salt tolerance compared to other bacteria such as pathogenic and food spoilage microorganisms (Fessard and Remize, 2019; Yao et al., 2020). This action makes salt a common preservative in a variety of foods, including fermented foods such as salami and cheese (Ho et al., 2016). During the processing of certain cheeses, salt can be present in concentrations as high as 4.0%, which can suddenly increase osmolality and reduce cell viability (Oh and Jung, 2015). The addition of NaCl disrupts the osmotic balance between the extracellular environment and the cytoplasm, exerting osmotic pressure on bacterial cells (Burgess et al., 2016). To tolerate such changes, during growth in high osmolality, LAB adjust their intracellular osmolality to maintain the turgor pressure and activate mechanisms preventing cell death. Moreover, it has been

Table 4
Resistance genes transfer before and after exposure to food-related stresses.

		Transfer Rate TC/R				Transferred resistance genes	MIC value of TCs [μg/mL]				Genotype of donor	MIC value of D [μg/mL]			
		S	CN	K	TE		S	CN	K	TE		S	CN	K	TE
LAB-45	C	–	–	6.07×10^{-6}	–	*aph(3′)-IIIa*	–	–	4	–	*tetO, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, cat*	0.125	0.25	6	32
	pH 4.0	–	6.13×10^{-6}	9.32×10^{-6}	–	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	–	1	12	–					
	4% NaCl	–	6.76×10^{-6}	1.93×10^{-5}	–	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	–	6	6	–					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	–	–	–	–	–	–					
	400 MPa	–	–	–	–	–	–	–	–	–					
LAB-43	C	–	–	2.32×10^{-6}	6.53×10^{-5}	*aph(3′)-IIIa, tetM*	–	–	6	32	*tetM, tetO, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, cat*	0.125	0.5	6	64
	pH 4.0	–	9.10×10^{-7}	6.43×10^{-6}	8.26×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	1.5	8	24					
	4% NaCl	–	8.90×10^{-7}	9.73×10^{-6}	9.32×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	4	8	24					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	6.71×10^{-5}	*tetM*	–	–	–	24					
	400 MPa	–	–	–	6.79×10^{-5}	*tetM*	–	–	–	24					
LAB-41	C	–	–	4.53×10^{-6}	–	*aph(3′)-IIIa*	–	–	3	–	*tetW, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, cat*	0.125	0.38	4	32
	pH 4.0	–	6.43×10^{-6}	8.02×10^{-6}	–	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	–	1.5	12	–					
	4% NaCl	–	5.03×10^{-6}	1.58×10^{-5}	–	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	–	4	12	–					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	–	–	–	–	–	–					
	400 MPa	–	–	–	–	–	–	–	–	–					
LAB-3	C	–	–	3.98×10^{-6}	6.62×10^{-5}	*aph(3′)-IIIa, tetM*	–	–	4	24	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, cat*	0.25	0.5	6	32
	pH 4.0	–	5.86×10^{-7}	8.02×10^{-6}	7.04×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	1	6	32					
	4% NaCl	–	5.75×10^{-7}	9.87×10^{-6}	7.23×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	8	6	24					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	6.49×10^{-5}	*tetM*	–	–	–	32					
	400 MPa	–	–	–	6.55×10^{-5}	*tetM*	–	–	–	32					
LAB-1	C	–	–	4.30×10^{-6}	7.75×10^{-5}	*aph(3′)-IIIa, tetM*	–	–	3	6	*tetM, tetW, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, bla_{OXA}*	0.19	0.75	6	12
	pH 4.0	–	4.49×10^{-6}	5.45×10^{-6}	8.50×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	1.5	4	12					
	4% NaCl	–	5.03×10^{-6}	5.87×10^{-6}	8.76×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	6	4	6					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	7.50×10^{-5}	*tetM*	–	–	–	4					
	400 MPa	–	–	–	7.87×10^{-5}	*tetM*	–	–	–	4					
LAB-8	C	–	–	4.36×10^{-6}	3.96×10^{-5}	*tetM*	–	–	1	1.5	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	0.125	0.19	0.19	0.19
	pH 4.0	–	5.32×10^{-6}	9.63×10^{-6}	8.10×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	1	6	4					
	4% NaCl	–	5.76×10^{-6}	1.45×10^{-5}	9.78×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	4	4	4					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	3.75×10^{-5}	*tetM*	–	–	–	4					
	400 MPa	–	–	–	3.59×10^{-5}	*tetM*	–	–	–	6					
LAB-46b	C	–	–	6.19×10^{-6}	5.17×10^{-5}	*aph(3′)-IIIa, tetM*	–	–	2	1.5	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia*	0.047	2	3	1
	pH 4.0	–	7.93×10^{-6}	9.97×10^{-6}	8.89×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	4	12	4					
	4% NaCl	–	8.03×10^{-6}	1.36×10^{-5}	8.97×10^{-5}	*aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, tetM*	–	4	12	6					

(continued on next page)

Table 4 (*continued*)

		Transfer Rate TC/R				Transferred resistance genes	MIC value of TCs [μg/mL]				Genotype of donor	MIC value of D [μg/mL]			
		S	CN	K	TE		S	CN	K	TE		S	CN	K	TE
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	4.97×10^{-5}	*tet*M	–	–	–	4					
	400 MPa	–	–	–	5.12×10^{-5}	*tet*M	–	–	–	4					
LAB-39	C	–	–	7.23×10^{-6}	5.03×10^{-5}	*aph(3′)-IIIa*, *tet*O	–	–	4	6	*tet*M, *tet*O, *aph(3′)-IIIa*, *aac (6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ia*	0.19	0.25	6	12
	pH 4.0	–	4.38×10^{-6}	8.79×10^{-6}	8.49×10^{-5}	*aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *tet*O	–	1.5	8	8					
	4% NaCl	–	4.97×10^{-6}	1.02×10^{-5}	8.97×10^{-5}	*aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *tet*O	–	4	8	6					
	7 °C	–	–	–	–	–	–	–	–	–					
	200 MPa	–	–	–	5.00×10^{-5}	*tet*O	–	–	–	6					
	400 MPa	–	–	–	5.98×10^{-5}	*tet*O	–	–	–	6					

Abbreviations: C – control, S – streptomycin, CN – gentamicin, K – kanamycin, TE – tetracycline, D – donors, TC – transconjugants, R – recipients, "-" – not checked.

reported that *Lb. acidophilus* and *Lb. casei* produce protective molecules, such as DnaK and HtrS operon proteins, which protect the cells from salt-induced injuries (Mbye et al., 2020). In the present study, an increase in the expression of genes encoding resistance to kanamycin, gentamicin and tetracycline was observed after exposure to increased NaCl concentration. Several studies have reported an association between osmotic stress and antibiotic resistance, although they were focused only on pathogenic microorganisms. Reported changes in antibiotic resistance mainly affected ampicillin, tetracycline, vancomycin and chloramphenicol (Komora et al., 2017; Ma et al., 2019).

Bacteria encounter cold stress during refrigerated food storage, for either ripening (e.g. cheeses) or safety (e.g. yogurt) (Liao et al., 2020). The physiological stress caused by lowering the temperature is manifested by reducing the fluidity of the membrane and changing DNA and RNA supercoiling. At low temperatures, cellular components and enzymes become rigid and highly concentrated (Louesdon et al., 2015; Mbye et al., 2020). Food fermentation is often executed at a relative low temperature (0–10 °C), where cold-resistant LAB strains surviving cold stress conditions are present. Over the past few decades, many studies have been carried out on the mechanisms of cold resistance in pathogenic bacteria such as *L. monocytogenes* and *S. aureus*, as they can survive in cold storage and pose a food safety hazard while only a few studies have focused on LAB. Cold-resistant LAB are known to adapt to cold environments, keeping cells viable and producing more lactic acid which improves the taste of the product and its properties (Chen et al., 2017; Liu et al., 2020). In the current study, decreased gene expression and MIC values were observed for gentamicin, kanamycin and tetracycline. There are currently no studies available analyzing the effect of cold stress on the antibiotic resistance of bacteria.

High-pressure processing is considered one of the most promising new methods of non-thermal preservation of food. Due to high pressure, it is possible to eliminate pathogens and spoilage microorganisms while maintaining the sensory and functional properties of the product (Li et al., 2020). Therefore, they are more often used to preserve food obtained using starter or protective cultures. High-pressure processing can stop or inhibit LAB growth by inactivating specific enzymes when biochemical reactions are altered (Modugno et al., 2018). LAB respond to HPP stress with various intensity, although a moderate pressure value does not break covalent bonds in most of them. The LAB response to HPP is more difficult to identify because the HPP response systems are very similar to the response to other stress factors, potentially due to the presence of cross-protection systems (Liu et al., 2018). Current research into the LAB response to stress induced by high pressure focuses on changes in the structure, metabolism, growth and viability of cells. High pressure can affect the bacterial proteome and induce conformational changes in proteins (Gayán et al., 2017). In response to high pressure, LAB can produce more unsaturated fatty acids, increase the fluidity of the cell membrane and produce more polysaccharides (Bucka-Kolendo et al., 2020; D. Yang et al., 2021). According to the literature, high-pressure processing might be able to effectively inactivate *L. plantarum* in different matrixes such as beer, milk, phosphate buffer and citrate buffer, achieving 5-log reductions at values of 400–500 MPa applied for 30 s to several minutes (Carreño et al., 2011; Putnik et al., 2020). In a study by Castro et al. (2015), exposure to high pressure has a significant effect on the viability of microorganisms, probably due to the multiple or accumulated injuries inside the cell. In the current study, changes in the tetracycline resistance gene expression were observed. There are currently few studies on the effects of HPP on changes in antibiotic resistance. The authors' pilot studies showed that high-pressure exposure increased the expression of the *tet*M gene in foodborne enterococci but in a study by Tabanelli et al. (2013) HPP treatment did not modify the antibiotic resistance of lactic acid bacteria.

In the present study, it was observed that after exposure to low pH and high NaCl concentration, the transfer rate of genes encoding resistance to gentamicin, kanamycin and tetracycline increased. The obtained results are reflected in the literature data, according to which environmental stresses during food production and storage can affect the rate of gene transfer and the number of plasmid copies in bacterial cells. The horizontal transfer of antibiotic resistance plasmids can be promoted by food preservation stresses such as high or low temperature, high NaCl concentration and low pH, although the mechanisms are as yet unknown (Mahon et al., 2007; McMahon et al., 2007; Klu et al., 2012; Obolski and Hadany, 2012; Polit et al., 2018). Stress can also contribute to resistance development by promoting the spread of resistance genes. A variety of stressors, including antibiotics, induce competence in Gram-positive bacteria, promoting the acquisition of resistance genes. Antibiotics that target DNA replication have been shown to increase the copy number of genes at or near the replication origin, including genes for competence, thereby increasing transformation and the potential for resistance gene acquisition (Slager et al., 2014).

This study may lead to a deeper understanding of the response of lactic acid bacteria to food-related stresses in relation to antibiotic resistance. This is noteworthy, as it may be suggested that environmental conditions during food production and/or storage may contribute changes in the expression and spreading intensity of the resistance genes. Based on the obtained results, it can be suggested that there is a need for further research in this area to assess changes in cells during factors influencing antibiotic resistance depending on the resistance mechanisms. Currently, there is no extensive discussion in the

literature on this topic, so it is important to understand in depth the mechanisms that may influence the results obtained, which is important in the context of food processing and antibiotic resistance.

5. Conclusions

The obtained results indicate that the conditions prevailing during food production and storage may influence changes in the expression of antibiotic resistance genes, resulting in changes in the resistance phenotype. Moreover, stress factors influenced the frequency of gene transfer. These results confirm the necessity to analyze the strains used in the form of starter and protective cultures for the presence of antibiotic resistance. There is a large knowledge gap regarding changes in antibiotic resistance in response to environmental alterations that are inevitable in the food industry. Although antibiotic-resistant strains from industrial cultures do not pose a direct threat to consumers, food processing-related stresses may lead to the survival of strains resistant and with increased expression and transfer of resistance genes, contributing to the spread of resistance in the environment.

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CRediT author statement

Urszula Zarzecka: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft, Visualization, Funding acquisition, Project administration. **Wioleta Chajęcka-Wierzchowska**: Methodology, Writing - review & editing, Supervision. **Arkadiusz Zakrzewski:** Methodology, Validation, Investigation. **Anna Zadernowska:** Conceptualization, Writing - review & editing, Supervision. **Maria João Fraqueza**: Supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Authors declared no conflict of interests.

Data availability

The dataset used during this study is available from the author upon reasonable request.

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Appendix A. Supplementary data

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Table S1 Primers used in qPCR reactions

Gene	Sequence of primers 5'-3'	Amplicon size (bp)	Annealing temperature	References
gap	F: GCAGGAGCAGAGATAACCACA R: TGTCTACGCAGAACCAGATGC	152	60ºC	Jaimee and Halami (2017)
aac(6')Ie-aph(2'')Ia	F: GCCAGAACATGAATTACACG R: GCCACACTATCATAACCACTAC	170		
aph(3')-IIIa	F: CGTCCTTTGCTCGGAAGAGTATG R: CGATGGAGTGAAAGAGCCTGATG	92		
16S rDNA	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	130		Flórez et al. (2014)
*tet*M	F: CAGAATTAGGAAGCGTGGACAA R: CCTCTCTGACGTTCTAAAAGCGTAT	67		

Table S2 Results of qPCR analysis – raw data

Strain		C_T values*±SD			$2^{-\Delta\Delta CT}$		C_T values*±SD		$2^{-\Delta\Delta CT}$
		gap	*aph(3')-IIIa*	*aac(6')Ie-aph(2'')Ia*	*aph(3')-IIIa*	*aac(6')Ie-aph(2'')Ia*	16S rDNA	*tet*M	*tet*M
Osmotic stress treatment									
LAB-45	C	26.313±0.12	27.040±0.08	29.770±0.09	1.000[a]	1.000[a]	n.a.		
	4% NaCl	26.323±0.10	25.033±0.05	28.070±0.05	4.046[b]	3.272[b]			
LAB-41	C	25.500±0.06	26.227±0.12	29.530±0.11	1.000[a]	1.000[a]			
	4% NaCl	25.590±0.15	24.023±0.11	27.130±0.20	4.902[b]	5.618[b]			
LAB-43	C	24.647±0.05	25.017±0.13	26.003±0.17	1.000[a]	1.000[a]			
	4% NaCl	23.787±0.08	22.037±0.04	22.480±0.09	4.347[b]	6.335[b]			
LAB-39	C	25.470±0.12	25.693±0.06	28.753±0.07	1.000[a]	1.000[a]			
	4% NaCl	25.580±0.11	24.050±0.09	26.663±0.11	3.371[b]	4.595[b]			
LAB-3	C	25.533±0.08	26.113±0.12	27.087±0.12	1.000[a]	1.000[a]			
	4% NaCl	25.137±0.07	23.547±0.14	24.657±0.10	4.500[b]	4.093[b]			
LAB-46b	C	25.397±0.11	26.110±0.08	27.857±0.09	1.000[a]	1.000[a]	11.797±0.12	16.997±0.10	1.000[a]
	4% NaCl	26.000±0.11	25.033±0.08	26.257±0.08	3.204[b]	4.605[b]	10.783±0.10	13.483±0.11	5.657[b]
LAB-8	C	25.250±0.07	25.563±0.04	19.983±0.12	1.000[a]	1.000[a]	28.590±0.07	30.360±0.12	1.000[a]
	4% NaCl	24.967±0.12	23.060±0.12	17.240±0.14	4.659[b]	5.502[b]	30.347±0.09	30.89±0.13	2.340[b]
LAB-1	C	25.370±0.20	25.650±0.21	30.963±0.05	1.000[a]	1.000[a]	n.a.		
	4% NaCl	24.727±0.18	23.120±0.04	28.370±0.06	3.698[b]	3.864[b]			
Acidic stress treatment									
LAB-45	C	26.313±0.12	27.040±0.10	29.770±0.04	1.000[a]	1.000[a]	n.a.		
	pH 4.0	27.130±0.09	26.143±0.12	29.230±0.06	3.279[b]	2.561[b]			
LAB-41	C	25.500±0.12	26.227±0.05	29.530±0.11	1.000[a]	1.000[a]			
	pH 4.0	25.407±0.05	24.720±0.07	27.533±0.04	2.664[b]	3.741[b]			
LAB-43	C	24.647±0.14	25.017±0.08	26.003±0.11	1.000[a]	1.000[a]			
	pH 4.0	25.030±0.10	24.093±0.06	24.910±0.10	2.474[b]	2.783[b]			
LAB-39	C	25.470±0.07	25.693±0.12	28.753±0.17	1.000[a]	1.000[a]			
	pH 4.0	24.867±0.06	23.680±0.10	26.677±0.18	2.657[b]	2.777[b]			
LAB-3	C	25.533±0.03	26.113±0.03	27.087±0.04	1.000[a]	1.000[a]			
	pH 4.0	24.690±0.06	24.147±0.06	25.017±0.06	2.178[b]	2.340[b]			
LAB-46b	C	25.397±0.10	26.110±0.11	27.857±0.07	1.000[a]	1.000[a]	11.797±0.10	16.997±0.05	1.000[a]
	pH 4.0	25.757±0.11	25.137±0.13	26.130±0.10	2.520[b]	4.248[b]	12.123±0.11	16.030±0.06	2.451[b]
LAB-8	C	25.250±0.20	25.563±0.15	19.983±0.17	1.000[a]	1.000[a]	28.590±0.13	30.360±0.10	1.000[a]
	pH 4.0	25.010±0.04	23.073±0.18	18.037±0.16	4.757[b]	3.264[b]	29.300±0.14	28.823±0.18	4.746[b]
LAB-1	C	25.370±0.05	25.650±0.03	30.963±0.06	1.000[a]	1.000[a]	n.a.		

