POLITECHNIKA BYDGOSKA
im. Jana i Jędrzeja Śniadeckich

RADA NAUKOWA DYSCYPLINY ROLNICTWO I OGRODNICTWO

ROZPRAWA DOKTORSKA

w formie zbioru opublikowanych i powiązanych tematycznie artykułów naukowych w dyscyplinie rolnictwo i ogrodnictwo

mgr inż. Piotr Kanarek

OPRACOWANIE INNOWACYJNEJ TECHNOLOGII BEZCHLOROWEGO UZDATNIANIA WÓD POPŁUCZNYCH PRZEMYSŁU ROLNO-SPOŻYWCZEGO Z WYKORZYSTANIEM SKŁADNIKÓW BIOAKTYWNYCH POCHODZENIA ROŚLINNEGO

Development of an innovative chlorine-free technology for the treatment of wash water from the agri-food industry using bioactive plant-based components

DZIEDZINA: Nauki rolnicze
DYSCYPLINA: Rolnictwo i ogrodnictwo

PROMOTOR

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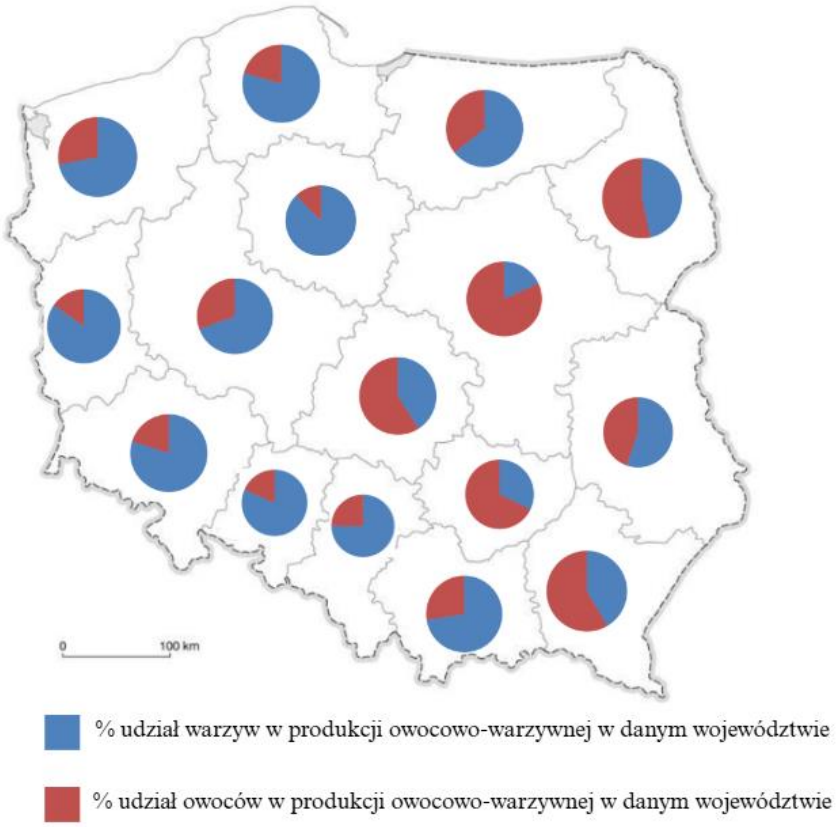
1. WSTĘP

W ostatnich latach na terenie Europy obserwuje się wzrost częstotliwości występowania okresów suszy (np.: 2003, 2007, 2011, 2012, 2015, 2017, 2018, 2019, 2020), co potwierdzają liczne analizy hydrologiczne i klimatyczne (Blauhut i wsp., 2022; Hanel i wsp., 2018). Zjawisko to negatywnie wpływa na szereg czynników, takich jak: lokalne stosunki hydrologiczne, erozję gleby, straty w produkcji roślinnej i zwierzęcej, degradację torfowisk, wzrost ryzyka pożarów, jak również straty energetyczne. Wymienione czynniki są szczególnie dotkliwe w regionach o ograniczonej zdolności adaptacyjnej (Intergovernmental Panel on Climate Change, 2023). Z perspektywy zdrowia publicznego również można wyróżnić zagrożenia związane ze wzrostem temperatur, takie jak zwiększone ryzyko dla nasilenia lub pojawiania się chorób związanych z patogenami środowiska wodnego, żywności i wektorami zwierzęcymi (np. stawonogami) (Semenza i wsp., 2012). Rosnąca średnioroczna temperatura może sprzyjać także częstszej detekcji zróżnicowanych patogenów (Dietrich i wsp., 2023). Obecnie intensyfikowane są wysiłki związane z wdrażaniem rozwiązań służących przeciwdziałaniu negatywnym skutkom zmian klimatu, dotykających również sektor produkcji żywności (Abbass i wsp., 2022; Dinar i wsp., 2019).

W tym kontekście szczególnie istotnym jest skupienie się na zabiegach związanych z retencją, oszczędzaniem i recyklingiem wody, która jest krytycznym czynnikiem w produkcji rolnej (He i Rosa, 2023; Nika i wsp., 2020). Rolnictwo jest najbardziej wodochłonnym sektorem gospodarki, odpowiadającym za około 70% rocznego wykorzystania słodkiej wody (Pimentel i wsp., 2004). Przykładowo, średni ślad wodny – rozumiany jako suma komponentu zielonego (deszczowego) i niebieskiego (wody pobranej do nawadniania) potrzebnej do wytworzenia 1 kg produktu – wynosi dla pomidorów przemysłowych (dorzecze rzeki Pinios, Grecja) średnio 61 l/kg, jednakże jednostkowy ślad wodny dla poszczególnych upraw wykazuje wyraźną zmienność temporalno-przestrzenną, zależną od warunków klimatycznych, systemu nawadniania oraz lokalnych praktyk rolniczych (Evangelou i wsp., 2016; Mialyk i wsp., 2024). Wśród środków zarządzania zrównoważonym wykorzystaniem wody w rolnictwie można wyróżnić rozwój sieci stawów retencyjnych, która poprawiłaby lokalne stosunki wodne, stanowiąc rezerwuar wody w okresie niedoboru i przeciwdziałając także postępującej degradacji środowiska (Staccione i wsp., 2021). Innym wdrażanym rozwiązaniem jest wykorzystanie wody odzyskanej ze ścieków (ang.: *reclaimed water*) w procesie nawadniania upraw (Truchado i wsp., 2021). Jednakże, zastosowanie tego rodzaju wody może budzić uzasadnione obawy społeczne związane z propagacją chorobotwórczych mikroorganizmów na produkowane warzywa i owoce (Gelting i wsp., 2011).

W świetle powyższych uwarunkowań, również w Polsce, istotnym zagadnieniem staje się ocena potencjału i struktury produkcji rolnej, ze szczególnym uwzględnieniem sektora ogrodniczego i sadowniczego, który cechuje się wysoką wodochłonnością oraz dużym znaczeniem gospodarczym. Na podstawie danych Głównego Urzędu Statystycznego za rok 2024 całkowite zbiory warzyw gruntowych w Polsce wyniosły 38 313 501 dt. Największy udział w tej kategorii odnotowano w województwie kujawsko-pomorskim, gdzie zbiory

osiągnęły poziom 6 521 516 dt, co stanowiło około 17,0 % krajowej produkcji warzyw gruntowych. W przypadku owoców z drzew, całkowita masa zbiorów wyniosła 37 315 811 dt. Najwyższe wartości zaobserwowano w województwie mazowieckim, gdzie zebrano 17 560 423 dt, co stanowiło 47,1 % całkowitych zbiorów tej grupy. Zbiory owoców z krzewów owocowych i plantacji jagodowych ukształtowały się na poziomie 4 817 056 dt. W tej kategorii dominowało województwo lubelskie z wynikiem 1 552 597 dt, co stanowiło 32,2 % krajowej produkcji owoców jagodowych (Główny Urząd Statystyczny, Departament Rolnictwa i Środowiska, 2025) (Rysunek 1).



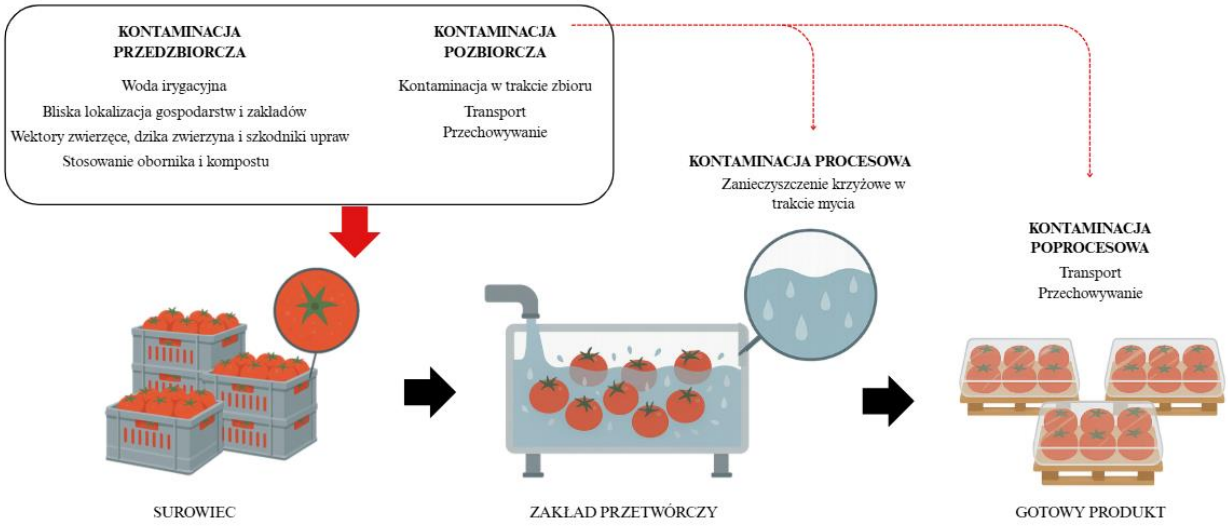
Rys. 1. Zestawienie udziału owoców i warzyw [%] w ogólnej produkcji owocowo-warzywnej w poszczególnych województwach (opracowanie własne na podstawie raportu GUS, 2025).

Jak zauważa Kuśmierek-Tomaszewska i wsp. (2021), susze w Polsce, choć występują bez wyraźnej regularności, są zjawiskiem powtarzalnym i znacząco wpływają na warunki produkcji rolnej. Przykładowo w regionie Pomorza i Kujaw w analizowanym trzydziestoletnim okresie (1981–2010), niedobory opadów w kluczowych miesiącach sezonu wegetacyjnego (maj–czerwiec) miały miejsce średnio co czwarty rok (Kuśmierek-Tomaszewska i Żarski, 2021). Biorąc pod uwagę rosnące zapotrzebowanie na wodę w całym sektorze rolnym, konieczne staje się również uwzględnienie w działaniach adaptacyjnych sektorów okołorolniczych, w tym zwłaszcza przetwórstwa rolno-spożywczego (Manzocco i wsp., 2015).

Sektor przetwórstwa owocowo-warzywnego, silnie związany z rolniczą produkcją pierwotną, również stoi przed istotnymi wyzwaniami w zakresie zarządzania wodą. Duże zużycie wody w procesach technologicznych, głównie mycia i płukania, skutkuje

powstawaniem znacznych ilości wód popłucznych, często obciążonych zanieczyszczeniami organicznymi (w tym mikrobiologicznymi) (Rasines i wsp., 2024). Zgodnie z estymacjami, przyjmuje się, że do przetworzenia 1 kilograma owoców bądź warzyw zużywa się około 5 litrów wody, która w wielu przypadkach nie jest ponownie wykorzystywana, a generowana jako ściek i zrzucana do sieci kanalizacyjnej (Lehto i wsp., 2014). Takie podejście nie wpisuje się w racjonalne i zrównoważone gospodarowanie zasobami, jednakże stanowi dogodną niszę do implementacji elementów gospodarki o obiegu zamkniętym, w tym oczyszczania wody.

Jednym z głównych zagrożeń związanych z ponownym wykorzystaniem nieoczyszczonej wody jest mikrobiologiczna kontaminacja krzyżowa pomiędzy partiami surowca. Pomimo stosowania środków dezynfekujących, woda używana do płukania warzyw i owoców pozostaje istotnym wektorem zakażeń krzyżowych (Possas i Pérez-Rodríguez, 2023). Duże obciążenie materią organiczną (cząstkami zawieszonymi i rozpuszczonymi), jak również zanieczyszczenie mikrobiologiczne już na etapie uprawy sprzyja propagacji patogenów w zakładach przetwórczych. W ramach analizy kontaminacji surowca i produktu wyróżnić można dwa główne rodzaje zanieczyszczenia. Pierwszym z nich jest kontaminacja przedzbiorcza, która może wynikać z wykorzystywania zanieczyszczonej wody do nawadniania, emisji patogenów z blisko zlokalizowanych gospodarstw i jednostek intensywnego tuczu zwierząt, stosowania nawozów naturalnych i wektorów odzwierzęcych, a także szeroko rozumianego rodzaju i systemu uprawy (Miceli i Settanni, 2019). Drugi rodzaj zanieczyszczenia obejmuje zbiór oraz nieprawidłowe warunki transportu, magazynowania i przetwarzania surowca w zakładzie przetwórczym (Rysunek 2).



Rys. 2. Schemat przedstawiający główne czynniki kontaminacji przedzbiorczej i pozbiorczej w produkcji owocowo-warzywnej (opracowanie własne).