	pH 4.0	25.443±0.07	23.603±0.06	29.413±0.05	4.347[b]	3.081[b]			
Cold stress treatment									
LAB-45	C	26.313±0.06	27.040±0.14	29.770±0.13	1.000[a]	1.000[a]	n.a.		
	7ºC	24.713±0.07	26.297±0.06	29.017±0.05	0.552[a]	0.556[a]			
LAB-41	C	25.500±0.13	26.227±0.07	29.530±0.19	1.000[a]	1.000[a]			
	7ºC	24.587±0.16	25.583±0.06	29.507±0.23	0.829[a]	0.540[a]			
LAB-43	C	24.647±0.08	25.017±0.12	26.003±0.10	1.000[a]	1.000[a]			
	7ºC	25.223±0.03	25.890±0.13	26.847±0.05	0.814[a]	0.831[a]			
LAB-39	C	25.470±0.21	25.693±0.19	28.753±0.07	1.000[a]	1.000[a]			
	7ºC	24.570±0.02	26.100±0.07	28.933±0.07	0.404[b]	0.473[b]			
LAB-3	C	25.533±0.16	26.113±0.09	27.087±0.10	1.000[a]	1.000[a]			
	7ºC	24.647±0.06	25.717±0.13	27.080±0.16	0.712[a]	0.543[a]			
LAB-46b	C	25.397±0.07	26.110±0.05	27.857±0.05	1.000[a]	1.000[a]	11.797±0.19	16.997±0.07	1.000[a]
	7ºC	25.237±0.08	25.967±0.07	28.233±0.06	0.989[a]	0.689[a]	11.757±0.08	17.170±0.09	0.863[a]
LAB-8	C	25.250±0.15	25.563±0.10	19.983±0.08	1.000[a]	1.000[a]	28.590±0.09	30.360±0.10	1.000[a]
	7ºC	24.827±0.08	25.297±0.06	20.207±0.04	0.897[a]	0.639[a]	29.323±0.06	31.950±0.13	0.552[a]
LAB-1	C	25.370±0.05	25.650±0.06	30.963±0.10	1.000[a]	1.000[a]	n.a.		
	7ºC	23.920±0.13	24.793±0.14	30.050±0.17	0.663[a]	0.689[a]			
High pressure stress treatment									
LAB-45	C	26.313±0.12	27.040±0.09	29.770±0.05	1.000[a]	1.000[a]	n.a.		
	200 MPa	26.213±0.10	27.450±0.02	30.550±0.10	0.702[a]	0.543[a]			
	400 MPa	26.243±0.19	27.047±0.06	30.357±0.07	0.948[a]	0.634[a]			
LAB-41	C	25.500±0.12	26.227±0.12	29.530±0.12	1.000[a]	1.000[a]			
	200 MPa	25.513±0.19	26.780±0.11	30.160±0.19	0.688[a]	0.652[a]			
	400 MPa	24.487±0.09	26.110±0.08	29.907±0.05	0.537[a]	0.382[b]			
LAB-43	C	24.647±0.10	25.017±0.04	26.003±0.18	1.000[a]	1.000[a]			
	200 MPa	22.780±0.09	25.017±0.10	24.933±0.13	0.274[b]	0.576[a]			
	400 MPa	24.313±0.05	24.847±0.12	26.023±0.13	0.893[a]	0.783[a]			
LAB-39	C	25.470±0.12	25.693±0.13	28.753±0.15	1.000[a]	1.000[a]			
	200 MPa	24.873±0.11	25.873±0.06	30.060±0.05	0.584[a]	0.267[b]			
	400 MPa	25.760±0.10	26.910±0.14	30.540±0.19	0.526[a]	0.354[b]			
LAB-3	C	25.533±0.12	26.113±0.19	27.087±0.09	1.000[a]	1.000[a]			
	200 MPa	24.263±0.08	25.673±0.17	27.407±0.09	0.563[a]	0.332[b]			
	400 MPa	25.037±0.14	25.913±0.15	26.970±0.10	0.814[a]	0.768[a]			
LAB-46b	C	25.397±0.19	26,110±0.19	27.857±0.17	1.000[a]	1.000[a]	11.797±0.05	16.997±0.13	1.000[a]
	200 MPa	24.933±0.06	26.373±0.10	29.713±0.04	0.604[a]	0.200[b]	10.437±0.09	13.213±0.12	5.364[b]
	400 MPa	25.543±0.08	26.967±0.05	28.817±0.06	0.611[a]	0.569[a]	10.343±0.10	13.077±0.10	5.528[b]

LAB-8	C	25.250±0.08	25.563±0.07	19.983±0.06	1.000[a]	1.000[a]	28.590±0.11	30.360±0.19	1.000[a]
	200 MPa	24.373±0.10	25.570±0.06	19.883±0.04	0.542[a]	0.584[a]	30.020±0.06	29.957±0.19	3.564[b]
	400 MPa	24.400±0.14	25.333±0.07	19.817±0.19	0.651[a]	0.623[a]	29.945±0.04	29.537±0.10	4.526[b]
LAB-1	C	25.370±0.19	25.650±0.05	30.963±0.10	1.000[a]	1.000[a]	n.a.		
	200 MPa	25.070±0.19	25.840±0.09	30.897±0.10	0.712[a]	0.851[a]			
	400 MPa	25.277±0.10	25.767±0.10	30.890±0.04	0.865[a]	0.986[a]			

* - The reported C_T value is the mean of values obtained from all independent reactions; "n.a." - non analyzed; „C„ – control sample - before stress treatment; [a-b] - mean values in rows with different letters differ significantly at $P<0.05$

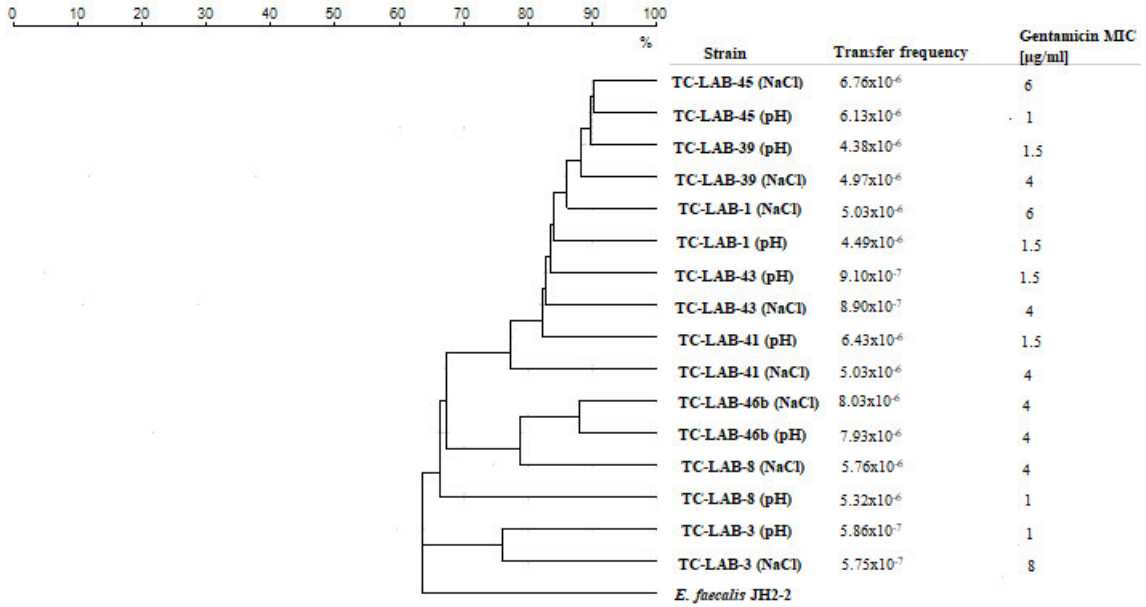


Figure S1 Unweighted pair group method with the arithmetic mean (UPGMA) dendrogram derived from genotyping of *E. faecalis* JH2–2 and transconjugants obtained in gentamycin resistance genes transfer.
Abbreviations: TC – tranconjugant.

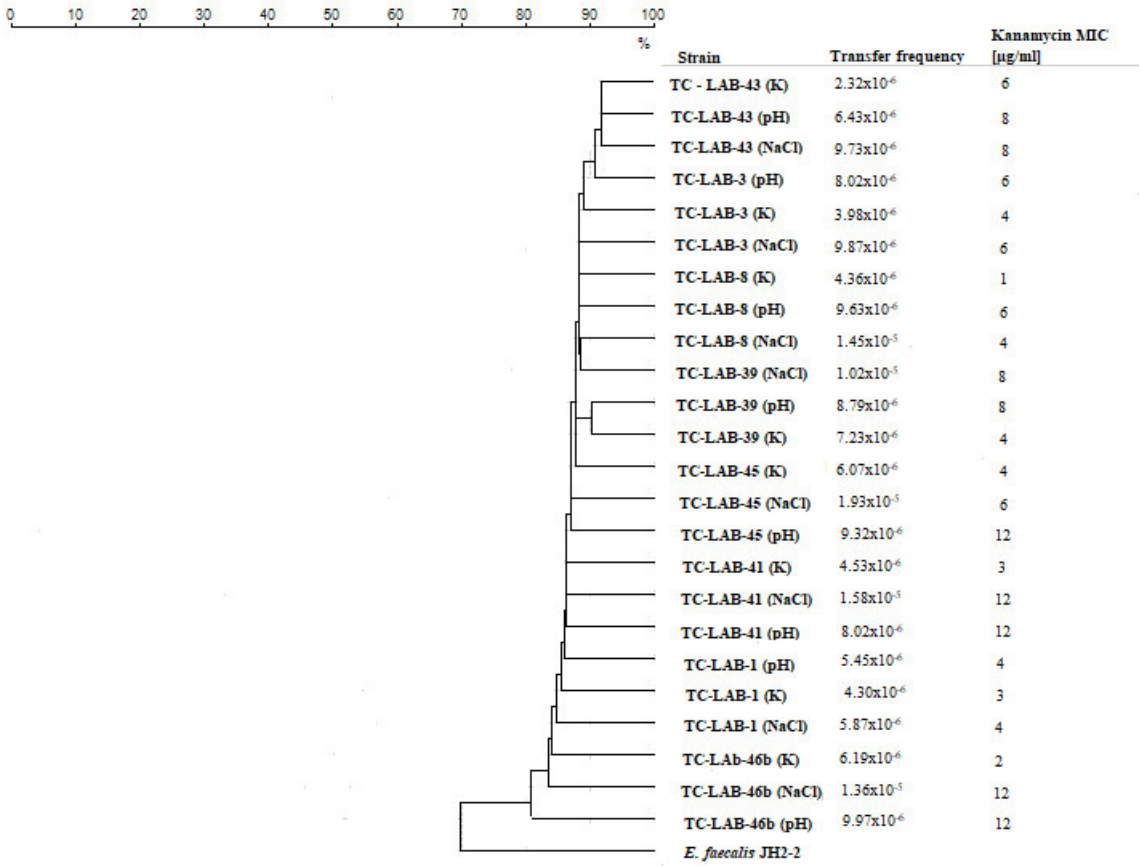


Figure S2 Unweighted pair group method with the arithmetic mean (UPGMA) dendrogram derived from genotyping of *E. faecalis* JH2–2 and transconjugants obtained in kanamycin resistance genes transfer.
Abbreviations: TC – tranconjugant.

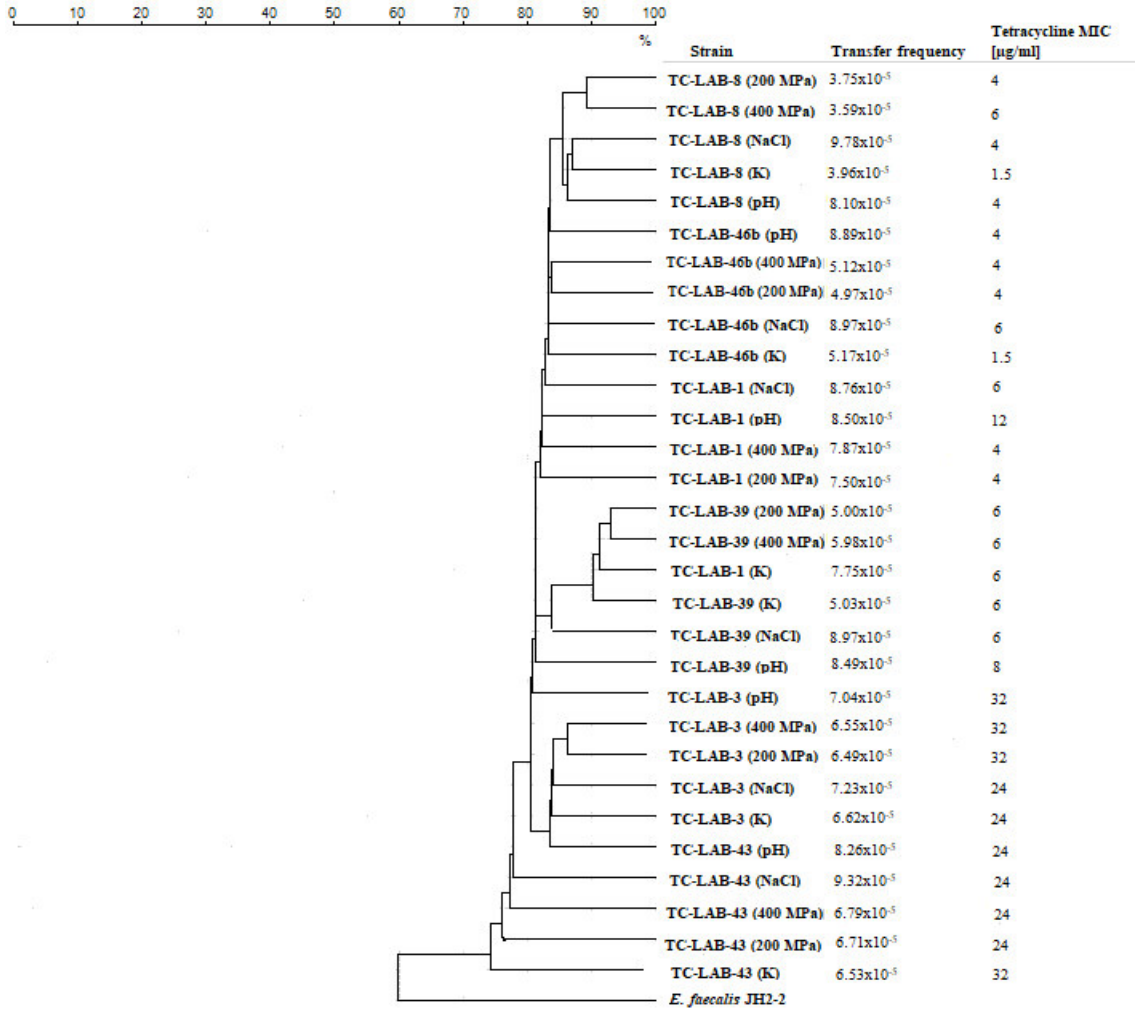


Figure S3 Unweighted pair group method with the arithmetic mean (UPGMA) dendrogram derived from genotyping of *E. faecalis* JH2–2 and transconjugants obtained in tetracycline resistance genes transfer.
Abbreviations: TC – tranconjugant.