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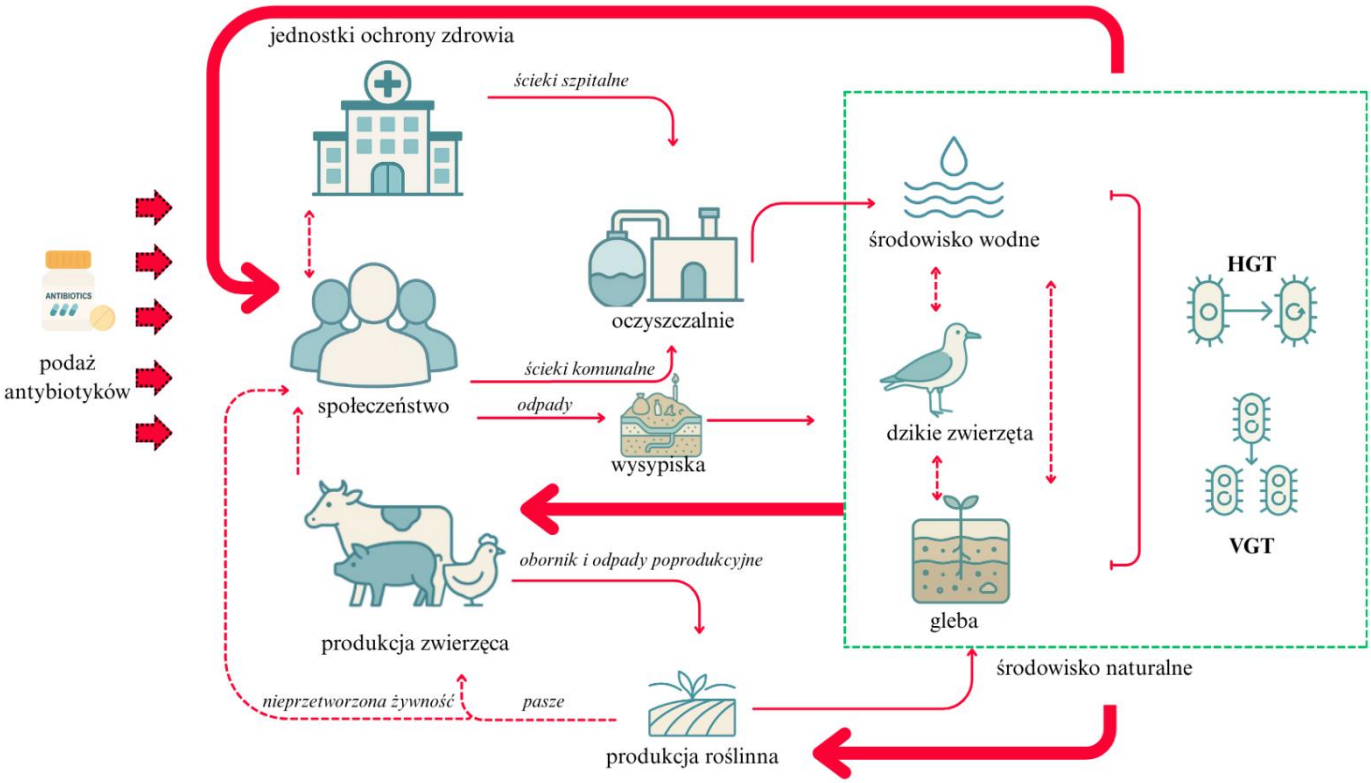
Nieprawidłowe praktyki w trakcie zbioru i przetwarzania surowca mogą znacząco zwiększyć liczbę drobnoustrojów, a zwłaszcza przyczynić się do obecności patogenów, w końcowym produkcie. Przykładem mogą być badania przeprowadzone przez Ijabadeniyi i wsp. (2011), w których stwierdzono występowanie licznych patogenów bakteryjnych, w tym *E. coli,* bakterii z rodzaju *Salmonella,* a także *Listeria monocytogenes, Staphylococcus aureus* i *Enterococcus* spp. w próbkach wody irygacyjnej oraz na nawadnianych nią warzywach. Łepecka i wsp. (2022) w oparciu o wykonane analizy mikrobiologiczne wykazali obecność m.in. grzybów pleśniowych, drożdży oraz bakterii z rodziny *Enterobacteriaceae* w gotowych do zjedzenia mieszankach różnych sałat. Pałeczki *Escherichia coli* wykryto w 50% wszystkich badanych próbek sałat, bakterie z rodzaju *Salmonella* – w 26,7% próbek, *Listeria monocytogenes* – w 33,3%, natomiast *Staphylococcus aureus* zidentyfikowano w trzech próbkach. Wśród bakterii wykrywanych na warzywach i owocach odnotowywano również inne odpowiedzialne za ciężkie zatrucia pokarmowe gatunki: *Bacillus cereus, Vibrio cholerae, Campylobacter* spp., *Shigella* spp. i *Clostridium* spp. (Thomas i wsp., 2024). Zagrożenie mikrobiologiczne wynika nie tylko z obecności patogenów na powierzchni warzyw i owoców, lecz także z ich zdolności do przenikania do wnętrza tkanek roślinnych. Penetracja tkanek może następować przez naturalne otwory (np. aparaty szparkowe, pory, połączenia korzeniowe) lub uszkodzenia mechaniczne i zmiany martwicze, co umożliwia ich kolonizację zarówno w okresie przed–, jak i pozbiorczym, również z możliwością tworzenia biofilmów (Kim i wsp., 2023; Melo i Quintas, 2023; Zhao i wsp., 2024).

Zanieczyszczenie mikrobiologiczne surowca nieodłącznie związane jest z ryzykiem występowania antybiotykoopornych szczepów. Rosnąca podaż antybiotyków do środowiska naturalnego wspiera rozwój antybiotykoopornych szczepów i występowanie wolnych genów antybiotykooporności w środowisku (Larsson i Flach, 2022). Wśród czynników przyczyniających się do antybiotykooporności można wymienić jednostki ochrony zdrowia, ścieki komunalne, jak również rolnictwo, związane z produkcją zwierzęcą i wykorzystywanie obornika (Muhammad i wsp., 2020; Omuferen i wsp., 2022) Następnie, w wyniku wertykalnego i horyzontalnego (koniugację, transformację i transdukcję) transferu genów dochodzi do utrwalenia tego wysoce niekorzystnego zjawiska. (Lin i wsp., 2021) (Rysunek 3).



Rys. 3. Schemat sprzężenia zwrotnego i cyrkulacji zjawiska antybiotykooporności w środowisku naturalnym i antropogenicznym (opracowanie własne).

Jak zauważa Zahra i wsp. (2025) występowanie antybiotykoopornych szczepów na powierzchni owoców i warzyw może stanowić poważne zagrożenie dla bezpieczeństwa zdrowia publicznego. W badaniach wykazano, że oporność wyizolowanych szczepów *E. coli* na antybiotyki, szczególnie na ampicylinę i erytromycynę, była powszechna, a poziom ich wielolekooporności (MDR, *multidrug resistance*), zależał od rodzaju warzyw i pory roku (Zahra i wsp., 2025). Zwiększająca się liczba doniesień naukowych sprawia, że coraz częściej postuluje się wzmocnienie monitoringu szczepów wykazujących oporność na środki przeciwdrobnoustrojowe (AMR, *antimicrobial resistance*) w produkcji owocowo-warzywnej. Badania przeprowadzone na Sycylii wykazały, że z 86,2% warzyw wyizolowano 53 szczepy AMR, w tym 10 MDR z różnymi genami oporności (m.in. *blaTEM*, *tetA–E*, *qnrS*, *sulI*) (Castello i wsp., 2023). Zagrożenia nie są wyłącznie związane ze sprzedażą detaliczną na targowiskach i rynkach, problem rozwoju antybiotykooporności dotyczy również warzyw i owoców pochodzących z zakładów przetwórczych. Badania nad próbkami sałaty i szpinaku pobranymi z komercyjnych gospodarstw z własnymi pakowalniami wykazały, że 64,7% izolatów *E. coli* charakteryzowało się wielolekoopornoscią (Ratshilingano i wsp., 2022). Również badania Iseppi i wsp., (2018) potwierdziły obecność bakterii Gram-ujemnych wytwarzających β-laktamazy (w tym metalo-β-laktamazy) w 20 z 80 testowanych miksów sałat gotowych do spożycia. Również w badaniach nad kontaminacją owoców i warzyw przez

S. aureus zaobserwowano występowanie wzorców MDR oraz rozległej oporności na leki (XDR- *extensively drug resistance*), co wskazuje na poważny problem oporności na antybiotyki wśród szczepów *S. aureus* izolowanych z owoców i warzyw gotowych do spożycia. Wyniki badań wykazały, że izolaty pochodzące z sałaty i kapusty czerwonej charakteryzowały się ogólnie niższym poziomem oporności, natomiast szczepy wyizolowane z kolendry wykazywały wyższy poziom MDR (Jia i wsp., 2024).

Jedną ze strategii zmniejszania ryzyka zanieczyszczenia mikrobiologicznego w przetwórstwie rolno-spożywczym jest stosowanie chemicznych i fizycznych metod uzdatniania wody procesowej pomiędzy szarżami produkcyjnymi. Z literatury przedmiotu wynika, że najczęstszą metodą oczyszczania wody po procesie płukania surowca jest aplikacja dezynfekcji chlorowej (w postaci podchlorynu sodu lub wapnia oraz w mniejszym stopniu chloru gazowego) (Gil i Allende, 2018). Wśród innych metod można wyróżnić stosowanie kwasu nadoctowego, elektrolizę wody, a także ozonowanie i promieniowanie UV (Lee i Huang, 2019; Rahman i wsp., 2016; Wang i wsp., 2019; Zhang i wsp., 2022). W przypadku metod opartych o związki chloru, pomimo wysokiej wydajności tego typu rozwiązań, istnieje możliwość powstawania negatywnych produktów ubocznych o zróżnicowanym wpływie na środowisko naturalne i zdrowie człowieka (np.: toksyczność, cytotoksyczność, neurotoksyczność, genotoksyczność, mutagenność i kancerogenność). Do negatywnych produktów ubocznych zalicza się m.in. chloroform, kwas dichlorooctowy, kwas trichlorooctowy, halonitrometany, haloacetamidy, haloacetonitryle, bromian, chloryn, chloran, kwas jodooctowy, jodoform, jodowe trihalometany, bromodichlorometan, bromoform, kwas dibromooctowy (Al-Otoum i wsp., 2016; Plewa i wsp., 2004; Richardson i wsp., 2007; Zimoch i Łobos, 2016). Stosowanie ozonu do oczyszczania wody na szeroką skalę, oprócz wyższych nakładów finansowych, jest utrudnione z uwagi na fakt, że wydajność dezynfekcji uzależniona jest od wielu czynników (rodzaj produktu, cechy powierzchni, powierzchnia, rodzaj drobnoustrojów i obciążenie mikrobiologiczne) (Jin i Adhikari, 2025). Aplikacja UV, z kolei, może być utrudniona z uwagi na niską zdolność penetracyjną promieniowania UV-C. Również obecny w wodzie mikroplastik odgrywa znaczącą rolę w niwelowaniu oddziaływania promieniowania, stwarza bowiem barierę ochronną dla mikroorganizmów (Chawla i wsp., 2021; Shen i wsp., 2021). W przypadku kwasu nadoctowego (PAA), pomimo coraz szerszego zastosowania tego dezynfektantu, warto zaznaczyć, że wzrost zawartości materii organicznej w wodzie (np. resztek roślinnych) powoduje szybki spadek efektywnego stężenia PAA (Banach i wsp., 2015). Narastająca potrzeba wdrażania rozwiązań związanych z recyrkulacją wody w układach zamkniętych, a także niepożądane skutki uboczne tradycyjnej dezynfekcji chlorowej, sprzyjają poszukiwaniu alternatywnych strategii w celu utrzymania zapewnienia bezpieczeństwa mikrobiologicznego wód procesowych.