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Effect of high pressure processing on changes in antibiotic resistance genes expression among strains from commercial starter cultures

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ABSTRACT

This study analyzed the effect of high-pressure processing on the changes in resistance phenotype and expression of antibiotic resistance genes among strains from commercial starter cultures. After exposure to high pressure the expression of genes encoding resistance to aminoglycosides (*aac(6′)Ie-aph(2″)Ia* and *aph(3′)-IIIa*) decreased and the expression of genes encoding resistance to tetracyclines (*tet*M and *tet*W), ampicillin (*bla*Z) and chloramphenicol (*cat*) increased. Expression changes differed depending on the pressure variant chosen. The results obtained in the gene expression analysis correlated with the results of the phenotype patterns. To the best of the authors' knowledge, this is one of the first studies focused on changes in antibiotic resistance associated with a stress response among strains from commercial starter cultures. The results suggest that the food preservation techniques might affect the phenotype of antibiotic resistance among microorganisms that ultimately survive the process. This points to the need to verify strains used in the food industry for their antibiotic resistance as well as preservation parameters to prevent the further increase in antibiotic resistance in food borne strains.

1. Introduction

Currently, the use of starter cultures is an important part of the food industry, as it allows a wide variety of fermented products to be obtained. The main group of microorganisms used to compose starter cultures are lactic acid bacteria (LAB). Due to the wide use of LAB in the food industry, it is important that these strains do not pose a risk to consumers (Laranjo et al., 2017; Zarzecka et al., 2020). It has been shown that strains used in the food industry might become carriers of antibiotic resistance genes and thus contribute to the spread of resistance in the environment (Casado Muñoz et al., 2016; Zarzecka et al., 2020, 2022a).

Microorganisms come into contact with many different stress factors during food production. Among the stress factors affecting microorganisms during food manufacturing is high-pressure processing (HPP), a promising non-thermal method of food preservation that reduces microorganisms but does not affect vitamins, amino acids or flavor compounds. This method has been known for many years, but due to high costs, it has only been used on a large scale for several years (Mbye et al., 2020; Ma et al., 2020). HPP is also used to preserve fermented foods, mainly dairy products (yoghurt, kefir, cheese) and fermented meats. Using thermal preservation techniques after fermentation would not keep the number of the lactic acid bacteria at desired levels, therefore the use of non-thermal preservation methods seems optimal. Research on the effect of high-pressure treatment on the microflora of yogurt showed that high-pressure treatment at 500 MPa does not significantly decreased the number of lactic acid bacteria. In addition, fermented foods have been found to maintain acidity throughout the shelf life after HPP treatment. It is worth mentioning that the survival of microorganisms in fermented foods subjected to high pressure treatment depends on the initial bacterial load (Nema et al., 2022). The evaluation of HPP response mechanisms is a valuable tool to assess the shelf life of food products preserved using this method. However, the LAB response to HPP is difficult to identify in comparison to the response to other stress factors. It is suggested that the HPP response mechanisms come from the cross-protection system (Bucka-Kolendo and Sokołowska, 2017; Yang et al., 2021).

An important (but rarely discussed) issue is the influence of physicochemical factors related to food production on the metabolic processes of microorganisms intentionally added to food, including in the form of starter cultures. Exposure to unfavorable conditions during the production process forces the LAB to adapt to these conditions. The

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ability to deal with a wide range of environmental factors changes the stress response mechanisms (Ma et al., 2020; Wu et al., 2013; Yang et al., 2021). Studies conducted to date indicate that the molecular mechanisms of the stress response can be species- or strain-specific and are usually associated with the induction of a large number of genes and synthesis of stress response proteins (Hussain et al., 2013). The activation of a stress response may result in the selection of strains with changed stress tolerance. Some authors indicate reduced sensitivity to several antibiotics, including antibiotics which are important in clinical practice (Al-Nabulsi et al., 2015; Giacometti et al., 2021; McMahon et al., 2007; Komora et al., 2017). Studies focused on stress-induced changes in antibiotic resistance have only been performed for pathogenic microorganisms, while currently there are no available studies refering to non-pathogenic microorganisms such as lactic acid bacteria.

The aim of this study was to determine the effect of high-pressure processing on resistance phenotype and the expression of *dna*K, *gro*EL and *clp*L genes as well as antibiotic resistance genes in strains derived from commercial starter cultures.

2. Materials and methods

2.1. Strains

The study was carried out using ten strains derived from commercial starter cultures. Strains were selected from the collection described previously (Zarzecka et al., 2022a). Seven of the strains belong to the former *Lactobacillus* genus (*Lactiplantibacillus paraplantarum* n = 2, *Lactocaseibacillus paracasei* n = 2, *Latilactobacillus curvatus* n = 1, *Lactobacillus helveticus*, *Lactobacillus* sp. N = 1 each) and three to the *Lactococcus* genus (*Lactococcus lactis* ssp. *lactis* n = 2 and *Lactococcus lactis* ssp. *cremoris* n = 1). The strains' characteristics are summarized in Table 1. The criterion for selecting the strains used in this study was to have at least one antibiotic resistance gene with a simultaneous low Minimal Inhibitory Concentration (MIC) for this antibiotic. The genus names of bacteria have been updated in line with the new nomenclature (Zheng et al., 2020).

2.2. High pressure treatment

Pressure values and pressurization time were chosen on the basis on the preliminary study (unpublished data) to guarantee the induction of the genes under the stress condition, and to avoid the inactivation of the bacterial cells. The survival rate of the strains was checked by the plate count method as described previously (Zarzecka et al., 2022c).

High pressure treatment was applied to cultures of selected strains from the stationary growth phase (10 mL). The strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany). The following pressure and time conditions were selected for the analysis: 300 MPa/3 min, 300 MPa/5 min, 400 MPa/1 min and 400 MPa/3 min. The pressurization times did not include the come-up and come-down times. The pressurization was carried out in glycol-water solution (1:1, v/v) using the high-pressure single-chamber U4040 (Unipress, Warsaw, Poland) as described previously (Zarzecka et al., 2022b). Temperature was kept at 20 ± 3 °C, the pressure rise rate was 300 MPa/min, and the pressure relief time was 2–4 s. For each tested strain, the assays were performed in three independent processes. Study design is presented on

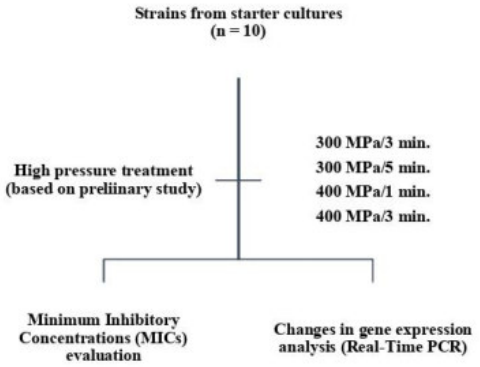


Fig. 1. Study design flowchart.

Table 1
Characteristic of strains analyzed in the study.

Strain	Identification	Origin	Antibiotic MICs [μg/mL]									Antibiotic resistance genes
			AMP	CN	C	TE	E	DA	K	S	VA	
LAB-1	*Lactobacillus paraplantarum*	Kefir starter culture	0.5	0.75	8	12	0.19	0.5	6	0.19	–	*tetM, tetW, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, aac(6′)-Ia, bla*$_{OXA}$
LAB-11	*Lactobacillus paraplantarum*	Cheese starter culture	0.25	0.38	32	>256	0.25	0.25	8	0.25	–	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aph(2′)-Ic, cat*
LAB-14	*Lactococcus lactis* ssp. *lactis*	Yoghurt starter culture	0.19	0.19	12	48	0.125	1.5	12	0.125	0.19	*tetK, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, cat*
LAB-16	*Lactococcus lactis* ssp. *lactis*	Cheese starter culture	0.19	1	8	32	0.125	0.25	8	0.125	0.094	*tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia*
LAB-26 b	*Lactococcus lactis* ssp. *cremoris*	Cheese starter culture	0.25	4	12	32	0.25	0.75	12	0.25	0.19	*tetM, tetW, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, blaZ, cat*
LAB-31	*Lactocaseibacillus paracasei*	Cheese starter culture	0.19	2	4	1	0.023	0.032	32	0.023	–	*tetM, tetW, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia*
LAB-41	*Lactobacillus paracasei*	Cheese starter culture	1	0.38	32	32	0.125	0.023	4	0.125	–	*tetW, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, aac(6′)-Ia, cat*
LAB-43	*Lactobacillus curvatus*	Meat starter culture	0.125	0.5	16	64	0.125	0.38	6	0.125	–	*tetM, tetO, aph(3′)-IIIa, aac(6′)-Ie-aph (2″)-Ia, cat*
LAB-45	*Lactobacillus helveticus*	Cheese starter culture	0.75	0.25	0.125	32	0.125	0.032	6	0.125	–	*tetO, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia, cat*
LAB-63	*Lactobacillus* sp.	Yoghurt starter culture	3	0.5	8	48	0.19	0.047	16	0.19	–	*blaZ*

Abbreviations: AMP – ampicillin, CN – gentamicin, C – chloramphenicol, TE – tetracycline, E – erythromycin, DA – klindamycin, K – kanamycin, S – streptomycin, VA – vancomycin, „–" – non-analyzed.
Based on the previous study (Zarzecka et al., 2022a).

Fig. 1.

After the stress treatment was completed, the samples were subjected to the next stages of the study. Unpressurized samples were used as a control.

2.3. Determination of antibiotic resistance phenotype

After the stress treatment, the MIC values were determined for following antibiotics – chloramphenicol (C), gentamycin (CN), kanamycin (K), tetracycline (TE) and ampicillin (AMP). The MIC values were determined by applying gradient strips (Liofilchem, Italy) on Mueller-Hinton agar (Merck, Germany) inoculated with suspensions in 0.85% NaCl solution at a density of 0.5° in the McFarland scale prepared from bacterial cultures. The MIC values were read after 18 h of anaerobic incubation at 30 °C (CLSI, 2019). The analysis was performed in three independent replications.

2.4. RNA extraction and reverse transcription into cDNA

Total RNA was extracted from control and pressurized cultures using the Total RNA Mini Plus kit (A&A Biotechnology, Gdansk, Poland) in duplicate for each sample. Then purification and concentration of RNA samples was carried out using Clean-Up RNA Concentrator kit (A&A Biotechnology, Gdansk, Poland). The purity and concentration of the RNA were measured using Spectrophotometer/Fluorometer DeNovix DS-11 FX (DeNovix Inc., Wilmington, USA) at wavelengths of 260 nm and 280 nm. The RNA integrity and quality were evaluated by electrophoresis on a 1.2% agarose gel in 0.5% TBE buffer, running at 90 V for 1 h and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, Cambridge, UK). The RNA samples were stored at −80 °C.

All the RNA samples were matrices for the synthesis of first cDNA strand using Transcriba Kit (A&A Biotechnology, Gdansk, Poland) with recombinant MMLV reverse transcriptase. DNA polymerase activity was at temperature of 42 °C, and random hexamers were used as primers. Obtained cDNA was used as a template to Real-Time PCR reactions.

2.5. Gene expression analysis

Gene expression analysis was performed using Real-Time PCR method with three independent reactions for each replicate. Reactions were conducted using the RotorGene Q system (Qiagen, Germany).

The study analyzed the expression of three genes related to the stress response: *clpL*, *gro*EL and *dna*K, and the following antibiotic resistance genes: *aac(6′)-Ie-aph(2″)-Ia*, *aph(3′)-IIIa*, *tet*M, *tet*W, *bla*Z and *cat*. The following genes have been selected as housekeeping genes: *gap* (encoding glyceraldehyde-3-phosphate), 23 S rRNA and 16 S rDNA. The expression of genes associated with the stress response was analyzed for each of the strains. The number of antibiotic resistance genes whose expression was analyzed for each strain was selected based on the baseline characteristics of the strains. Despite the wide variety of resistance genes carried by the strains, only genes encoding resistance to antibiotics for which the strain showed a low MIC value were analyzed for gene expression. On the basis of preliminary studies, it was assumed that if a strain initially has a high MIC value for a given antibiotic, no significant changes in the expression of the resistance gene occur under the influence of the stress factor, because the expression level is high before the start of the analysis (Table 2). The gene expression was determined fluorometrically using SYBR green with the RotorGene Q system (Qiagen, Germany) and calculated using relative quantification, in which relevant gene expression is normalized to the expression of the housekeeping gene. A threshold was determined using the software for Real-Time PCR reactions. Comparative CT ($2^{-\Delta\Delta CT}$) method, proposed by Livak and Schmittgen (2001) was used for the analysis of results. Expression ratios ≥2.00 represented a significant increase in gene expression, while expression ratios ≤0.50 represented a significant decrease in gene expression. Primer sequences were obtained from Lenz et al. (2010) (23 S rRNA, *gro*EL and *dna*K), Flórez et al. (2014) (16 S rDNA, *tet*M and *tet*W), George and Halami (2017) (*gap*, *aac(6′)Ie-aph(2″)Ia* and *aph(3′)IIIa*), Ferreira et al. (2013) (*clp*L), Dehbashi et al. (2021) (*bla*Z) and Casanovas-Massana et al. (2014) (*cat*) (Table 3). All the primers were synthesized by the Genomed (Gdansk, Poland). Gene expression analysis was performed with three independent reactions. The relevant gene expression of each gene and each HPP condition was considered significant ($p < 0.05$) on the basis a Student's *t*-test performed using the control (unpressurized) sample as reference.

Table 2
Antibiotic resistance genes analyzed in each strain.

Strain	Analyzed antibiotic resistance genes					
	aph (3′)-IIIa	aac (6′)Ie-aph (2″)Ia	*tet*M	*tet*W	*bla*Z	cat
LAB-1		x	x	x		x
LAB-11	x	x				x
LAB-14	x	x				x
LAB-16	x	x			x	x
LAB-26 b	x	x				
LAB-31	x	x	x	x		x
LAB-41	x	x				x
LAB-43	x	x				x
LAB-45	x	x				
LAB-63					x	

Based on the previous study (Zarzecka et al., 2022a).

Real-time PCR reactions for 16 S rDNA, *tet*M and *tet*W genes were performed in a final volume of 10 μL containing 5 μl of PowerUp SYBR Green MasterMix (ThermoFischer Scientific, Waltham, Massachusetts, USA), 0.45 μL of each primer (10 pmol/1 μL) and 100 ng of template cDNA, filled up with ddH_2O to a final volume. Cycling conditions were following: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The qPCR reactions for remaining genes were performed in a volume of 15 μl containing 7.5 μl of PowerUp SYBR Green MasterMix (ThermoFischer Scientific, Waltham, Massachusetts, USA), 1.05 μl of each primer (10 pmol/μl) and 10 ng of template cDNA, filled up with ddH_2O to a final volume. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 60 s at 72 °C.

The specificity of the each qPCR product was checked based on the melting curve constructed by heating in a slow ramp between 60 and 95 °C in increments of 0.5 °C for 5 s.

2.6. Statistical analysis

Statistical analyses were performed using Student's t-test and/or the one-way ANOVA. For the analyses, $p \leq 0.05$ was considered significant. The statistical analyses and visualization were performed using Prism 9.3.1. (Prism, GraphPad Software, San Diego).

3. Results

3.1. Changes in the antibiotic resistance phenotype

The analysis of the obtained results showed that the influence of high pressure caused changes in the phenotype of resistance to antibiotics. It was observed that the MIC value for chloramphenicol increased significantly after applying a pressure of 300 MPa for 3 min (e.g. from 32 μg/mL to 192 μg/mL). As the duration of the process and the pressure value increased, the MIC value also increased from baseline, but the MIC value was lower than after 300 MPa/3 min treatment. Similar results were obtained for ampicillin resistance, where a significant increase in MIC was demonstrated with each pressurization conditions, with the increase in MIC being the greatest when the 300 MPa pressure was applied. The MIC value of the aminoglycoside antibiotics (gentamicin and kanamycin) decreased from baseline with each of the pressure parameters (e.

Table 3
Primers used in qPCR reactions.

Gene	Sequence of primers 5′-3′	Amplicon size (bp)	Annealing temperature	References
Housekeeping genes				
gap	F: GCAGGAGCAGAGATAACCACA R: TGTCTACGCAGAACCAGATGC	152	60 °C	George and Halami (2017)
23 S rRNA	F: CCTATCGGCCTCGGCTTAG R: AGCGAAAGACAGGTGAGAATCC	101		Lenz et al. (2010)
16 S rDNA	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	130		Flórez et al. (2014)
Stress-response genes				
clpL	F: GGGACGTGCCGGAAGCAATCA R: GCCTCTGTCCTTGACTTCCTTGACC	109	60 °C	Ferreira et al. (2013)
groEL	F: CTGTTTCAGTTGCAGCAC R: CAAATCGGCGAAACAACG	109		Lenz et al. (2010)
dnaK	F: CAGTTAACCCTGACGAAG R: TGACAACGGTGTTACGTC	104		
Antibiotic resistance genes				
aac(6′)Ie-aph(2″)Ia	F: GCCAGAACATGAATTACACG R: GCCACACTATCATAACCACTAC	170	60 °C	George and Halami (2017)
aph(3′)IIIa	F: CGTCCTTTGCTCGGAAGAGTATG R: CGATGGAGTGAAAGAGCCTGATG	92		
tetM	F: CAGAATTAGGAAGCGTGGACAA R: CCTCTCTGACGTTCTAAAAGCGTAT	67		Flórez et al. (2014)
tetW	F: ACGGCAGCGCAAAGAGAA R: CGGGTCAGTATCCGCAAGTT	60		
blaZ	F: AAGAGATTTGCCTATGCTTC R: GCTTGACCACTTTTATCAGC	115		Dehbashi et al. (2021)
cat	F: CTAATGTTCATGGTCTCTTCCCGAAA R: CTAGTCCAAGGTATCCCAGAAATCG	103		Casanovas-Massana et al. (2014)

g. from 2 μg/mL to 0.75 μg/mL). Importantly, no differences in MIC values were observed between the samples irrespective of the pressure values used and the duration of the process. In the case of tetracycline resistance, it has been shown that, with a high initial MIC value (>30 μg/mL), no change in MIC was observed regardless of the pressure and duration of the process. Two strains with low MIC values for tetracycline (1 μg/mL and 12 μg/mL) were analyzed. For these strains, a significant increase in the MIC value was observed after the application of the high-pressure treatment (e.g. from 12 μg/mL to 64 μg/mL). It is worth adding that the MIC values after high pressure treatment did not differ significantly, regardless of the pressure value used and the duration of the process (Table 4).

3.2. Changes in antibiotic resistance genes expression

The effect of high pressure processing on the expression level of genes encoding stress response proteins: *dnaK*, *groEL* and *clpL* as well as genes encoding resistance to gentamicin: *aac(6′)-Ie-aph(2″)-Ia*,

Table 4
Changes in resistance phenotype after high pressure treatment.

		Strain	LAB-1	LAB-11	LAB-14	LAB-16	LAB-26 b	LAB-31	LAB-41	LAB-43	LAB-45	LAB-63
MIC values [μg/mL]	C	K	8	32	12	8	12	4	32	16	0.125	8
		300 MPa/3 min	–	128	48	–	48	–	192	96	0.75	–
		300 MPa/5 min	–	64	32	–	24	–	96	48	0.5	–
		400 MPa/1 min	–	48	24	–	16	–	48	32	0.38	–
		400 MPa/3 min	–	48	24	–	16	–	48	32	0.38	–
	CN	K	0.75	0.38	0.19	4	1	2	0.38	0.5	0.25	0.5
		300 MPa/3 min	0.38	0.125	0.064	1.5	0.5	0.75	0.19	0.25	0.125	–
		300 MPa/5 min	0.38	0.125	0.064	1.5	0.5	0.75	0.19	0.25	0.125	–
		400 MPa/1 min	0.38	0.125	0.064	1.5	0.5	0.75	0.19	0.25	0.125	–
		400 MPa/3 min	0.38	0.125	0.064	1.5	0.5	0.75	0.19	0.25	0.125	–
	K	K	6	8	12	12	8	32	4	6	6	16
		300 MPa/3 min	3	6	6	6	4	1.5	2	4	2	–
		300 MPa/5 min	3	6	6	6	4	1.5	2	4	2	–
		400 MPa/1 min	3	6	6	6	4	1.5	2	4	2	–
		400 MPa/3 min	3	6	6	6	4	1.5	2	4	2	–
	TE	K	12	>256	48	32	32	1	32	64	32	48
		300 MPa/3 min	64	>256	48	32	32	16	32	64	32	–
		300 MPa/5 min	64	>256	48	32	32	16	32	64	32	–
		400 MPa/1 min	64	>256	48	32	32	16	32	64	32	–
		400 MPa/3 min	64	>256	48	32	32	16	32	64	32	–
	AMP	K	0.5	0.25	0.19	0.25	0.19	0.19	1	0.125	0.75	3
		300 MPa/3 min	–	–	–	–	0.75	–	–	–	–	8
		300 MPa/5 min	–	–	–	–	0.5	–	–	–	–	6
		400 MPa/1 min	–	–	–	–	0.38	–	–	–	–	4
		400 MPa/3 min	–	–	–	–	0.38	–	–	–	–	4

Abbreviations: C - chloramphenicol, CN – gentamicin, K – kanamycin, TE – tetracycline, AMP – ampicillin, "-" – non-analyzed.
Based on obtained results.

kanamycin: *aph(3')-IIIa*, tetracycline: *tet*M and *tet*W, chloramphenicol: *cat* and ampicillin: *blaZ* was analyzed. Relevant quantification showed accordance between the results obtained in the phenotypic analysis and results of gene expression.

Analysis of the expression of stress response genes showed that the expression of each of the three analyzed genes increased significantly in response to high pressure treatment. The expression levels of the *clp*L gene after applying each of the pressure variants did not differ significantly from each other. However, differences were observed between the expression levels of the *dna*K and *gro*EL genes depending on the pressure conditions used.

Obtained results indicate that the expression of aminoglycoside resistance genes decreased significantly (relative gene expression level < 0.5). For any of the genes, no significant differences were observed between the expression levels depending on the pressure conditions. Simultaneously, a significant increase in the expression of tetracycline resistance genes was observed, of which the highest increase in the level of expression was noticed for the *tet*W gene. In the case of the *tet*M gene, no differences were observed between the expression levels depending on the pressure variant. However, for the *tet*W gene it was noted that the expression level after applying the 400 MPa/3 min it was significantly lower than the expression levels in the other variants. The expression of genes encoding ampicillin and chloramphenicol resistance also increased after the application of high pressure. Importantly, the increase in expression was the highest after the application of the 300 MPa/3 min variant, and with the increase in pressure value and the duration of the process, the level of expression slightly decreased (Fig. 2, Tables S1–S2).

4. Discussion

It is widely reported that food production processes represent a change in environmental conditions and can therefore be a stress factor for microorganisms in food, including lactic acid bacteria (LAB) found in commercial starter cultures. High pressure processing (HPP) is one of the most promising techniques used in food production for non-thermal food preservation, including fermented food. Its main advantage is the effective reduction of the number of microorganisms while maintaining nutritional quality (Li et al., 2020). There are many studies are focused on the resistance to heat, cold, osmotic or acidic stress, but there is a limited number of studies regarding molecular mechanisms of the high-pressure response (Bucka-Kolendo et al., 2021). The ability of microorganisms to survive unfavorable conditions is considered as an individual feature, species- or even strain-specific. Under stressful conditions all microorganisms activate response mechanisms allowing them to survive, influencing other microbial features. According to the literature, HPP-response results from cross-protection to other stress factors and it is reported to be difficult to characterize. As a result, the changes related to the response to high pressure stress are similar to the changes caused by other stress factors, among the LAB strains the most frequently reported are high and low temperature treatments. Since LAB are very important to the food industry, it should be important to understand all aspects of their stress response (Bucka-Kolendo and Sokołowska, 2017; Song et al., 2014). Under the influence of stress factors various genes encoding stress-response proteins are activated. As a result, cells are able to express a particular set of proteins including molecular chaperones and proteases. Among them, Clp proteins, which are widely conserved in Gram-positive bacteria and play a key role in the protein quality control system are most frequently reported (Ferreira et al., 2013). It is reported that in the case of stress related to high pressure, the *clp*L gene is most often activated. Moreover, according to the literature data, genes encoding the chaperone protein DnaK (*dna*K gene) and genes changing translation or chaperones (*gro*EL) are also frequently activated (Bucka-Kolendo et al., 2021).

Regulation of stress response genes have been investigated among several Gram-positive bacteria and the genetic model for detailed

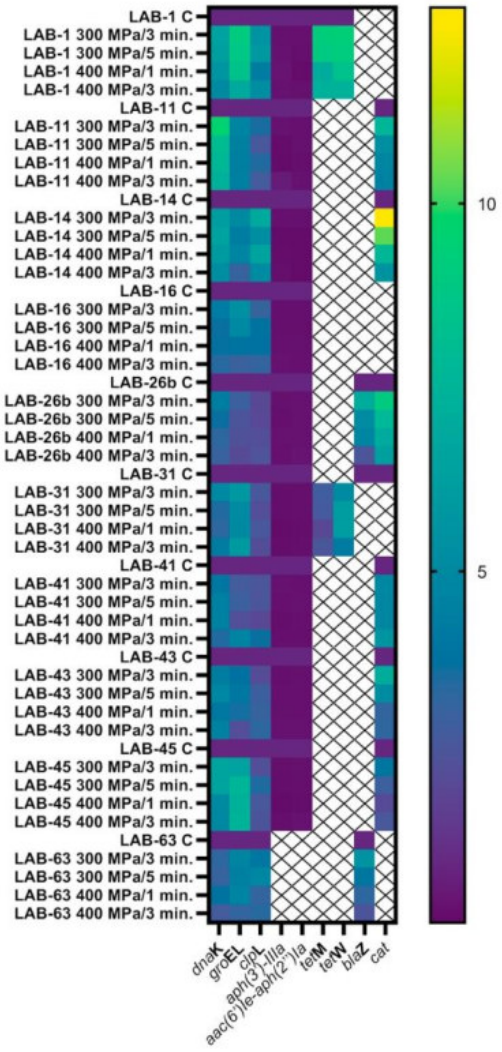


Fig. 2. Relative genes expression changes in response to high pressure treatment. Abbreviation: C – control sample. Based on own study.

studies was *Bacillus subtilis*. However, there is still not enough literature data about the transcriptional regulation of stress-related genes in LAB strains in response to high pressure treatment. It is suggested that under the high pressure stress the mRNA amount of many genes is changing, and that can be a result of the selective transcription or mRNA stability (Guan et al., 2022; Palud et al., 2018). This study analyzed changes in the expression of *dna*K, *gro*EL and *clp*L genes in response to various parameters of high pressure treatment. A significant increase in the expression of the analyzed genes in response to high pressure treatment, regardless of strain identification, was shown. In the study by Bucka-Kolendo et al. (2021) it was shown that not all strains belonging to the

former *Lactobacillus* genus showed the same change in *gro*EL and *dna*K gene expression in response to high pressure stress. A suggested explanation of these results is complexity of the stress response mechanisms, consisting of many different pathways that can be activated.

An interesting issue seems to be the influence of food-related stresses on changes in antibiotic resistance in microorganisms. Currently, such studies are mainly related to pathogenic microorganisms or opportunistic pathogens, e.g. *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis* (Al Nabulsi et al., 2015; Komora et al., 2017; Woode et al., 2020; Ma et al., 2020). Little is said about the changes in antibiotic resistance among LAB. This is most likely due to the fact that they are non-pathogenic microorganisms that do not pose a direct threat to the health of consumers. However, recently published studies have shown that lactic acid bacteria can also carry antibiotic resistance genes as well as take part in their distribution in the environment (Fraqueza, 2015; Rozman et al., 2020; Zarzecka et al., 2022a). Therefore, it is important to check how high-pressure treatment, recently one of the most common food-related stresses, affects the level of antibiotic resistance of LAB. In the current study, it was observed that high pressure exposure caused an increase in the minimum inhibitory concentration for three antibiotics: chloramphenicol, tetracycline and ampicillin. Simultaneously, it was observed that the MIC values for the aminoglycoside antibiotics (kanamycin and gentamicin) decreased slightly after the application of high-pressure treatment. Similar results were obtained in other studies conducted by our research team (Unpublished data). The results obtained in the phenotypic analysis were consistent with the results of the gene expression analysis. It was observed that of the two tetracycline resistance genes: *tet*M and *tet*W, when tetracycline MIC was increased, the *tet*W gene relative expression level was higher than the expression of *tet*M gene. Importantly, in the case of chloramphenicol and ampicillin, the highest increase in MIC values was observed after the mildest pressure variant (300 MPa/3 min) was used. This may suggest that milder conditions are more likely to stimulate changes in resistance to these antibiotics. Due to the lack of literature data with which to compare the results obtained in this study, other Gram-positive bacteria will also be referred to due to their similar response mechanisms. Another study conducted by the authors showed that high-pressure exposure increased the expression of the *tet*M gene in foodborne enterococci (Unpublished data). However Tabanelli et al. (2013) indicated that HPP treatment did not modify the antibiotic resistance of lactic acid bacteria. There are currently only a few studies on the effects of HPP on changes in antibiotic resistance because the effect of high-pressure treatment on LAB is difficult to identify and the HPP response systems are very similar to the response to other stress factors, potentially due to the presence of cross-protection systems (Liu et al., 2020). It is suggested that other stress factors, mainly temperature stress, may cause similar changes in the level of antibiotic resistance. Woode et al. (2020) reported decreasing in resistance to several antibiotics in response to heat stress. Similar results obtained McMahon et al. (2007) reporting lower MIC values for *Staphylococcus* strains after heat stress. However, Poole (2012) indicated that heat stress that has similar effects as high pressure treatment, is linked to β-lactam resistance, which is related to ClpL chaperone, found in Gram-positive microorganisms. Author indicated that in *Streptococcus pneumoniae clp*L gene expression is associated with increased penicillin resistance. This could possibly explain the increase in MIC values for ampicillin, observed in this study, which is also β-lactam antibiotic. In the same study, Poole (2012) indicated also decreasing in aminoglycoside resistance among Gram-positive microorganisms as an effect of heat stress, what is in accordance with results obtained in this study. Moreover, Dorrian et al. (2011) reported that induction of a heat stress caused increasing in macrolide resistance in *Lactococcus lactis* strains, although no mechanism was proposed.

Undoubtedly, this topic is still poorly understood and requires further research in this area. This will allow a full understanding of the mechanisms linking the stress response to changes in antibiotic resistance.

This is noteworthy, as it may be suggested that environmental conditions during food production may contribute to changes in the expression of the resistance genes. Based on the obtained results, there is a need for further research in this area to assess changes in cells during exposure to factors influencing antibiotic resistance depending on the resistance mechanisms. Currently, there is no extensive discussion in the literature on this topic, so it is important to understand in depth the mechanisms that may influence the results obtained, which is significant in the context of food processing and antibiotic resistance.

5. Conclusions

Understanding the complexity of stress-related gene regulation networks among LAB strains is important for the improvement of the industrial strategies using LAB strains and HPP.

The obtained results indicate that the food preservation techniques may influence changes in the expression of antibiotic resistance genes, resulting in changes in the resistance phenotype. These results confirm that it is necessary to further analyze the strains used in the form of starter cultures for the presence of antibiotic resistance and mechanisms of their regulation. There is a large knowledge gap in the area of antibiotic resistance and food-related stresses. Although antibiotic-resistant strains from industrial cultures do not pose a direct threat to consumers, food processing-related stresses may lead to the survival of strains with increased expression and intensive spread of resistance in the environment.

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CRediT authors statement

Urszula Zarzecka: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Anna Zadernowska:** Conceptualization, Writing - review & editing, Supervision. **Wioleta Chajęcka-Wierzchowska**: Writing - review & editing, Supervision. **Patryk Adamski**: Investigation, Data curation. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Authors declared no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104169.