W ostatnim czasie rośnie zainteresowanie implementacją nowych, nieinwazyjnych metod oczyszczania wody, w ramach zintegrowanego podejścia. Do takich zabiegów można zaliczyć np.: stosowanie nanocząstek srebra, miedzi, dwutlenku tytanu, tlenku grafenu, nanorurek węglowych, tlenku cynku oraz tlenku żelaza, które wykazują działanie przeciwdrobnoustrojowe (Olawade i wsp., 2024). Interesującym rozwiązaniem jest również stosowanie naturalnych, roślinnych koagulantów w celu ograniczenia stosowania ich chemicznych odpowiedników, jak również wykorzystywanie materiału roślinnego do syntezy nanocząstek (Diver i wsp., 2023; Khalaf i wsp., 2025).

Część substancji i związków fitochemicznych może negatywnie oddziaływać na mikroorganizmy, co jest wynikiem adaptacji roślin wobec stresorów biologicznych (Álvarez-Martínez i wsp., 2020; Chassagne i wsp., 2021). Do kluczowych grup wtórnych metabolitów roślin zalicza się m.in. alkaloidy, olejki eteryczne oraz różnorodne związki fenolowe, w tym garbniki, kwasy fenolowe i flawonoidy. Choć związki te nie warunkują bezpośrednio przeżycia roślin, pełnią istotne funkcje obronne, zwłaszcza w ochronie przed mikroorganizmami chorobotwórczymi (Fialova i wsp., 2017). Mechanizm działania alkaloidów opiera się głównie na blokowaniu aktywności pomp efluksowych, zahamowaniu biosyntezy białek oraz interferencji procesu transkrypcji DNA (Alghazeer i wsp., 2022). Flawonoidy wykazują aktywność przeciwbakteryjną poprzez szereg odmiennych mechanizmów, m.in. mogą one hamować syntezę kwasów nukleinowych oraz zakłócać procesy metaboliczne odpowiedzialne za wytwarzanie energii w komórkach bakteryjnych. Związki te wpływają również na etapy adhezji oraz formowanie biofilmów, co utrudnia kolonizację powierzchni przez drobnoustroje. Ponadto zdolne są do blokowania poryn w błonie komórkowej i modyfikowania jej przepuszczalności. Efektem tych działań jest także zmniejszenie potencjału patogennego mikroorganizmów (Fernández-Rojas i Gutiérrez-Venegas, 2018; Kováč i wsp., 2023). Również garbniki wykazują działanie przeciwdrobnoustrojowe poprzez uszkadzanie błony komórkowej, wiązanie białek i składników ściany komórkowej, chelatowanie jonów oraz hamowanie adhezji i agregacji patogenów (Huang i wsp., 2024). Kwasy fenolowe wpływają na komórki bakteryjne poprzez uszkodzenie błony komórkowej, prowadząc do zmiany hydrofobowości oraz ładunku powierzchniowego. W następstwie tych modyfikacji dochodzi do wycieku zawartości cytoplazmy i śmierci komórki bakteryjnej (Kauffmann i Castro, 2023). Dużym zainteresowaniem naukowym cieszą się również olejki eteryczne, rozumiane jako lotne mieszaniny wtórnych metabolitów roślinnych (zawierające w swym składzie m.in. aldehydy, alkohole, ketony, terpeny i proste związki fenolowe) (Bunse i wsp., 2022). Olejki eteryczne również oddziałują holistycznie, poprzez zaburzenie struktury fosfolipidowej błony komórkowej, a tym samym, zwiększenie jej przepuszczalności, symultanicznie może dojść również do inhibicji enzymów (Li i wsp., 2022; Mangalagiri i wsp., 2021).

Ekstrakty roślinne najczęściej badane są w warunkach *in vitro* i celowane wobec konkretnego gatunku bakterii (np.: szczepów klinicznych lub wobec patogenów żywności) (Morguette i wsp., 2023; Plaskova i Mlcek, 2023)**. Możliwości wykorzystania ekstraktów roślinnych do złóż biologicznych w technologii bezchlorowego oczyszczania wody jest ciekawą i proekologiczną alternatywą w przetwórstwie warzywno-owocowym. Ponadto zaletą takiego rozwiązania jest wtórne wykorzystanie wody, co przekłada się na redukcję śladu wodnego, zmniejszenie produkcji ścieków przemysłowych i optymalizację ekonomiczną. Rozwój tej ścieżki wymaga przejścia z modeli in vitro do rzeczywistych systemów poprzez identyfikację aktywnych związków i testy w warunkach zbliżonych do naturalnych.**

2. WYKAZ ARTYKUŁÓW NAUKOWYCH STANOWIĄCYCH CYKL PUBLIKACJI ROZPRAWY DOKTORSKIEJ

Osiągnięciem naukowym niniejszej rozprawy jest opracowanie i opublikowanie w recenzowanych, międzynarodowych czasopismach cyklu czterech artykułów z listy *Journal Citation Reports* (JCR), które w sposób komplementarny opisują wyniki uzyskane w kolejnych etapach badań nad problematyką zanieczyszczenia mikrobiologicznego wody w przemyśle rolno-spożywczym i opracowaniem systemu biologicznego uzdatniania wody. W skład cyklu prac wchodzą:

1. Kanarek Piotr, Breza-Boruta Barbara, Rolbiecki Roman, Microbial composition and formation of biofilms in agricultural irrigation systems- a review, *Ecohydrology & Hydrobiology* (Elsevier), 2024, 24(3), 583 590, https://doi.org/10.1016/j.ecohyd.2023.10.004, 100 pkt MNiSW[1], Impact Factor: 2,2

2. Kanarek Piotr, Breza-Boruta Barbara, Bogiel Tomasz. In the Depths of Wash Water: Isolation of Opportunistic Bacteria from Fresh-Cut Processing Plants, *Pathogens* (MDPI). 2024;13(9):768, https://doi.org/10.3390/pathogens13090768, 100 pkt MNiSW[1], Impact Factor: 3,3

3. Kanarek Piotr, Breza-Boruta Barbara, Stocki Marcin. Antimicrobial Activity and Phytochemical Profiling of Natural Plant Extracts for Biological Control of Wash Water in the Agri-Food Industry. *Applied Sciences* (MDPI). 2025;15(9):5199. https://doi.org/10.3390/app15095199, 100 pkt MNiSW[1], Impact Factor: 2,5

4. Kanarek Piotr, Breza-Boruta Barbara, Poćwiardowski Wojciech, Pilot-Scale Evaluation of a Filter Prototype for Bacterial Inactivation in Agro-Food Processing Wastewater, *Water* (MDPI), 2025;17(7):2631. https://doi.org/10.3390/w17172631, 100 pkt MNiSW[1], Impact Factor: 3,0

Łączna wartość punktacji MEiN/MNiSW[1]: **400**
Łączna wartość Impact Factor: **11**

[1]Liczba punktów wg Komunikatu Ministra Edukacji i Nauki w sprawie wykazu czasopism naukowych z dnia 5 stycznia 2024 roku.

3. UZASADNIENIE SPÓJNOŚCI TEMATYCZNEJ CYKLU PUBLIKACJI ROZPRAWY

Cykl publikacji, stanowiący podstawę do ubiegania się o stopień doktora, opiera się na badaniach nad opracowaniem systemu oczyszczania wód popłucznych pochodzących z przemysłu rolno-spożywczego z wykorzystaniem materiałów pochodzenia roślinnego. W obliczu rosnących potrzeb ponownego wykorzystania wody w obiegu zamkniętym oraz negatywnych skutków ubocznych tradycyjnej dezynfekcji chlorowej poszukuje się alternatywnych metod dla zapewnienia bezpieczeństwa mikrobiologicznego wód w sektorze rolno-spożywczym.

W publikacji nr 1 przedstawiono aktualny stan wiedzy dotyczący zanieczyszczeń mikrobiologicznych w systemach wodnych stosowanych w rolnictwie oraz metod ograniczania rozwoju biofilmu i patogenów. Woda stosowana do nawadniania jest ważnym wektorem transmisji patogenów, które docelowo mogą znajdować się na powierzchni, a także w tkankach miękkich warzyw i owoców. Artykuł ten analizuje m.in. problemy skażenia wody i tworzenia biofilmów w instalacjach nawadniających oraz wskazuje na ograniczenia tradycyjnych środków dezynfekcyjnych. Tym samym **publikacja ta uzasadnia potrzebę poszukiwania nowych, przyjaznych środowisku metod dezynfekcji, stanowiąc podbudowę teoretyczną dla kolejnych etapów badań.**

W publikacji nr 2 skupiono się na identyfikacji mikroorganizmów obecnych w wodach popłucznych z wybranych zakładów przetwórstwa owocowo-warzywnego na terenie Polski. Scharakteryzowano wyizolowane szczepy bakterii z pobranych próbek wody popłucznej czterech zakładach przemysłu rolno–spożywczego, a uzyskane wyniki badań potwierdziły występowanie bakterii potencjalnie patogennych. Wszystkie izolaty poddano identyfikacji gatunkowej i do dalszych badań wytypowano sześć reprezentatywnych, potencjalnie chorobotwórczych szczepów. Badania obejmowały również ocenę wybranych fenotypowych profili antybiotykooporności dla bakterii testowych, co pozwoliło na ocenę ryzyka rozwoju antybiotykooporności

Publikacja nr 3 obejmuje badania nad naturalnymi surowcami roślinnymi pod kątem ich zdolności do ograniczania wzrostu bakterii testowych. Przeprowadzono przesiewową ocenę aktywności przeciwdrobnoustrojowej ekstraktów roślinnych. Przebadano ekstrakty z 13 różnych gatunków i części roślin (liście, kwiaty, kora, owoce, korzenie, pędy), kierując się ich znanymi właściwościami antybakteryjnymi i zróżnicowanym składem fitochemicznym. W wyniku testów, połączonych z jakościowym i ilościowym profilowaniem fitochemicznym, wytypowano sześć ekstraktów wykazujących działanie hamujące wobec wyizolowanych wcześniej patogenów. Wyniki publikacji nr 3 potwierdzają założenie o współdziałaniu komponentów roślinnych i stanowiły podstawę do kolejnego etapu badań, ukierunkowanego na stworzenie kompozytu filtracyjnego z wybranych materiałów.

Publikacja nr 4, dotyczy wdrożenia i oceny prototypowego filtra z wypełnieniem roślinnym w warunkach półtechnicznych. Na tym etapie nastąpiła integracja wcześniejszych wyników – wybrane w trzeciej publikacji bioaktywne surowce roślinne zostały immobilizowane w hydrożelu i posłużyły do skonstruowania wielowarstwowego złoża filtracyjnego, przez które przepuszczano skażoną mikrobiologicznie wodę popłuczną.

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Podsumowując, wszystkie cztery publikacje tworzą spójny tematycznie cykl, ukierunkowany na rozwiązanie zdefiniowanego problemu. Każda z prac odpowiada kolejnym etapom realizacji celu głównego – od diagnozy problemu mikrobiologicznego, przez opracowanie rozwiązań, po ich weryfikację w warunkach zbliżonych do rzeczywistych.

Nowatorski charakter rozprawy wyraża się w czterech wzajemnie powiązanych osiągnięciach (Rysunek 4). Osiągnięcie pierwsze, związane jest z zestawieniem doniesień literaturowych na temat źródeł kontaminacji wody irygacyjnej, ważnego wektora w propagacji drobnoustrojów na owoce i warzywa. Drugi artykuł rozszerza obecny stan wiedzy na temat zanieczyszczeń mikrobiologicznych wód popłucznych przemysłu rolno-spożywczego, z uwagi na fakt, że do tej pory nie przeprowadzono próbkowania w kilku różnych zakładach przetwórczych, powiązanych z identyfikacją gatunkową i analizą wybranych fenotypowych profili oporności. Trzecia praca opisuje wyniki aktywności przeciwdrobnoustrojowej ekstraktów z 13 surowców roślinnych przeciw szczepom bezpośrednio wyizolowanym z wód popłucznych, łącząc wyniki z analizą fitochemiczną GC-MS. Odpowiada to na zidentyfikowaną lukę dotyczącą praktycznego zastosowania ekstraktów w kontekście biologicznych metod oczyszczania wód procesowych. Czwarta publikacja prezentuje wdrożenie złoża filtracyjnego, w którym bioaktywne ekstrakty są immobilizowane w hydrożelu i łączone z warstwami węgla aktywnego oraz żywicy jonowymiennej, stanowiąc alternatywę dla dominujących metod chlorowych.

Jako autor niniejszej rozprawy doktorskiej odegrałem większościową rolę w opracowaniu koncepcji prac, planowaniu i realizacji badań naukowych, analizie uzyskanych wyników, a także w przygotowaniu i redagowaniu publikacji naukowych. Jestem również pierwszym autorem we wszystkich publikacjach i autorem korespondencyjnym w trzech z nich.

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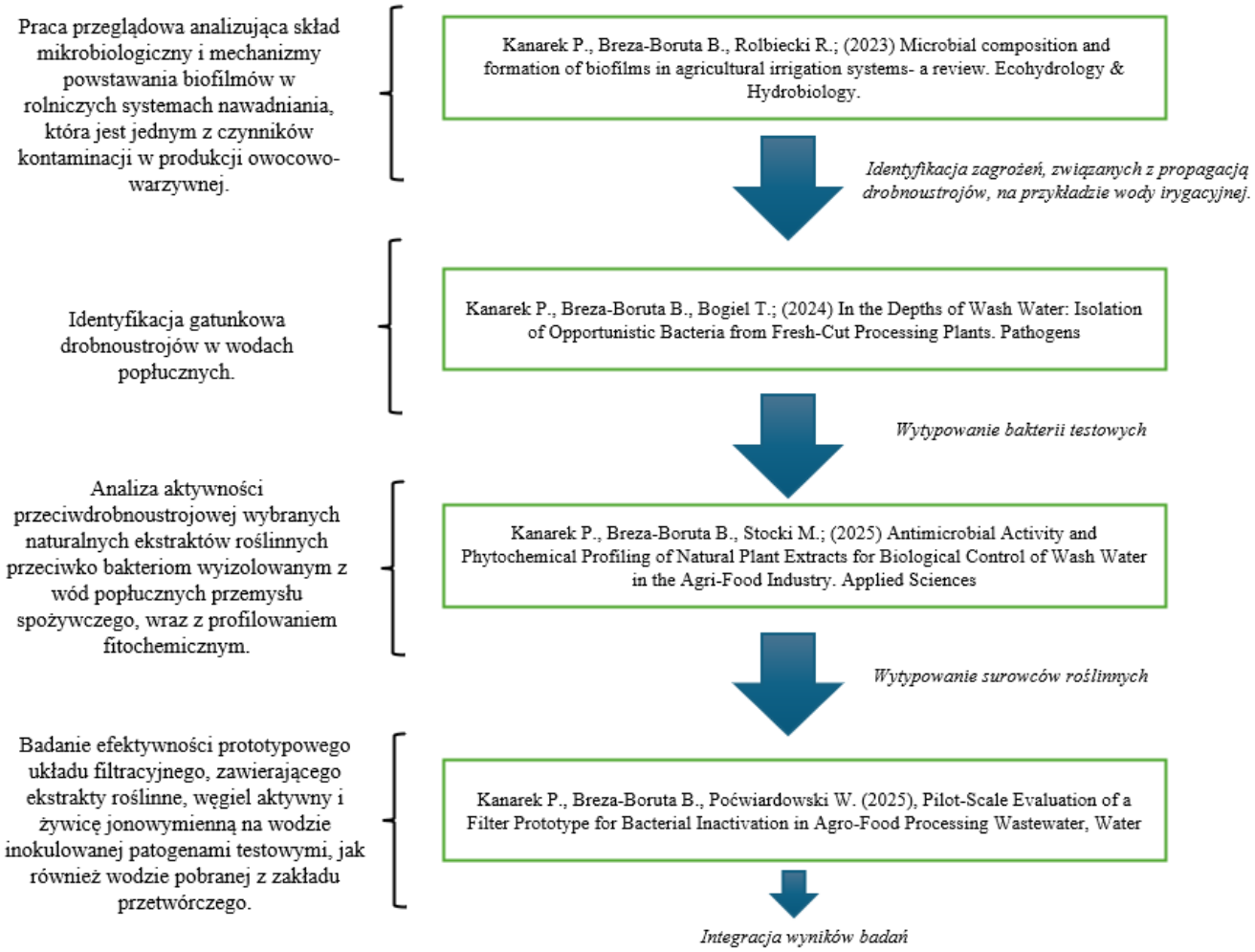