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Table S1 Results of qPCR analysis – raw data (mean C_T values)

Strain	HPP conditions	C_T values* ± SD											
		Housekeeping genes			Stress response genes			Aminoglycosides resistance genes		Tetracycline resistance genes		Ampicillin resistance gene	Chloramphenicol resistance gene
		23S rRNA	*gap*	16S rDNA	*dna*K	*gro*EL	*clp*L	*aph(3')-IIIa*	*aac(6')Ie-aph(2'')Ia*	*tet*M	*tet*W	*bla*Z	*cat*
LAB-1	K	21.82±0.09	25.37±0.19	25.82±0.04	25.13±0.10	25.91±0.05	25.13±0.03	26.43±0.04	30.96±0.11	26.65±0.11	26.65±0.13	-	-
	300 MPa/3 min.	22.23±0.15	25.44±0.14	25.23±0.09	22.86±0.11	23.16±0.08	22.63±0.07	27.75±0.05	32.41±0.07	22.86±0.06	22.60±0.09	-	-
	300 MPa/5 min.	22.17±0.10	23.92±0.20	26.17±0.11	22.84±0.21	23.13±0.11	22.93±0.10	26.13±0.11	31.05±0.12	23.84±0.14	23.61±0.10	-	-
	400 MPa/1 min.	21.92±0.08	24.73±0.13	24.92±0.03	22.71±0.19	22.93±0.14	23.13±0.11	26.84±0.12	32.37±0.16	22.93±0.12	22.42±0.16	-	-
	400 MPa/3 min.	22.22±0.07	25.07±0.15	23.23±0.03	23.19±0.13	23.53±0.12	23.13±0.11	27.36±0.09	31.89±0.10	21.19±0.09	21.19±0.10	-	-
LAB-11	K	21.32±0.17	21.37±0.10	-	25.91±0.07	25.51±0.12	24.41±0.11	25.65±0.12	30.96±0.10	-	-	-	23.15±0.10
	300 MPa/3 min.	21.23±0.08	21.44±0.12	-	22.56±0.11	23.19±0.12	22.50±0.10	26.90±0.09	32.41±0.19	-	-	-	20.13±0.08
	300 MPa/5 min.	21.17±0.03	21.92±0.08	-	22.81±0.10	23.19±0.09	22.84±0.12	27.93±0.11	32.75±0.20	-	-	-	20.61±0.09
	400 MPa/1 min.	21.13±0.03	22.73±0.07	-	22.72±0.09	23.19±0.14	22.43±0.19	28.91±0.12	33.87±0.14	-	-	-	20.71±0.11
	400 MPa/3 min.	22.20±0.13	22.07±0.09	-	23.91±0.14	24.23±0.15	23.80±0.09	26.84±0.09	32.89±0.15	-	-	-	21.84±0.13
LAB-14	K	19.32±0.16	21.13±0.07	-	23.90±0.02	24.91±0.17	25.15±0.12	24.65±0.12	29.96±0.20	-	-	-	22.52±0.08
	300 MPa/3 min.	20.20±0.28	21.30±0.11	-	22.16±0.04	23.45±0.12	23.25±0.19	26.13±0.11	31.41±0.17	-	-	-	19.73±0.10
	300 MPa/5 min.	19.15±0.05	21.65±0.05	-	21.35±0.05	22.63±0.16	22.59±0.14	27.13±0.14	32.35±0.19	-	-	-	18.98±0.11
	400 MPa/1 min.	20.53±0.20	21.75±0.12	-	22.42±0.09	23.85±0.14	23.65±0.08	27.12±0.09	32.67±0.21	-	-	-	20.79±0.09
	400 MPa/3 min.	20.12±0.11	22.15±0.11	-	22.13±0.02	24.08±0.12	23.90±0.10	27.20±0.10	32.99±0.19	-	-	-	20.84±0.11
LAB-16	K	22.52±0.17	23.56±0.10	-	24.20±0.08	22.13±0.19	25.00±0.14	25.15±0.13	28.95±0.12	-	-	18.60±0.05	24.76±0.12
	300 MPa/3 min.	21.92±0.11	23.13±0.08	-	21.49±0.10	19.10±0.15	22.75±0.16	26.19±0.16	29.96±0.14	-	-	16.20±0.07	21.13±0.07
	300 MPa/5 min.	22.15±0.07	22.97±0.09	-	21.95±0.19	19.45±0.16	22.70±0.17	26.12±0.14	29.89±0.12	-	-	16.90±0.14	21.90±0.18
	400 MPa/1 min.	21.34±0.13	23.25±0.07	-	21.12±0.15	19.00±0.14	21.90±0.14	26.19±0.10	29.89±0.21	-	-	17.15±0.04	21.90±0.06
	400 MPa/3 min.	22.10±0.06	23.19±0.13	-	22.00±0.14	20.10±0.17	22.90±0.09	25.99±0.09	29.95±0.15	-	-	18.85±0.10	22.86±0.08
LAB-A	K	19.50±0.07	20.20±0.11	-	23.12±0.11	25.10±0.07	23.60±0.10	18.56±0.21	25.95±0.04	-	-	-	25.90±0.14

	300 MPa/3 min.	19.90±0.11	20.10±0.10	-	21.39±0.13	24.19±0.18	22.95±0.13	19.72±0.16	26.95±0.09	-	-	-	23.13±0.12
	300 MPa/5 min.	20.15±0.17	20.27±0.11	-	21.90±0.17	24.50±0.12	23.17±0.14	20.10±0.15	27.19±0.06	-	-	-	23.59±0.18
	400 MPa/1 min.	20.30±0.07	21.05±0.14	-	22.20±0.12	24.80±0.14	23.20±0.11	20.54±0.09	27.89±0.06	-	-	-	23.90±0.21
	400 MPa/3 min.	21.10±0.12	21.19±0.11	-	23.10±0.14	25.10±0.11	23.90±0.10	20.90±0.06	27.95±0.08	-	-	-	24.86±0.22
LAB-31	**K**	20.50±0.12	22.12±0.21	23.15±0.12	23.00±0.17	24.13±0.17	20.90±0.15	20.12±0.04	26.16±0.09	19.21±0.18	19.12±0.11	-	-
	300 MPa/3 min.	20.32±0.14	22.26±0.08	23.07±0.10	20.50±0.09	21.40±0.18	19.30±0.17	21.90±0.05	27.90±0.10	17.60±0.10	16.70±0.07	-	-
	300 MPa/5 min.	20.19±0.02	22.19±0.20	23.19±0.14	20.90±0.11	21.00±0.12	19.40±0.14	21.87±0.12	27.80±0.14	17.80±0.15	16.55±0.09	-	-
	400 MPa/1 min.	20.15±0.09	21.88±0.15	22.98±0.16	20.94±0.10	21.45±0.15	19.12±0.09	22.00±0.11	27.60±0.15	18.00±0.14	16.33±0.05	-	-
	400 MPa/3 min.	19.90±0.09	21.90±0.18	22.80±0.19	20.32±0.14	20.97±0.18	19.14±0.16	21.75±0.14	27.50±0.18	17.50±0.09	16.70±0.14	-	-
LAB-41	**K**	23.40±0.12	17.50±0.03	-	25.30±0.13	26.10±0.14	20.19±0.15	19.10±0.10	20.17±0.19	-	-	-	22.29±0.16
	300 MPa/3 min.	23.54±0.10	17.80±0.05	-	23.41±0.16	24.76±0.11	18.95±0.14	20.50±0.12	21.87±0.14	-	-	-	20.13±0.14
	300 MPa/5 min.	23.80±0.15	18.00±0.09	-	23.90±0.17	24.90±0.09	19.24±0.08	20.95±0.19	21.77±0.16	-	-	-	20.42±0.10
	400 MPa/1 min.	23.18±0.06	17.90±0.08	-	22.93±0.09	24.65±0.16	18.92±0.07	21.15±0.18	21.98±0.20	-	-	-	19.80±0.15
	400 MPa/3 min.	23.48±0.21	18.20±0.09	-	23.20±0.10	23.90±0.14	18.40±0.13	21.29±0.16	22.39±0.21	-	-	-	19.86±0.12
LAB-43	**K**	25.60±0.11	20.30±0.11	-	16.80±0.09	18.60±0.12	21.93±0.08	17.09±0.10	18.30±0.14	-	-	-	19.32±0.09
	300 MPa/3 min.	25.12±0.12	20.18±0.19	-	14.20±0.06	16.20±0.15	20.32±0.10	18.39±0.15	19.42±0.18	-	-	-	16.03±0.15
	300 MPa/5 min.	25.32±0.05	20.45±0.17	-	14.22±0.10	16.29±0.12	20.10±0.07	18.60±0.14	19.77±0.19	-	-	-	16.67±0.10
	400 MPa/1 min.	24.96±0.05	20.60±0.16	-	14.15±0.16	16.10±0.18	19.64±0.07	18.64±0.14	20.00±0.10	-	-	-	16.98±0.11
	400 MPa/3 min.	25.00±0.03	21.00±0.21	-	14.00±0.02	16.80±0.18	19.60±0.12	19.00±0.02	20.19±0.14	-	-	-	17.00±0.14
LAB-45	**K**	23.20±0.05	17.55±0.12	-	21.20±0.04	21.32±0.12	21.02±0.09	21.20±0.04	17.45±0.18	-	-	-	16.20±0.08
	300 MPa/3 min.	23.17±0.09	17.98±0.22	-	18.45±0.06	18.60±0.19	19.76±0.13	23.13±0.08	19.12±0.09	-	-	-	14.16±0.10
	300 MPa/5 min.	22.90±0.11	18.00±0.19	-	18.60±0.07	18.10±0.12	18.90±0.14	23.28±0.14	19.46±0.07	-	-	-	14.30±0.11
	400 MPa/1 min.	22.78±0.15	17.45±0.18	-	18.45±0.08	18.03±0.14	19.21±0.19	22.98±0.23	19.00±0.14	-	-	-	14.70±0.09
	400 MPa/3 min.	23.00±0.12	18.10±0.10	-	18.30±0.10	18.22±0.10	19.50±0.20	23.34±0.14	19.20±0.11	-	-	-	14.58±0.11
LAB-	**K**	22.30±0.06	-	-	21.20±0.14	20.72±0.20	22.30±0.11	-	-	-	-	19.43±0.13	-

300 MPa/3 min.	22.16±0.07	-	-	19.32±0.11	18.90±0.14	20.13±0.21	-	-	-	-	16.83±0.21	-
300 MPa/5 min.	21.98±0.08	-	-	19.23±0.12	18.14±0.12	20.32±0.16	-	-	-	-	17.10±0.22	-
400 MPa/1 min.	22.43±0.04	-	-	19.40±0.18	18.70±0.16	20.14±0.09	-	-	-	-	17.83±0.19	-
400 MPa/3 min.	22.10±0.13	-	-	19.52±0.21	18.26±0.17	20.32±0.07	-	-	-	-	17.90±0.26	-

***** - The reported C_T value is the mean of values obtained from all independent reactions, "-"- non analyzed; „K„ – control sample - unpressurized

Table S2 Results of relative gene expression analysis ($2^{-\Delta\Delta CT}$ values)

Strain	HPP conditions	Relative gene expression level								
		Stress response genes			Aminoglycosides resistance genes		Tetracycline resistance genes		Ampicillin resistance gene	Chloramphenicol resistance genes
		*dna*K	*gro*EL	*clp*L	*aph(3')-IIIa*	*aac(6')Ie-aph(2'')Ia*	*tet*M	*tet*W	*bla*Z	*cat*
LAB-1	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-
	300 MPa/3 min.	6.39[b]	8.96[b]	5.52[b]	0.42[b]	0.39[b]	9.19[b]	9.32[b]	-	-
	300 MPa/5 min.	6.22[b]	8.75[b]	5.84[b]	0.45[b]	0.35[b]	8.94[b]	9.06[b]	-	-
	400 MPa/1 min.	5.71[c]	8.45[b]	4.28[b]	0.48[b]	0.24[b]	7.04[b]	8.33[b]	-	-
	400 MPa/3 min.	5.06[c]	6.90[c]	5.29[b]	0.43[b]	0.42[b]	7.28[b]	7.38[c]	-	-
LAB-11	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	1.00[a]
	300 MPa/3 min.	9.60[b]	4.70[b]	3.55[b]	0.44[b]	0.39[b]	-	-	-	7.62[b]
	300 MPa/5 min.	8.03[b]	4.50[b]	2.68[b]	0.30[b]	0.42[b]	-	-	-	5.23[c]
	400 MPa/1 min.	7.72[c]	4.39[b]	3.46[b]	0.27[c]	0.34[b]	-	-	-	4.76[c]
	400 MPa/3 min.	7.36[c]	4.47[b]	2.81[b]	0.71[d]	0.43[b]	-	-	-	4.56[c]
LAB-14	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	1.00[a]
	300 MPa/3 min.	6.13[b]	5.07[b]	6.87[b]	0.40[b]	0.41[b]	-	-	-	12.66[b]
	300 MPa/5 min.	6.45[b]	4.32[b]	5.26[b]	0.26[c]	0.27[c]	-	-	-	10.28[b]
	400 MPa/1 min.	5.92[b]	4.83[b]	6.53[b]	0.28[c]	0.23[c]	-	-	-	7.64[c]
	400 MPa/3 min.	5.21[c]	3.11[c]	5.14[b]	0.35[b]	0.25[c]	-	-	-	5.56[c]
LAB-16	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	-
	300 MPa/3 min.	4.32[b]	5.39[b]	3.14[b]	0.36[b]	0.37[b]	-	-	-	-
	300 MPa/5 min.	3.68[b]	4.96[b]	3.81[b]	0.34[b]	0.35[b]	-	-	-	-
	400 MPa/1 min.	3.73[b]	3.86[c]	3.78[b]	0.39[b]	0.42[b]	-	-	-	-
	400 MPa/3 min.	3.43[c]	3.05[c]	3.20[b]	0.43[b]	0.39[b]	-	-	-	-
LAB-26b	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	1.00[a]	1.00[a]
	300 MPa/3 min.	4.38[b]	3.03[b]	2.07[b]	0.42[b]	0.47[b]	-	-	6.96[b]	8.98[b]
	300 MPa/5 min.	3.65[b]	2.48[b]	2.11[b]	0.36[b]	0.44[b]	-	-	5.10[c]	7.78[b]
	400 MPa/1 min.	3.29[b]	2.38[b]	2.30[b]	0.46[b]	0.47[b]	-	-	4.76[c]	6.96[c]
	400 MPa/3 min.	3.07[c]	2.14[c]	2.46[b]	0.39[b]	0.49[b]	-	-	2.55[d]	6.23[c]
LAB-31	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-
	300 MPa/3 min.	4.99[b]	5.86[b]	2.68[b]	0.32[b]	0.33[b]	2.89[b]	5.06[b]	-	-
	300 MPa/5 min.	3.46[b]	5.06[b]	2.28[b]	0.31[b]	0.33[b]	2.73[b]	6.10[b]	-	-
	400 MPa/1 min.	3.27[b]	5.03[b]	2.69[b]	0.23[b]	0.31[b]	2.06[b]	6.15[b]	-	-
	400 MPa/3 min.	4.23[c]	5.90[b]	2.23[b]	0.28[b]	0.34[b]	2.57[b]	4.20[c]	-	-
LAB-41	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	1.00[a]
	300 MPa/3 min.	4.08[b]	2.79[b]	2.60[b]	0.47[b]	0.38[b]	-	-	-	4.91[b]
	300 MPa/5 min.	4.44[b]	3.03[b]	2.55[b]	0.39[b]	0.47[b]	-	-	-	4.82[b]
	400 MPa/1 min.	4.53[b]	2.35[b]	2.07[b]	0.32[b]	0.38[b]	-	-	-	4.82[b]
	400 MPa/3 min.	3.48[c]	4.86[c]	3.66[b]	0.36[b]	0.35[b]	-	-	-	5.69[b]
LAB-43	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	1.00[a]
	300 MPa/3 min.	4.35[b]	3.78[b]	2.19[b]	0.37[b]	0.42[b]	-	-	-	7.01[b]
	300 MPa/5 min.	4.93[b]	4.08[b]	2.93[b]	0.39[b]	0.40[b]	-	-	-	5.17[c]
	400 MPa/1 min.	4.04[b]	3.63[b]	3.14[b]	0.42[b]	0.38[b]	-	-	-	3.25[d]
	400 MPa/3 min.	4.59[b]	2.30[c]	3.32[b]	0.43[b]	0.44[b]	-	-	-	3.29[d]

LAB-45	**K**	1.00[a]	1.00[a]	1.00[a]	1.00[a]	1.00[a]	-	-	-	1.00[a]
	300 MPa/3 min.	6.59[b]	6.45[b]	2.35[b]	0.35[b]	0.42[b]	-	-	-	4.03[b]
	300 MPa/5 min.	6.50[b]	7.57[b]	3.53[b]	0.32[b]	0.34[b]	-	-	-	3.03[b]
	400 MPa/1 min.	5.02[c]	7.31[b]	2.62[b]	0.27[b]	0.32[b]	-	-	-	2.11[c]
	400 MPa/3 min.	4.93[c]	7.46[b]	2.50[b]	0.33[b]	0.43[b]	-	-	-	2.68[c]
LAB-63	**K**	1.00[a]	1.00[a]	1.00[a]	-	-	-	-	1.00[a]	-
	300 MPa/3 min.	3.34[b]	4.79[b]	4.08[b]	-	-	-	-	5.50[b]	-
	300 MPa/5 min.	3.14[b]	4.44[b]	4.89[b]	-	-	-	-	4.03[c]	-
	400 MPa/1 min.	3.81[b]	4.79[b]	3.16[b]	-	-	-	-	3.32[c]	-
	400 MPa/3 min.	2.79[c]	3.20[c]	3.43[b]	-	-	-	-	2.51[d]	-

a, b, c, d - values in columns within each attribute with different superscripts are different ($P < 0.05$), „-„ – non-analyzed

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High-pressure processing effect on conjugal antibiotic resistance genes transfer *in vitro* and in the food matrix among strains from starter cultures

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ABSTRACT

This study analyzed the effect of high-pressure processing (HPP) on the frequency of conjugal gene transfer of antibiotic resistance genes among strains obtained from starter cultures. Gene transfer ability was analyzed *in vitro* and *in situ* in the food matrix. It was found that the transfer of aminoglycoside resistance genes did not occur after high-pressure treatment, either *in vitro* or *in situ*. After exposure to HPP, the transfer frequencies of tetracycline, ampicillin and chloramphenicol resistance genes increased significantly compared to the control sample, both *in vitro* and *in situ*. The frequency of resistance genes transfer in the food matrix in the pressurized samples did not differ significantly from the *in vitro* transfer rate. Minimum Inhibitory Concentrations (MICs) for these antibiotics determined for transconjugants were lower or equal to MICs determined for the donors. No significant differences were observed between the MIC values determined for the transconjugants obtained *in vitro* and *in situ*. The results suggest that HPP may contribute to the spread of antibiotic resistance. This points to the need to verify starter cultures strains for their antibiotic resistance and pressurization parameters to avoid spreading antibiotic resistance genes.