Rys. 4. Zestawienie powiązań pomiędzy poszczególnymi pracami cyklu publikacyjnego.

Wynikiem prowadzonych badań są również zgłoszenia dotyczące ochrony własności intelektualnej:

- **Patent: Filtr modułowy do uzdatniania wody z wykorzystaniem hydrożeli z ekstraktami roślinnymi, żywicy jonowymiennej oraz węgla aktywnego (numer zgłoszenia: P.453104, załącznik w sekcji 7.4. Kopie potwierdzające zgłoszenie patentowe i wzór użytkowy).**
- **Wzór użytkowy: Sposób otrzymywania poliakrylanowego hydrożelu uwodnionego sterylnymi, zagęszczonymi ekstraktami roślinnymi o właściwościach przeciwdrobnoustrojowych (numer zgłoszenia: P.453128, załącznik w sekcji 7.4. Kopie potwierdzające zgłoszenie patentowe i wzór użytkowy).**

3.1. WYKAZ SKRÓTÓW, SYMBOLI I JEDNOSTEK

AMC	amoksycylina z kwasem klawulanowym (amoxicillin with clavulanic acid)
AMK	amikacyna (amikacin)
AMP	ampicylina (ampicillin)
AMR	oporność na środki przeciwdrobnoustrojowe (antimicrobial resistance)
C	złoże z węglem aktywnym (activated carbon bed)
CAZ	ceftazydym (ceftazidime)
CIP	cyprofloksacyna (ciprofloxacin)
EUCAST-	Europejski Komitet ds. Oznaczania Lekowrażliwości (European Committe on Antimicrobial Susceptibility)
FDC	cefiderokol (cefiderocol)
FEP	cefepim (cefepime)
GEN	gentamycyna (gentamicin)
H	hydrożel z ekstraktami (hydrogel with extracts)
H–C–IER	złoże hydrożel–wegiel – żywica jonowymienna (hydrogel–carbon–ion exchange resin bed)
HGT	horyzontalny transfer genów (horizontal gene transfer)
I	pośredni ze zwiększoną ekspozycją (intermediate with increased exposure)
R	oporny (resistant)
S	wrażliwy (susceptible)
IER	żywica jonowymienna (ion exchange resin)
IPM	imipenem (imipenem)
JTK	jednostka tworząca kolonie (colony forming unit)
LOG_{10}	logarytm dziesiętny; w niniejszej pracy używany do wyrażenia zmian w liczebności populacji bakterii w skali logarytmicznej (decimal logarithm; in this work used to express changes in bacterial population size on a logarithmic scale)
LVX	lewofloksacyna (levofloxacin)
LZD	linezolid (linezolid)
MALDI-TOF	spektrometria mas z desorpcją/ jonizacją wspomaganą matrycą i analizą czasu przelotu (Matrix-Assisted Laser Desorption/Ionization – Time of Flight mass spectrometry)

MDR	wielolekooporność (multidrug resistance)
MEM	meropenem (meropenem)
MXF	moksyfloksacyna (moxifloxacin)
PAA	kwas nadocotowy (peracetic acid)
PRL	piperacylina (piperacillin)
TGC	tygecyklina (tigecycline)
TIC	tykarcylina (ticarcillin)
TIC/CLA	tykarcylina z kwasem klawulanowym (ticarcillin with clavulanic acid)
TMP	trimetoprim (trimethoprim)
TOB	tobramycyna (tobramycin)
TSA	agar tryptozowo-sojowy (tryptic soy agar)
VAN	wankomycyna (vancomycin)
VGT	werykalny transfer genów (vertical gene transfer)
XDR	rozległa oporność na leki (extensively drug resistance*)*

3.2. HIPOTEZA BADAWCZA, CEL I ZAKRES BADAŃ

Problematyka racjonalnego gospodarowania zasobami wodnymi, ukierunkowana na ograniczanie śladu wodnego, stanowi jedno z kluczowych narzędzi w przeciwdziałaniu negatywnym skutkom zmian klimatycznych. W tym kontekście szczególne znaczenie ma sektor rolno-spożywczy, który charakteryzuje się dużym zużyciem wody procesowej i generowaniem znacznych ilości ścieków popłucznych. Jednocześnie w branży tej z powodzeniem można wdrożyć innowacyjne rozwiązania oparte na wykorzystaniu naturalnych związków fitochemicznych, które mogą pełnić funkcję bioaktywnych środków wspierających procesy uzdatniania i ponownego wykorzystania wody.

Celem głównym pracy była ocena przydatności materiałów pochodzenia roślinnego, które mogą zostać wykorzystane do wypełnienia złoża antybakteryjnego w bezchlorowym systemie filtracyjnym do odzyskiwania i uzdatniania wód popłucznych przemysłu rolno-spożywczego. W ramach osiągnięcia celu głównego określono następujące cele pośrednie:

1. Wyizolowanie ze środowiska produkcyjnego i wytypowanie bakterii testowych, a także określenie wybranych profili fenotypowej antybiotykooporności.

Wyizolowanie bakterii ze środowiska produkcyjnego oraz charakterystyka wybranych profili fenotypowej oporności stanowiło pierwszy oraz kluczowy etap badań i pozwoliło na dobór reprezentatywnych dla środowiska wód popłucznych szczepów testowych. Dzięki temu możliwe było uzyskanie bardziej wiarygodnych wyników, a uzyskane dane miały większą wartość aplikacyjną.

2. Sprawdzenie zdolności antybakteryjnych wybranych materiałów pochodzenia roślinnego w warunkach laboratoryjnych, z uwzględnieniem ich skuteczności w środowisku wodnym.

Na podstawie danych literaturowych dotyczących surowców roślinnych, wykazujących właściwości przeciwdrobnoustrojowe oraz badań wstępnych z wykorzystaniem metody studzienkowo-dyfuzyjnej dokonano oceny potencjału antybakteryjnego wybranych materiałów pochodzenia roślinnego. Umożliwiło to również porównanie aktywności poszczególnych ekstraktów wobec szczepów testowych oraz wytypowanie tych, które stanowiły najbardziej obiecujące komponenty złoża filtracyjnego.

3. Wytypowanie materiałów roślinnych jako wypełnienia złoża filtracyjnego do dalszych badań, sprawdzenie ich efektywności antybakteryjnej w różnych konfiguracjach, pozwalających na optymalizację procesu.

Analiza skuteczności ekstraktów z uwzględnieniem efektu ich skojarzonego działania, pozwoliła wskazać układy o najwyższej efektywności przeciwbakteryjnej i stanowiła podstawę do dalszej optymalizacji procesu filtracji.