1. Introduction

Starter cultures play a key role in many areas of the food industry, mainly in the dairy and meat industries. These are selected preparations of bacterial strains, single or multi-species, responsible for the most important biochemical changes (Bintsis, 2018; Zarzecka et al., 2022a). The microorganisms included in the starter cultures, mainly lactic acid bacteria (LAB), are introduced into food in large numbers of the order of up to 10^7–10^9 CFU/g, so it is important to ensure that they do not pose a risk to consumers, *e.g.* by carrying antibiotic resistance genes. Although EU guidelines indicate that food-added strains should not be carriers of acquired resistance to antibiotics, there are reports of their detection in these strains (Laulund et al., 2017; Zarzecka et al., 2020).

Currently, antibiotic resistance is one of the greatest challenges facing public health. According to the World Health Organization, the increasing occurrence of antibiotic resistance is a serious threat and is becoming one of the most serious public health problems. The WHO also warns of the lack of new antibiotics to fight the growing resistance. Most of the available antibiotics are modifications of existing ones, which in this case are only *ad hoc* solutions. Without urgent action, we are heading towards a post-antibiotic era where common infections and minor injuries will become fatal. Identifying and cutting the pathways for spreading resistance should become one of the priority tasks. It is known that one of the resistance-spreading routes is the food chain. Microorganisms included in the starter cultures are generally considered as safe, but recent studies show that they might be carriers of antibiotic resistance genes and may also be involved in their horizontal spread (Dušková et al., 2020; Nunziata et al., 2022; Ojha et al., 2021; Rozman et al., 2020; Zarzecka et al., 2022b). The efficiency and frequency of gene transfer are influenced by many factors, in particular the appropriate cell density and physiological state of bacterial cells that might be influenced by many physicochemical factors (James et al., 2021; Lerner et al., 2017). Li and Zhang (2022) showed that the frequency of conjugal gene transfer might also be influenced by environmental conditions. In the case of food-related microorganisms, the most important factors are related to the production and/or storage of food (Wang et al., 2022). Currently, limited data is available on the occurrence of conjugal gene transfer *in situ* among food-related microorganisms and how food-related conditions affect antibiotic resistance transfer during the fermentation process (Gazzola et al., 2012).

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One of the food preservation techniques, known for many years, but recently more often used in the food industry, is high-pressure processing (HPP), which involves the application of pressure from 100 MPa to 600 MPa for 30 s to several minutes (Abera and Giorgis, 2017; Huang et al., 2017; Yang et al., 2021). This method is able to improve food quality and increase food safety and its shelf-life by the inactivation of microorganisms (Nabi et al., 2021). HPP has advantages over thermal food preservation methods from the point of view of sensory and nutritional food quality. The possibility of using high-pressure processing in a hurdle technology to reduce food additives and salt amounts is also indicated (Pottier et al., 2017). The application of HPP in the food industry has become more popular in recent years and is now applied in various food industries mainly in dairy, meat, juice, and jam production (Tsevdou et al., 2019; Vieira et al., 2020). If HPP is used to preserve food obtained with starter cultures, the process parameters should be selected in a way that prevents the complete elimination of bacterial cells. However, it has been proven that cells that survive the pressure process may show physiological changes, and the expression of various genes may also change. It has also been suggested that pressurized cells may show changes in their ability to transfer resistance genes (Daryaei et al., 2008; Nuñez et al., 2020).

Thus, the aim of this study was to determine the effect of high-pressure processing on the changes in the frequency of antibiotic resistance genes transfer among strains derived from commercial starter cultures.

2. Materials and methods

2.1. Selection of donor and recipient strains

Donor strains ($n = 5$) were received from the collection of the Department of Industrial and Food Microbiology described previously (Zarzecka et al., 2022b). These strains were obtained from commercial starter cultures and were found to carry plasmid-located genes encoding resistance to aminoglycosides: *aac(6′)-Ie-aph(2″)-Ia* and *aph(3′)-IIIa*; tetracyclines: *tet*M, chloramphenicol: *cat* and β-lactams: *bla*Z. Three of the strains belong to the former *Lactobacillus* genus (*Lactocaseibacillus paracasei*, *Latilactobacillus curvatus* and *Lactobacillus sp.* $n = 1$ each) and two to the *Lactococcus* genus (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* $n = 1$ each). The strains' characteristics are summarized in Table 1. The genus names of bacteria have been updated in line with the new nomenclature (Zheng et al., 2020).

Enterococcus faecalis JH2–2 (LMG 19456) strain, free from plasmids, resistant to rifampicin (MIC >256 μg/mL) and fusidic acid (MIC >256 μg/mL) was the standard recipient in the experiment. Recipient strain is susceptible for ampicillin (MIC 0.038 μg/mL), tetracycline (MIC 0.125 μg/mL), chloramphenicol (MIC 4 μg/mL), gentamicin (MIC 0.5 μg/mL) and kanamycin (MIC 2 μg/mL).

2.2. High pressure treatment

Conditions of high-pressure processing (pressure values and pressurization time) were chosen on the basis on the preliminary study (Data not shown) to avoid the inactivation of the bacterial cells and to ensure a sufficient number of viable cells.

High-pressure treatment was applied to 10 mL of strains' cultures from the stationary growth phase. The strains were cultured in the Brain Heart Infusion (BHI) broth (Merck, Germany). The pressurization conditions were selected for the analysis as follows: 300 MPa/3 min, 300 MPa/5 min, 400 MPa/1 min and 400 MPa/3 min. Selected pressurization time did not include the come-up and come-down time. High pressure treatments were carried out in glycol-water solution (1:1, *v*/v) in the high-pressure single-chamber U4040 (Unipress, Warsaw, Poland) as described previously (Zarzecka et al., 2022c). Temperature of the processes was kept at 20 ± 3 °C, with the pressure rise rate 300 MPa/min, and the pressure relief time 2–4 s. For each donor-recipient pair, the assays were performed in three independent processes.

Both for *in vitro* and *in situ* matings after the stress treatment was completed, the samples were subjected to the next stages of the study. Unpressurized samples were used as a control.

2.3. Conjugal transfer of resistance genes

2.3.1. In vitro filter mating

The experiment was conducted by the membrane filter method described by Nawaz et al. (2011). For this purpose, 1 mL of each of the tested (pressurized) and control (non-pressurized) donor and recipient cultures was added to 5 mL of sterile BHI medium and incubated for 4 h at 37 °C until obtaining the midexponential growth phase. Then the 1 mL of donor culture was mixed with the 1 mL recipient culture and filtered through a sterile cellulose membrane filter with a pore diameter of 0.45 μm. Then the filters were rinsed with 2 mL of sterile peptone physiological saline solution (PPS) (Merck, Germany). The filters were placed on BHI-agar medium and incubated aerobically overnight at 37 °C. After incubation, colonies grown on the filter were resuspended in 2 mL PPS, series of tenfold dilutions were made from the obtained suspension and plated on three types of selective media. Transconjugants were grown on T-BHI medium with the addition of fusidic acid (100 μg/mL), rifampicin (50 μg/mL) and one of the following antibiotics: tetracycline (10 μg/mL), chloramphenicol (10 μg/mL), gentamicin (1 μg/mL), kanamycin (1 μg/mL) or ampicillin (1 μg/mL). Donors were grown on TD-BHI medium with the addition one of the following antibiotics: tetracycline (10 μg/mL), chloramphenicol (10 μg/mL), gentamicin (1 μg/mL), kanamycin (1 μg/mL) or ampicillin (1 μg/mL). Recipients were grown on B–BHI medium with the addition of fusidic acid (100 μg/mL) and rifampicin (50 μg/mL). To exclude the possibility of transformation DNase I (ThermoFisher Scientific, Waltham, Massachusetts, USA) was

Table 1
Characteristic of strains analyzed in the study.

Strain	Identification	Origin	Antibiotic MICs [μg/mL]									Antibiotic resistance genes
			AMP	CN	C	TE	E	DA	K	S	VA	
LAB-16	*Lactococcus lactis ssp. lactis*	Cheese starter culture	0.19	1	8	32	0.125	0.25	8	0.125	0.094	***tetM*, *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *aac(6′)-Ia***
LAB-26b	*Lactococcus lactis ssp. cremoris*	Cheese starter culture	0.25	4	12	32	0.25	0.75	12	0.25	0.19	***tetM***, *tetW*, *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, ***blaZ***, *cat*
LAB-41	*Lactocaseibacillus paracasei*	Cheese starter culture	1	0.38	32	32	0.125	0.023	4	0.125	–	*tetW*, *aph(3′)-IIIa*, ***aac(6′)-Ie-aph(2″)-Ia***, *aac(6′)-Ia*, ***cat***
LAB-43	*Latilactobacillus curvatus*	Meat starter culture	0.125	0.5	16	64	0.125	0.38	6	0.125	–	*tetM*, *tetO*, ***aph(3′)-IIIa***, *aac(6′)-Ie-aph(2″)-Ia*, ***cat***
LAB-63	*Lactobacillus* sp.	Yoghurt starter culture	3	0.5	8	48	0.19	0.047	16	0.19	–	***blaZ***

Abbreviations: MICs – Minimum Inhibitory Concentrations, AMP – ampicillin, CN – gentamicin, C – chloramphenicol, TE – tetracycline, E – erythromycin, DA – klindamycin, K – kanamycin, S – streptomycin, VA – vancomycin, "-" – non-analyzed; The names of plasmid-encoded genes, the transfer of which were analyzed in the study, are shown in bold.

added to the mating plates at a concentration of 100 μg/mL. Incubation was carried out at 37 °C for 24–48 h. The transfer frequency was expressed as the ratio of the number of transconjugants to the number of recipients.

2.3.2. In situ mating in the food matrix

In order to assess the possibility of *in situ* conjugal transfer in food matrix, a model of yoghurt was prepared as described by Toomey et al. (2009). Skim-milk powder (4 % *w/v*) was mixed with 10 mL of pasteurized milk, heated at 63 °C for 30 min in a preheated water bath and cooled to 42 °C. Donor and recipient strains were cultured on BHI broth medium (Merck, Germany) for 48 h in 30 °C in anaerobic conditions. Then 1 mL of donor and recipient culture were added to milk and incubated at 42 °C until the pH 4.3 was obtained. The donor and recipient cultures were then mixed together 1:1 (*v/v*) in sterile LDPE bottle to final volume of 10 mL and the test samples were subjected to high-pressure treatment. The tested and control samples were then incubated at 4 °C for 48 h. After incubation, serial tenfold dilutions of fermented milk samples were plated onto selective media as described above. Plates were incubated aerobically for 24–48 h at 37 °C and the transfer frequency was expressed as described above.

2.3.3. Confirming the presence of antibiotic resistance in transconjugants

Obtained transconjugants were screened for their antibiotic resistance. The Minimum Inhibitory Concentrations (MICs) for following antibiotics: tetracycline, chloramphenicol, kanamycin, gentamicin and ampicillin were determined by applying gradient strips (Liofilchem, Italy) on an inoculated Mueller-Hinton agar plate (Merck, Germany). The MIC values were read after 18 h of aerobic incubation at 30 °C.

Then, genomic DNA was isolated from the transconjugants using the Genomic Mini kit (A&A Biotechnology, Poland) and plasmid DNA was isolated using Plasmid Mini kit (A&A Biotechnology, Poland) according to manufacturer's instructions. Then PCR reactions were performed using primers and conditions listed in Table 2 to confirm gene transfer (Goksel et al., 2016; Kaase et al., 2008; Padmasini et al., 2014; Roberts, 1994). PCR reactions were provided using genomic DNA and plasmid DNA. PCR reactions were conducted in a thermal cycler Mastercycler nexus GX2 (Eppendorf, Germany). The PCR products were separated by electrophoresis on 1.5 % agarose gels in 1 × Tris-borate-EDTA (TBE) buffer stained by 0.5 μg/mL of Midori green (ABO, Poland) 100 V for 6 min and then at 80 V for 90 min and visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene, UK).

2.3.4. Genotyping of transconjugants

The fingerprints of transconjugants found to have acquired resistance genes were obtained by Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR) according to Pusparini et al. (2018). The PCR reactions were performed in a total volume of 25 μL containing 12.5 μL of DreamTaq Green PCR Master Mix (2×) (ThermoFisher Scientific, Waltham, Massachusetts, USA), 1.25 μL of 10 pmol primers (Table 2) and 1.5 μL of DNA template filled up with ddH_2O. Cycling conditions were as follows: initial denaturation at 95 °C/7 min, followed by 30 cycles of denaturation at 94 °C/1 min, annealing at 52 °C/1 min and extension at 65 °C/8 min and a final elongation at 65 °C/15 min. The PCR products were separated by electrophoresis on 1.5 % agarose gels in 1 × Tris-borate-EDTA (TBE) buffer stained by 0.5 μg/mL of Midori green (ABO, Poland) at 150 V for 150 min and visualized as described above.

Then, fingerprints were compared to the fingerprints of recipient strain for confirmation purposes by cluster analysis with BioNumerics ver. 7.6.3. software (Applied Maths/bioMérieux, Sint-Martens-Latem, Belgium). The similarity distances between ERIC-PCR profiles were calculated using the Dice coefficient, and the dendrogram was based on the unweighted pair group method with arithmetic mean (UPGMA).

Moreover, all transconjugant strains were identified to the species level phenotypically using MALDI-TOF technique and genotypically using PCR method as described previously (Chajęcka-Wierzchowska et al., 2020) for confirmation purposes.

2.4. Statistical analysis

Statistical analyses were performed using the one-way ANOVA. For the analyses, $p \leq 0.05$ was considered significant. The statistical analyses and visualization were performed using Prism 9.3.1. (Prism, GraphPad Software, San Diego).

3. Results

3.1. Changes in resistance genes transfer frequency

This study analyzed changes in frequency of antibiotic resistance genes transfer after exposure to high-pressure treatment among strains from commercial starter cultures. As a result of the frequency of resistance gene transfer analysis before and after exposure to high-pressure processing, it was found that the transfer of genes *aph(3′)-IIIa* and *aac (6′)-Ie-aph(2″)-Ia* encoding aminoglycoside resistance did not occur

Table 2
Primers used in the study.