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4. Określenie trwałości i efektywności części składowych złoża filtracyjnego.

Określenie trwałości oraz efektywności poszczególnych komponentów złoża filtracyjnego, z uwzględnieniem zmian ich aktywności w czasie użytkowania pozwoliło na ocenę wydajności systemu.

5. Implementacja zaprojektowanego systemu w skali półtechnicznej.

Wdrożenie skonstruowanego układu filtracyjnego w warunkach półtechnicznych, z wykorzystaniem wody procesowej pochodzącej z zakładu przetwórstwa owocowo-warzywnego pozwoliło na ocenę działania systemu filtracyjnego w warunkach operacyjnych.

Hipoteza badawcza zakładała, że wykorzystanie materiałów pochodzenia roślinnego w wypełnieniu złoża antybakteryjnego pozwala na skuteczne unieczynnienie lub znaczne ograniczenie liczby bakterii występujących w wodach popłucznych zakładów owocowo-warzywnych, co stanowi skuteczną alternatywę dla dezynfekcji chemicznej w przemyśle spożywczym.

Tworząc model doświadczenia przyjęto następujące założenie (A) i hipotezy pomocnicze (H), których weryfikacja pozwala na potwierdzenie lub odrzucenie hipotezy badawczej:

A1: Wytypowane bakterie testowe cechują się dużą przydatnością do tego typu badań.

H1: Głównym czynnikiem eliminującym mikroorganizmy wskaźnikowe w trakcie filtracji są substancje zawarte w materiale roślinnym.

H2: Celowe jest łączenie różnych surowców roślinnych zawierających zróżnicowane związki bioaktywne o właściwościach przeciwbakteryjnych, ze względu na ograniczenie rozwoju jak największego spektrum drobnoustrojów.

H3: Stopień mikrobiologicznej kontaminacji wody po przeprowadzonej filtracji na zastosowanym złożu nie ulegnie pogorszeniu, a liczba bakterii w wodzie spadnie.

Weryfikacja przyjętej hipotezy badawczej, hipotez pomocniczych i założenia eksperymentu pozwoliła na realizację celów poznawczych, jak i utylitarnych.

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3.3. MATERIAŁY I METODY BADAŃ

3.3.1. Publikacja 2

Materiał badawczy w publikacji numer 2 stanowiły próbki wody pobrane we wrześniu 2022 r. z czterech zakładów przetwórstwa owocowo-warzywnego zlokalizowanych w woj. kujawsko-pomorskim i wielkopolskim (Tabela 1).

Tabela 1 Wytypowane zakłady przetwórstwa owocowo-warzywnego.

Oznaczenie zakładu	Lokalizacja	Typ mytego surowca
A	powiat bydgoski	śliwka, ogórek
B	powiat inowrocławski	cebula
C	powiat włocławski	pomidor
D	powiat kaliski	burak, ogórek

Próbki wody pobrano zgodnie z normą PN-EN ISO 19458:2007. Wodę pozyskano w trakcie procesu mycia surowca w odstępach jednogodzinnych, do sterylnych butelek szklanych po 1 litrze. Następnie próbki łączono, uzyskując próbę zbiorczą (3 l) dla każdej partii surowca.

Do izolacji mikroorganizmów zastosowano filtrację membranową (przy użyciu sączków o średnicy porów 0,22 µm i filtracji 100 ml wody na każde podłoże hodowlane). Po filtracji sączki zostały wyłożone i inkubowane na selektywnych podłożach stałych w kierunku następujących grup bakterii: *Escherichia coli* i inne *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas* spp., *Legionella* spp., *Enterococcus* spp., *Salmonella* spp. Do oznaczenia ogólnej liczby bakterii wykonano posiewy powierzchniowe wody na agarze odżywczym (Nutrient Agar I, Merck). Wykaz wykorzystanych podłoży i warunki hodowli drobnoustrojów przedstawiono w Tabeli 3.

Po inkubacji zliczano kolonie charakterystyczne dla poszczególnych grup bakterii, wyrażając wyniki jako jednostki tworzące kolonie (jtk) na 100 ml wody oraz jtk/ml dla całkowitej liczby bakterii. Następnie kolonie bakterii przesiano techniką posiewu redukcyjnego na agar tryptonowo-sojowy (Tryptic Soy Agar, TSA; Merck), a uzyskane czyste kultury poddano identyfikacji gatunkowej metodą spektrometrii masowej MALDI-TOF przy użyciu aparatu MALDI Biotyper (Bruker Daltonik GmbH, Brema, Niemcy; certyfikat CE/IVD, zgodnie z dyrektywą 98/79/WE).

Spośród oznaczonych bakterii do kolejnego etapu badań tj. określenia cechy lekooporności wytypowano: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus vulgaris*, *Enterococcus faecalis, Serratia marcescens.* Badanie wrażliwości bakterii przeprowadzono metodą dyfuzyjno–krążkową zgodnie z zaleceniami EUCAST (EUCAST, 2024). Zastosowano antybiotyki (producent: Oxoid; Thermo Scientific) dedykowane dla poszczególnych gatunków: piperacylina (PRL, 30 µg), ceftazydym (CAZ, 10 µg), cefiderokol (FDC, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), tobramycyna (TOB, 10 µg), lewofloksacyna (LVX, 5 µg), cyprofloksacyna (CIP, 5 µg),

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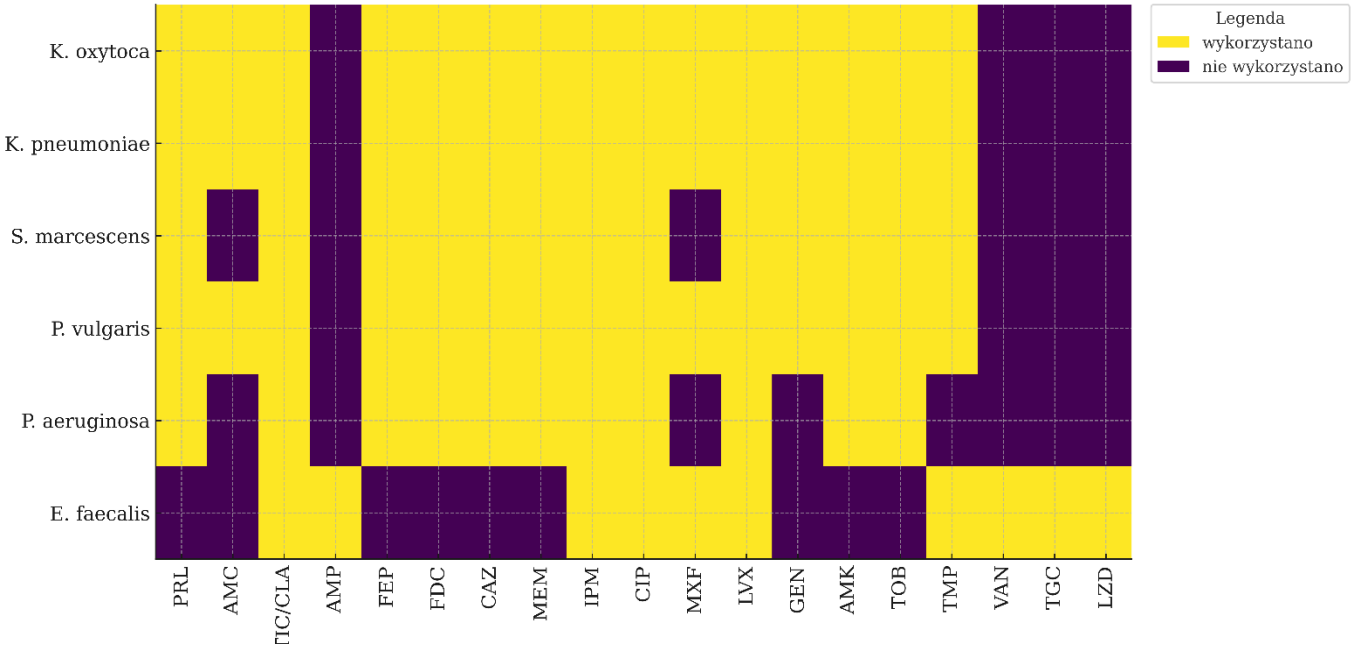
tikarcylina/kwas klawulanowy (TIC/CLA, 75–10 µg), cefepim (FEP, 30 µg), amikacyna (AMK, 30 µg), gentamycyna (GEN, 10 µg), moksyfloksacyna (MXF, 5 µg), amoksycylina/kwas klawulanowy (AMC, 20–10 µg), trimetoprim (TMP, 5 µg), ampicylina (AMP, 2 µg), tygecyklina (TGC, 15 µg), linezolid (LZD, 10 µg), wankomycyna (VAN, 5 µg) (Rysunek 5). Testy przeprowadzono na podłożu Mueller-Hinton Agar (Merck). Średnice stref zahamowania wzrostu (mm) mierzono i podawano jako średnią z dwóch powtórzeń. Na tej podstawie szczepy klasyfikowano jako oporne (R), wrażliwe o zwiększonej ekspozycji (I) lub wrażliwe (S).