Species/gene	Primer	Primer sequence 5′-3′	Amplicon size (bp)	Annealing temperature	Reference
Tetracyclines resistance gene					
tetM	tetM-F	GTGGACAAAGGTACAACGAG	406	62 °C	Roberts, 1994
	tetM-R	CGGTAAAGTTCGTCACACAC			
β-Lactams resistance gene					
blaZ	blaZ-F	CAAAGATGATATAGTTGCTTATTCTC	400	50 °C	Kaase et al., 2008
	blaZ-R	TGCTTGACCACTTTTATCAGC			
Aminoglycosides resistance genes					
aac(6′)-Ie-aph(2″)-Ia	Gent1-F	CAGGAATTTATCGAAAATGGTAGAAAAG	369	58 °C	Padmasini et al., 2014
	Gent1-R	CACAATCGACTAAAGAGTACCAAT			
aph(3″)-IIIa	Amino1-F	GGCTAAAATGAGAATATCACCGG	523		
	Amino1-R	CTTTAAAAAATCATACAGCTCGCG			
Fingerprinting					
cat	cat-F	CATATCAAATGAACTTTAATA	718	52 °C	Goksel et al., 2016
	cat-R	CGTTTTGTGAAGTAGTACACT			
ERIC-PCR	ERIC1	ATGTAAGCTCCTGGGGATTCA		52 °C	Pusparini et al., 2018
	ERIC2	AAGTAAGTGACTGGGGTGAGC			

after any of the high-pressure treatment variant, either *in vitro* or *in situ*. Transfer of these genes was observed only in control, unpressurized samples. The *aph(3')-IIIa* gene transfer rate in the control samples ranged from 5.37×10^{-6} to 9.05×10^{-5} and the *aac(6')-Ie-aph(2'')-Ia* gene transfer in the control samples ranged from 4.67×10^{-6} to 8.02×10^{-6}. It was also observed that the *in situ* transfer rate was not significantly different than the *in vitro* transfer rate.

Regarding the tetracycline resistance gene (*tet*M), transfer frequency in the control samples ranged from 5.43×10^{-6} to 8.91×10^{-5}. The frequency of ampicillin resistance gene (*bla*Z) transfer in control samples ranged from 1.74×10^{-6} to 3.24×10^{-6} while the frequency of chloramphenicol resistance gene (*cat*) transfer in the control samples ranged from 3.23×10^{-6} to 5.02×10^{-6}. The *in vitro* transfer rates for these antibiotics were not significantly different than transfer rate in the food matrix. After exposure to high pressures, the transfer frequencies, both *in vitro* and *in situ* changed significantly compared to the control sample. Transfer frequencies of *bla*Z and *cat* genes encoding resistance to ampicillin and chloramphenicol increased significantly after high-pressure treatment *in vitro* as well as *in situ*. However frequency of the tetracycline resistance gene *tet*M decreased after exposure to analyzed HPP variants *in vitro* and increased in the food matrix (Table 3).

3.2. Confirming the presence of antibiotic resistance in transconjugants

After analysis of resistance genes transfer frequency MIC values for tested antibiotics were determined among obtained transconjugants to confirm gene transfer. MIC values noticed for the transconjugants ranged as follows: from 0.38 to 0.75 μg/mL for gentamicin, from 4 to 6 μg/mL for kanamycin, from 24 to 32 μg/mL for tetracycline, from 12 to 32 μg/mL for chloramphenicol and from 0.125 to 0.25 μg/mL for ampicillin. These MIC values were lower or equal to MICs determined for the donors. What is noteworthy, MIC values for ampicillin, tetracycline and chloramphenicol among transconjugants were higher than in recipient strain, while MIC values for gentamicin and kanamycin were not significantly different than in recipient strains. No significant differences were observed between the MIC values determined for the transconjugants obtained *in vitro* and *in situ*. Occurrence of antibiotic resistance genes, the transfer of which was analyzed, were found in the genomic as well as plasmid DNA of transconjugants (Table 3). These results show that transconjugants carried antibiotic resistance genes which expressed. All of the transconjugant strains were also identified as *E. faecalis* both with MALDI-TOF technique and PCR method. Fingerprinting using ERIC-PCR showed similar recipient and transconjugants profiles (Fig. 1). The three exceptions were noticed (TC – LAB26b (*in situ*) 400 MPa/3 min, TC – LAB26b (*in situ*) 300 MPa/5 min and TC – LAB26b (*in vitro*) 300 MPa/5 min), but these strains were identified as *E. faecalis* both phenotypically and genotypically, what confirms that they are transconjugant strains. Such discrepancies might result from the limitations of genotyping method.

4. Discussion

For several decades, the food chain was considered one of the routes of transmission of antibiotic resistance in the environment. Currently, it is known that conjugal gene transfer may take place in various matrices. Moreover, while gene transfer has been demonstrated in a stable laboratory environment, still little is known about gene transfer in natural niches, like food matrix (Fletcher, 2015; Rossi et al., 2014; Thumu and Halami, 2018). That is why it is so important to understand the mechanisms determining the spread of genes in other conditions to identify any modifiable interactions to reduce or interrupt the spread of resistance in the environment. Since LAB are being added to food as starter cultures, studies of conjugal transfer among bacteria in the food matrix will lead to the understanding of genetic exchange mechanisms between bacteria in natural environments. Knowledge about the ability of LAB to transfer antibiotic resistance in food matrices is still limited. One example might be a study by Leroy et al. (2019), who reported the transfer of plasmids carrying antibiotic resistance during sausage fermentation, between *Lactobacillus* strains.

Food preservation techniques are considered environmental changes and might be stress factors for food-related microorganisms, such as lactic acid bacteria used in the food industry as starter cultures (Bucka-Kolendo and Sokołowska, 2017). It is noteworthy that microbial stress adaptation abilities depend on their individual characteristics and can also originate from cross-protection systems. Under adverse conditions, LAB activate response mechanisms, which may affect their features (Bucka-Kolendo et al., 2020).

One of the most promising food preservation techniques is high-pressure processing (HPP), which is also used to preserve fermented food (Jadhav et al., 2021; Nabi et al., 2021). There are many studies focused on the response to other physiochemical stressors, but there are a limited number of studies regarding the molecular mechanisms of the response to high-pressure treatment (Rossnerova et al., 2020).

To the best of the authors' knowledge, there are currently no studies on the impact of food-related stresses, such as HPP, on the possibility of the horizontal transfer of antibiotic resistance genes with non-pathogenic strains intentionally introduced into food. However, literature data indicate that in the food chain antibiotic resistance genes might be acquired by bacteria challenged with many various food-related stresses (Lopez et al., 2020; Obolski and Hadany, 2012; Oniciuc et al., 2019). Skandalis et al. (2021) also report that bacterial cells affected by stress factors may still participate in the horizontal gene transfer. Our previous study showed that the frequency of aminoglycoside and tetracycline resistance gene transfer changed after exposure to food-related stresses (Zarzecka et al., 2022d). These results are only partially in line with the results obtained in the current study. Results of aminoglycoside resistance genes transfer are the same in the both studies. In the current study tetracycline resistance gene transfer decreased after exposure to high pressure, what is in the opposite to the results obtained previously. This suggests that changes in the ability to transfer genes may result from individual strain characteristics and be a strain-specific feature. According to the literature, food-related stresses, such as those encountered during food preservation, may affect the rates of exchanging genetic material and the number of plasmids copies in bacterial cell. For example, exposure to heat, low pH or starvation has been reported to influence the inter- and intraspecies ability to spread resistance genes. The results obtained by Mc Mahon et al. (2007) indicate that environmental stresses, such as low pH, high osmolarity, low or high temperature or combinations of such factors are able to increase the rates of horizontal transfer of antibiotic resistance plasmids at a level comparable to the results obtained in this study. This might suggest that many food preservation techniques may result in the facilitated transfer of plasmids carrying antibiotic resistance genes among bacteria in food.

Similar studies have been conducted with regard to environmental strains and have shown that the selection pressure associated with environmental stress results in an increased frequency of gene transfer in strains that survived exposure to stress factors. The acquisition of antibiotic resistance genes seems to be a response to selection pressure. Exposure to stress factors in the environment exerts pressure, inducing a stress response and promoting horizontal gene transfer, which induces the spreading of resistance genes (Bombaywala et al., 2021; Chen et al., 2019; Lin et al., 2019).

Currently, the knowledge of the influence of stress factors, especially those related to food processing, on the possibility of horizontal gene transfer by conjugation is limited. For this reason, in the author's opinion, the present study fills a significant knowledge gap and could open the door to deepening research in this area, helping to understand how environmental conditions acting on microbes can influence the spread of resistance in the environment.

Table 3
Resistance genes transfer before and after exposure to high pressure processing *in vitro* and *in situ*.

		Genotype of donor	MIC value of D [μg/mL]					Transfer rate TC/R[a] ± SD					MIC value of TC [μg/mL]					Transferred resistance genes
			C	CN	K	TE	AMP	C	CN	K	TE	AMP	C	CN	K	TE	AMP	
In vitro filter mating																		
LAB-16	Control sample	***tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia,*** *aac(6′)-Ia*	n.a.	1	8	32	n.a.	n.a.	$6.32 \times 10^{-6} \pm 0.10^{a}$	$6.13 \times 10^{-6} \pm 0.070^{a}$	$5.43 \times 10^{-6} \pm 0.11^{a}$	n.a.	n.a.	0.75	6	24	n.a.	*tetM, aph(3′)-IIIa, aac (6′)-Ie-aph(2″)-Ia*
	300 MPa/ 3 min							n.a.	n.d.	n.d.	$3.61 \times 10^{-6} \pm 0.11^{b}$	n.a.	n.a.	n.a.	n.a.	24	n.a.	*tetM*
	300 MPa/ 5 min							n.a.	n.d.	n.d.	$3.21 \times 10^{-6} \pm 0.04^{b}$	n.a.	n.a.	n.a.	n.a.	32	n.a.	*tetM*
	400 MPa/ 1 min							n.a.	n.d.	n.d.	$3.89 \times 10^{-6} \pm 0.16^{b}$	n.a.	n.a.	n.a.	n.a.	24	n.a.	*tetM*
	400 MPa/ 3 min							n.a.	n.d.	n.d.	$2.61 \times 10^{-6} \pm 0.07^{b}$	n.a.	n.a.	n.a.	n.a.	32	n.a.	*tetM*
LAB-26b	Control sample	***tetM,*** *tetW, aph(3′)-IIIa, aac (6′)-Ie-aph(2″)-Ia,* ***blaZ,*** ***cat***	n.a.	n.a.	n.a.	32	0.25	n.a.	n.a.	n.a.	$8.91 \times 10^{-5} \pm 0.16^{a}$	$3.24 \times 10^{-6} \pm 0.09^{a}$	n.a.	n.a.	n.a.	24	0.25	*tetM, blaZ*
	300 MPa/ 3 min							n.a	n.a.	n.a.	$5.03 \times 10^{-5} \pm 0.07^{b}$	$5.43 \times 10^{-6} \pm 0.20^{b}$	n.a.	n.a.	n.a.	32	0.125	*tetM, blaZ*
	300 MPa/ 5 min							n.a.	n.a.	n.a.	$5.11 \times 10^{-5} \pm 0.10^{b}$	$5.31 \times 10^{-6} \pm 0.07^{b}$	n.a.	n.a.	n.a.	24	0.25	*tetM, blaZ*
	400 MPa/ 1 min							n.a.	n.a.	n.a.	$3.93 \times 10^{-5} \pm 0.11^{b}$	$5.49 \times 10^{-6} \pm 0.06^{b}$	n.a.	n.a.	n.a.	24	0.25	*tetM, blaZ*
	400 MPa/ 3 min							n.a.	n.a.	n.a.	$4.08 \times 10^{-5} \pm 0.03^{b}$	$5.16 \times 10^{-6} \pm 0.09^{b}$	n.a.	n.a.	n.a.	32	0.125	*tetM, blaZ*
LAB-41	Control sample	*tetW, aph(3′)-IIIa,* ***aac(6′)-Ie-aph(2″)-Ia,*** *aac(6′)-Ia,* ***cat***	32	0.38	n.a.	n.a.	n.a.	$3.32 \times 10^{-6} \pm 0.07^{a}$	$8.02 \times 10^{-6} \pm 0.07^{a}$	n.a.	n.a.	n.a.	24	0.38	n.a.	n.a.	n.a.	*cat, aac(6′)-Ie-aph(2″)-Ia*
	300 MPa/ 3 min							$8.12 \times 10^{-6} \pm 0.09^{b}$	n.d.	n.a.	n.a.	n.a.	24	n.a.	n.a.	n.a.	n.a.	*cat*
	300 MPa/ 5 min							$8.72 \times 10^{-6} \pm 0.16^{b}$	n.d.	n.a.	n.a.	n.a.	32	n.a.	n.a.	n.a.	n.a.	*cat*
	400 MPa/ 1 min							$8.56 \times 10^{-6} \pm 0.06^{b}$	n.d.	n.a.	n.a.	n.a.	32	n.a.	n.a.	n.a.	n.a.	*cat*
	400 MPa/ 3 min							$8.83 \times 10^{-6} \pm 0.09^{b}$	n.d.	n.a.	n.a.	n.a.	24	n.a.	n.a.	n.a.	n.a.	*cat*
LAB-43	Control sample	*tetM, tetO,* ***aph(3′)-IIIa,*** *aac (6′)-Ie-aph(2″)-Ia,* ***cat***	16	n.a.	6	n.a.	n.a.	$3.23 \times 10^{-6} \pm 0.03^{a}$	n.a.	$9.62 \times 10^{-5} \pm 0.09^{a}$	n.a.	n.a.	16	n.a.	4	n.a.	n.a.	*cat, aph(3′)-IIIa*
	300 MPa/ 3 min							$8.42 \times 10^{-6} \pm 0.10^{b}$	n.a.	n.d.	n.a.	n.a.	16	n.a.	n.a.	n.a.	n.a.	*cat*
	300 MPa/ 5 min							$7.75 \times 10^{-6} \pm 0.06^{b}$	n.a.	n.d.	n.a.	n.a.	12	n.a.	n.a.	n.a.	n.a.	*cat*

(continued on next page)

Table 3 (*continued*)

		Genotype of donor		MIC value of D [μg/mL]					Transfer rate TC/R^x ± SD				MIC value of TC [μg/mL]					Transfered resistance genes
				C	CN	K	TE	AMP	C	CN	K	TE	AMP	C	CN	K	TE	AMP	
	400 MPa/ 1 min								$7.56 \times 10^{-6} \pm 0.16^{b}$	n.a.	n.d.	n.a.	n.a.	12	n.a.	n. a.	n. a.	n.a.	*cat*
	400 MPa/ 3 min								$7.83 \times 10^{-6} \pm 0.07^{b}$	n.a.	n.d.	n.a.	n.a.	16	n.a.	n. a.	n. a.	n.a.	*cat*
LAB-63	Control sample	***blaZ***		n. a.	n.a.	n. a.	n. a.	3	n.a.	n.a.	n.a.	n.a.	$2.14 \times 10^{-6} \pm 0.05^{a}$	n. a.	n.a.	n. a.	n. a.	2	*blaZ*
	300 MPa/ 3 min								n.a.	n.a.	n.a.	n.a.	$4.43 \times 10^{-6} \pm 0.08^{b}$	n. a.	n.a.	n. a.	n. a.	3	*blaZ*
	300 MPa/ 5 min								n.a.	n.a.	n.a.	n.a.	$4.37 \times 10^{-6} \pm 0.06^{b}$	n. a.	n.a.	n. a.	n. a.	2	*blaZ*
	400 MPa/ 1 min								n.a.	n.a.	n.a.	n.a.	$4.43 \times 10^{-6} \pm 0.11^{b}$	n. a.	n.a.	n. a.	n. a.	2	*blaZ*
	400 MPa/ 3 min								n.a.	n.a.	n.a.	n.a.	$4.16 \times 10^{-6} \pm 0.04^{b}$	n. a.	n.a.	n. a.	n. a.	3	*blaZ*
In situ mating in the food matrix																			
LAB-16	Control sample	***tetM, aph(3′)-IIIa, aac(6′)-Ie-aph(2″)-Ia,*** *aac(6′)-Ia*	n. a.	1	8	32	n.a.	n.a.	$4.67 \times 10^{-6} \pm 0.06^{a}$	$5.37 \times 10^{-6} \pm 0.04^{a}$	$5.03 \times 10^{-6} \pm 0.08^{a}$	n.a.	n. a.	0.75	6	32	n.a.	*tetM, aph(3′)-IIIa, aac (6′)-Ie-aph(2″)-Ia*
	300 MPa/ 3 min								n.a.	n.d.	$1.91 \times 10^{-5} \pm 0.13^{b}$	n.a.	n. a.	n.a.	n. a.	24	n.a.	*tetM*
	300 MPa/ 5 min								n.a.	n.d.	$2.14 \times 10^{-5} \pm 0.08^{b}$	n.a.	n. a.	n.a.	n. a.	24	n.a.	*tetM*
	400 MPa/ 1 min								n.a.	n.d.	$2.24 \times 10^{-5} \pm 0.05^{b}$	n.a.	n. a.	n.a.	n. a.	32	n.a.	*tetM*
	400 MPa/ 3 min								n.a.	n.d.	$2.13 \times 10^{-5} \pm 0.04^{b}$	n.a.	n. a.	n.a.	n. a.	24	n.a.	*tetM*
LAB-26b	Control sample	***tetM****, tetW, aph(3′)-IIIa, aac (6′)-Ie-aph(2″)-Ia,* ***blaZ****, cat*	n. a.	n.a.	n. a.	32	0.25	n.a.	n.a.	n.a.	$8.31 \times 10^{-6} \pm 0.07^{a}$	2.98 × $10^{-6} \pm 0.04^{a}$	n. a.	n.a.	n. a.	32	0.25	*tetM, blaZ*
	300 MPa/ 3 min								n.a.	n.a.	$2.23 \times 10^{-5} \pm 0.04^{c}$	$5.87 \times 10^{-6} \pm 0.04^{b}$	n. a.	n.a.	n. a.	24	0.125	*tetM, blaZ*
	300 MPa/ 5 min								n.a.	n.a.	$2.41 \times 10^{-5} \pm 0.14^{c}$	$5.99 \times 10^{-6} \pm 0.02^{b}$	n. a.	n.a.	n. a.	32	0.125	*tetM, blaZ*
	400 MPa/ 1 min								n.a.	n.a.	$2.23 \times 10^{-5} \pm 0.03^{c}$	$5.79 \times 10^{-6} \pm 0.04^{b}$	n. a.	n.a.	n. a.	32	0.25	*tetM, blaZ*
	400 MPa/ 3 min								n.a.	n.a.	$2.23 \times 10^{-5} \pm 0.03^{c}$	$5.86 \times 10^{-6} \pm 0.13^{b}$	n. a.	n.a.	n. a.	24	0.25	*tetM, blaZ*
LAB-41	Control sample	*tetW, aph(3′)-IIIa,* ***aac(6′)-Ie-aph(2″)-Ia,*** *aac(6′)-Ia,* ***cat***	32	0.38		n. a.	n. a.	n.a.	$5.02 \times 10^{-6} \pm 0.06^{a}$	$7.22 \times 10^{-6} \pm 0.07^{a}$	n.a.	n.a.	n.a.	32	0.38	n. a.	n. a.	n.a.	*cat, aac(6′)-Ie-aph(2″)-Ia*

(*continued on next page*)

Table 3 (*continued*)

		Genotype of donor	MIC value of D [μg/mL]					Transfer rate TC/R* ± SD					MIC value of TC [μg/mL]					Transferred resistance genes
			C	CN	K	TE	AMP	C	CN	K	TE	AMP	C	CN	K	TE	AMP	
	300 MPa/ 3 min							$9.15 \times 10^{-6} \pm 0.04^{b}$	n.d.	n.a.	n.a.	n.a.	24	n.a.	n. a.	n. a.	n.a.	*cat*
	300 MPa/ 5 min							$9.42 \times 10^{-6} \pm 0.08^{b}$	n.d.	n.a.	n.a.	n.a.	32	n.a.	n. a.	n. a.	n.a.	*cat*
	400 MPa/ 1 min							$9.56 \times 10^{-6} \pm 0.05^{b}$	n.d.	n.a.	n.a.	n.a.	32	n.a.	n. a.	n. a.	n.a.	*cat*
	400 MPa/ 3 min							$9.63 \times 10^{-6} \pm 0.12^{b}$	n.d.	n.a.	n.a.	n.a.	24	n.a.	n. a.	n. a.	n.a.	*cat*
LAB-43	Control sample	*tetM*, *tetO*, ***aph(3′)-IIIa***, *aac (6′)-Ie-aph(2″)-Ia*, ***cat***	16	n.a.	6	n. a.	n.a.	$3.99 \times 10^{-6} \pm 0.07^{b}$	n.a.	$9.05 \times 10^{-5} \pm 0.07^{a}$	n.a.	n.a.	16	n.a.	4	n. a.	n.a.	*cat*, *aph(3′)-IIIa*
	300 MPa/ 3 min							$7.91 \times 10^{-6} \pm 0.05^{b}$	n.a.	n.d.	n.a.	n.a.	16	n.a.	n. a.	n. a.	n.a.	*cat*
	300 MPa/ 5 min							$8.03 \times 10^{-6} \pm 0.08^{b}$	n.a.	n.d.	n.a.	n.a.	12	n.a.	n. a.	n. a.	n.a.	*cat*
	400 MPa/ 1 min							$8.29 \times 10^{-6} \pm 0.02^{b}$	n.a.	n.d.	n.a.	n.a.	12	n.a.	n. a.	n. a.	n.a.	*cat*
	400 MPa/ 3 min							$8.33 \times 10^{-6} \pm 0.04^{b}$	n.a.	n.d.	n.a.	n.a.	16	n.a.	n. a.	n. a.	n.a.	*cat*
LAB-63	Control sample	***blaZ***	n. a.	n.a.	n. a.	n. a.	3	n.a.	n.a.	n.a.	n.a.	$1.74 \times 10^{-6} \pm 0.09^{a}$	n. a.	n.a.	n. a.	n. a.	3	*blaZ*
	300 MPa/ 3 min							n.a.	n.a.	n.a.	n.a.	$5.03 \times 10^{-6} \pm 0.05^{b}$	n. a.	n.a.	n. a.	n. a.	2	*blaZ*
	300 MPa/ 5 min							n.a.	n.a.	n.a.	n.a.	$5.31 \times 10^{-6} \pm 0.06^{b}$	n. a.	n.a.	n. a.	n. a.	2	*blaZ*
	400 MPa/ 1 min							n.a.	n.a.	n.a.	n.a.	$5.23 \times 10^{-6} \pm 0.09^{b}$	n. a.	n.a.	n. a.	n. a.	3	*blaZ*
	400 MPa/ 3 min							n.a.	n.a.	n.a.	n.a.	$5.17 \times 10^{-6} \pm 0.03^{b}$	n. a.	n.a.	n. a.	n. a.	3	*blaZ*

Abbreviations: C - chloramphenicol, CN – gentamicin, K – kanamycin, TE – tetracycline, AMP – ampicillin, TC – transconjugants, R – recipients, D – donors, "n.a." – not analyzed, "n.d." – non detected; The names of plasmid-encoded genes, the transfer of which were analyzed in the study, are shown in bold; a, b – values in columns within each attribute with different superscripts are different ($P < 0.05$); * - mean of three independent experiments; SD – standard deviation.

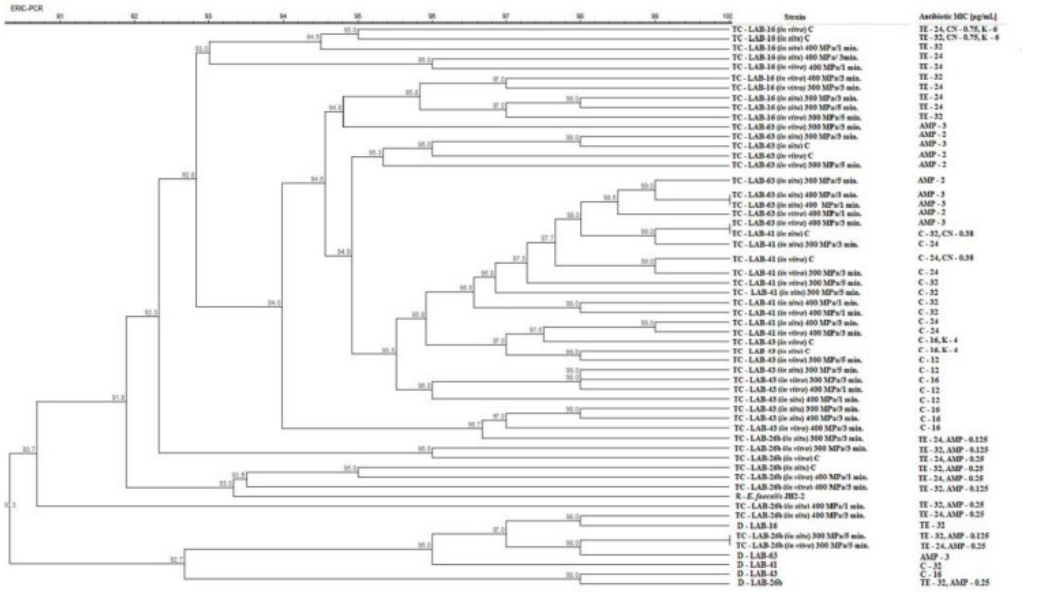


Fig. 1. Unweighted pair group method with the arithmetic mean (UPGMA) dendrogram derived from genotyping of *E. faecalis* JH2–2 and transconjugants obtained in resistance genes transfer.
Abbreviations: TC – tranconjugant, R – recipient, D – donor, TE – tetracycline, AMP – amplicillin, K – kanamycin, CN – gentamycin, C - chloramphenicol.

5. Conclusions

The results obtained in this study indicate that high-pressure processing may cause changes in the frequency of resistance gene transfer. These results confirm the necessity to analyze the strains used in the form of starter cultures for their abilities to spread antibiotic resistance as well as conditions of food preservation. There is a large knowledge gap in the field of the response of lactic acid bacteria to food-related stresses. Although antibiotic-resistant lactic acid bacteria do not pose a direct threat to consumers, food-related stresses may lead to the increasing transfer of resistance genes, contributing to the spread of resistance in the environment.

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CRediT authorship contribution statement

Urszula Zarzecka: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Anna Zadernowska:** Conceptualization, Writing - review & editing, Supervision. **Wioleta Chajęcka-Wierzchowska**: Writing - review & editing, Supervision. **Patryk Adamski**: Investigation, Data curation. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Authors declared no conflict of interests.

Data availability

Data will be made available on request.

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Zarzecka, U., Chajęcka-Wierzchowska, W., Zadernowska, A., 2022b. Microorganisms from starter and protective cultures - occurrence of antibiotic resistance and conjugal transfer of tet genes in vitro and during food fermentation. LWTFood Sci. Technol. 153, 112490 https://doi.org/10.1016/J.LWT.2021.112490.

Zarzecka, U., Chajęcka-Wierzchowska, W., Zakrzewski, A., Zadernowska, A., Fraqueza, M.J., 2022d. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiol. 107, 104090 https://doi.org/10.1016/J.FM.2022.104090.

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Z.2 OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI WCHODZĄCYCH W SKŁAD PRACY

mgr Urszula Zarzecka
Katedra Mikrobiologii Przemysłowej i Żywności
Wydział Nauki o Żywności
Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

Zarzecka U., Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424.

Mój wkład w powstanie publikacji był wiodący, polegał na współudziale w opracowaniu tematu pracy i przygotowaniu konspektu publikacji, samodzielnym zebraniu literatury i przygotowaniu wstępnej wersji manuskryptu oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 65%.

Urszula Zarzecka

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Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

Zarzecka U., Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424.

Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu tematu pracy i przygotowaniu konspektu publikacji, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 20%.

Anna Zadernowska

dr inż. Wioleta Chajęcka-Wierzchowska
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Wydział Nauki o Żywności
Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

Zarzecka U., Zadernowska A., Chajęcka-Wierzchowska W. 2020. Starter cultures as a reservoir of antibiotic resistant microorganisms. LWT-Food Science and Technology, 127, 109424. https://doi.org/10.1016/j.lwt.2020.109424.

Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu tematu pracy, nadzorze merytorycznym, udziale w redakcji manuskryptu po recenzji oraz pełnieniu roli autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 15%.

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Katedra Mikrobiologii Przemysłowej i Żywności
Wydział Nauki o Żywności
Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

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Zarzecka U., Chajęcka-Wierzchowska W., Zadernowska A. 2022. Microorganisms from starter and protective cultures - Occurrence of antibiotic resistance and conjugal transfer of *tet* genes *in vitro* and during food fermentation. LWT–Food Science and Technology, 153, 112490. https://doi.org/10.1016/j.lwt.2021.112490.

Mój wkład w powstanie publikacji był wiodący, polegał na współudziale w opracowaniu koncepcji badań, zebraniu i przeglądzie literatury, udziale w walidacji metodyki, wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu oraz udziale w redakcji manuskryptu po recenzji. Pełniłam także rolę autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 60%.

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Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, udziale w walidacji metodyki, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 20%.

Wioleta Chajęcka-Wierzchowska

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Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, udziale w walidacji metodyki, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 20%.

Anna Zadernowska

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Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji był wiodący, polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu i przeglądzie literatury, udziale w walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu oraz udziale w redakcji manuskryptu po recenzji. Pełniłam także rolę autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 55%.

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Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, udziale w walidacji metodyki, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 10%.

Wioleta Chajęcka-Wierzchowska

mgr inż. Arkadiusz Zakrzewski
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Wydział Nauki o Żywności
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Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji polegał na walidacji metodyki i wykonaniu analiz laboratoryjnych w zakresie analiz cytometrycznych. Mój udział procentowy w powstaniu publikacji szacuję na 15%.

Arkadiusz Zakrzewski

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Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 10%.

Anna Zadernowska

Prof. Maria João Fraqueza
CIISA—Centro de Investigação Interdisciplinar em Sanidade Animal
Faculdade de Medicina Veterinária
Universidade de Lisboa

Lisboa, 08.05.2023

STATEMENT

I hereby declare that I am the co-author of the publication:

Zarzecka U., Chajęcka-Wierzchowska W., Zakrzewski A., Zadernowska A., Fraqueza M.J. 2022. High pressure processing, acidic and osmotic stress increased resistance to aminoglycosides and tetracyclines and the frequency of gene transfer among strains from commercial starter and protective cultures. Food Microbiology, 107, 104090. https://doi.org/10.1016/J.FM.2022.104090.

My contribution to the creation of the publication consisted of supervision and participation in the editing of the manuscript. My percentage share in the creation of the publication is estimated at 10%.



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Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

Zarzecka U., Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. Effect of high pressure processing on changes in antibiotic resistance genes expression among strains from commercial starter cultures. Food Microbiology, 110, 104169. https://doi.org/10.1016/j.fm.2022.104169.

Mój wkład w powstanie publikacji był wiodący, polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu i przeglądzie literatury, walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu, udziale w redakcji manuskryptu po recenzji oraz pełnieniu roli autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 60%.

Urszula Zarzecka

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Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 20%.

Anna Zadernowska

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Wydział Nauki o Żywności
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Mój wkład w powstanie publikacji polegał na nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 15%.

Wioleta Chajęcka-Wierzchowska

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Uniwersytet Warmińsko-Mazurski w Olsztynie

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Mój wkład w powstanie publikacji polegał na współudziale w wykonaniu analiz laboratoryjnych i opracowaniu wyników. Mój udział procentowy w powstaniu publikacji szacuję na 5%.

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Uniwersytet Warmińsko-Mazurski w Olsztynie

Olsztyn, 08.05.2023

OŚWIADCZENIE

Niniejszym oświadczam, że jestem współautorem publikacji:

Zarzecka U., Zadernowska A., Chajęcka-Wierzchowska W., Adamski P. 2023. High-pressure processing effect on conjugal antibiotic resistance genes transfer in vitro and in the food matrix among strains from starter cultures. International Journal of Food Microbiology, 388, 110104. https://doi.org/10.1016/j.ijfoodmicro.2023.110104.

Mój wkład w powstanie publikacji był wiodący, polegał na zdobyciu finansowania badań, opracowaniu koncepcji badań, zebraniu i przeglądzie literatury, walidacji metodyki, współudziale w wykonaniu analiz laboratoryjnych, analizie i opracowaniu wyników, przygotowaniu wstępnej wersji manuskryptu, udziale w redakcji manuskryptu po recenzji oraz pełnieniu roli autora korespondencyjnego. Mój udział procentowy w powstaniu publikacji szacuję na 65%.

Urszula Zarzecka

dr hab. inż. Anna Zadernowska, prof. UWM
Katedra Mikrobiologii Przemysłowej i Żywności
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Mój wkład w powstanie publikacji polegał na współudziale w opracowaniu koncepcji badań, nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 15%.

Anna Zadernowska

dr inż. Wioleta Chajęcka-Wierzchowska
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Wydział Nauki o Żywności
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Mój wkład w powstanie publikacji polegał na nadzorze merytorycznym oraz udziale w redakcji manuskryptu po recenzji. Mój udział procentowy w powstaniu publikacji szacuję na 15%.

Wioleta Chajęcka-Wierzchowska

Patryk Adamski
Katedra Mikrobiologii Przemysłowej i Żywności
Wydział Nauki o Żywności
Uniwersytet Warmińsko-Mazurski w Olsztynie

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Mój wkład w powstanie publikacji polegał na współudziale w wykonaniu analiz laboratoryjnych i opracowaniu wyników. Mój udział procentowy w powstaniu publikacji szacuję na 5%.