Rys. 5. Zestawienie antybiotyków wykorzystanych w analizie fenotypowych profili antybiotykooporności dla poszczególnych szczepów.

3.3.2. Publikacja 3

Do badań wykorzystano materiał roślinny, który obejmował liście, kwiaty, korę, owoce, pędy oraz fusy z kawy i herbaty (Tabela 2, Fotografia 1). Wybór 13 gatunków roślin oparto na doniesieniach literaturowych, w których uzyskane wyniki potwierdzały działanie ekstraktów w badaniach *in vitro*. Surowce roślinne wytypowane do badań prowadzonych w niniejszej pracy nie były dotąd stosowane w zwalczaniu oportunistycznych patogenów wyizolowanych z wód popłucznych.

Tabela 2 Zestawienie surowców wybranych do przygotowania ekstraktów.

Nr	**Nazwa gatunkowa**	**Nazwa polska**	**Rodzaj materiału**
1	*Coffea* L.	kawowiec (rodzaj)	fusy kawowe
2	*Camellia sinensis*	herbata chińska	fusy herbaciane
3	*Urtica dioica* L.	pokrzywa zwyczajna	liście
4	*Tilia cordata* Mill.	lipa drobnolistna	kwiaty
5	*Quercus robur* L.	dąb szypułkowy	kora
6	*Rosa canina* L.	dzika róża	owoce
7	*Betula pendula* Roth	brzoza brodawkowata	kora
8	*Taraxacum officinale* F.H. Wiggers coll.	mniszek lekarski	korzenie
9	*Quercus suber* L.	dąb korkowy	kora
10	*Rubus idaeus* L.	malina właściwa	pędy
11	*Salix alba* L.	wierzba biała	kora
12	*Verbena officinalis*	werbena pospolita	pędy
13	*Pinus sylvestris* L.	sosna zwyczajna	pędy

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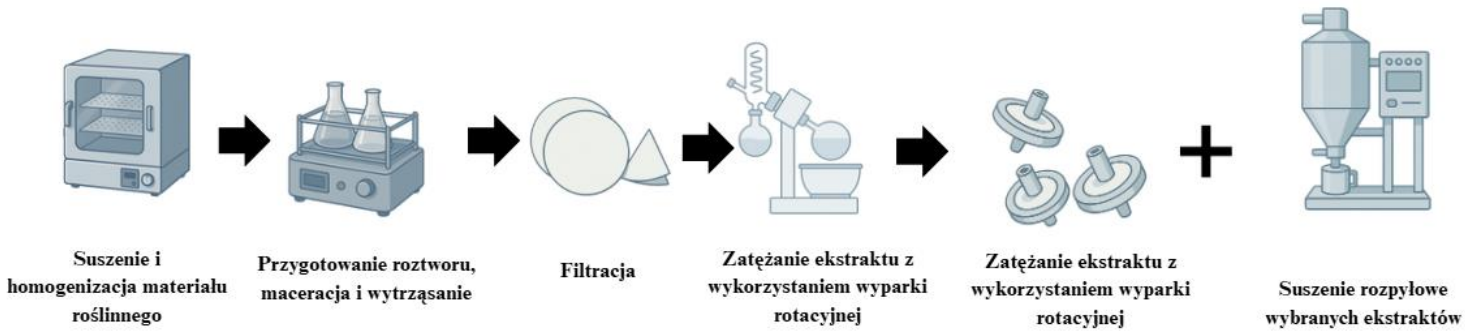
Fot. 1. Surowce roślinne wykorzystane do przygotowania ekstraktów (kolejność zgodnie z Tabelą 2).

Przygotowanie ekstraktów obejmowało 6 głównych etapów, które zostały zilustrowane na rysunku 6.



Rys. 6. Etapy przygotowania ekstraktów roślinnych do analiz (opracowanie własne).

Ekstrakty wodno-etanolowe (70%) sporządzono z 25 g uprzednio wysuszonego (12 h, 30 °C) i rozdrobnionego surowca roślinnego, zalewając go 250 ml rozpuszczalnika i wytrząsając przez 24 h (150 rpm). Po filtracji ekstrakty (150 ml) zatężano trzykrotnie (do 50 ml) przy użyciu wyparki rotacyjnej (65 °C, 90 rpm), a następnie sterylizowano przez sączenie (filtry strzykawkowe o średnicy porów 0,22 µm).

Do oceny przeciwbakteryjnych właściwości ekstraktów wykorzystano metodę studzienkowo-dyfuzyjną. Gęstość inokulum bakteryjnego standaryzowano densytometrycznie (1,0–2,0 × 10^8 jtk/ml; skala McFarlanda) i dodawano do upłynnionego agaru Mueller-Hintona (Merck), który po wymieszaniu wylewano na jałowe szalki Petriego. Po zestaleniu podłoża, wycięto jałowym korkoborem sześć studzienek (ø 8 mm), do których nanoszono po 100 µl badanego ekstraktu. Inkubację prowadzono przez 24 h w 36 ± 0,5 °C, po czym zmierzono strefy zahamowania wzrostu. Następnie ekstrakty, które wykazały właściwości przeciwbakteryjne łączono parami i testowano w układzie skojarzonym, analizując średnie z dwóch powtórzeń.

W kolejnym etapie badań ekstrakty, które wykazały aktywność przeciwdrobnoustrojową w teście dyfuzyjnym poddano suszeniu rozpyłowemu (180 °C – wlot, 91,9 °C – wylot). Stopień odzysku ekstraktu wyrażano jako stosunek masy suchego ekstraktu (C) do masy materiału roślinnego (D).

Następnie, wytypowane ekstrakty pozyskane z surowców bogatych w związki lotne (pędy, kwiaty, fusy) poddano analizie chromatografii gazowej sprzężonej ze spektrometrią mas. Próbki wysuszonych ekstraktów (10 mg) poddano derywatyzacji przez rozpuszczenie w 1 mL bezwodnej pirydyny (99,8%), dodanie 100 µL N,O-bis(trimethylsilyl) trifluoroacetamidu (BSTFA) i inkubację w temperaturze 60 °C przez 30 min, uzyskując pochodne trimetylosililowe (TMS). Po ochłodzeniu roztwory analizowano metodą chromatografii gazowej sprzężonej ze spektrometrią mas (GC–MS) na zestawie Agilent 7890A/5975C z autosamplerem 7693A. Rozdział prowadzono na kolumnie HP-5MS (30 m × 0,25 mm × 0,25 µm) z helem jako gazem nośnym. Program pieca obejmował wzrost temperatury z 50 do 325 °C z gradientem 3 °C/min oraz izotermą końcową 10 min. Udział składników wyznaczano na podstawie całkowitego prądu jonowego (TIC), a wartości podawano jako % TIC.

Identyfikację jakościową prowadzono na podstawie widm masowych oraz indeksów retencji, które wyznaczano w oparciu o czas retencji homologicznej serii n-alkanów analizowanych w tych samych warunkach chromatograficznych. Widma masowe, wraz z obliczonymi wartościami RI (Retention Index), porównywano z bazą NIST oraz biblioteką widm i indeksów retencji pochodnych trimetylosilylowych (TMS).

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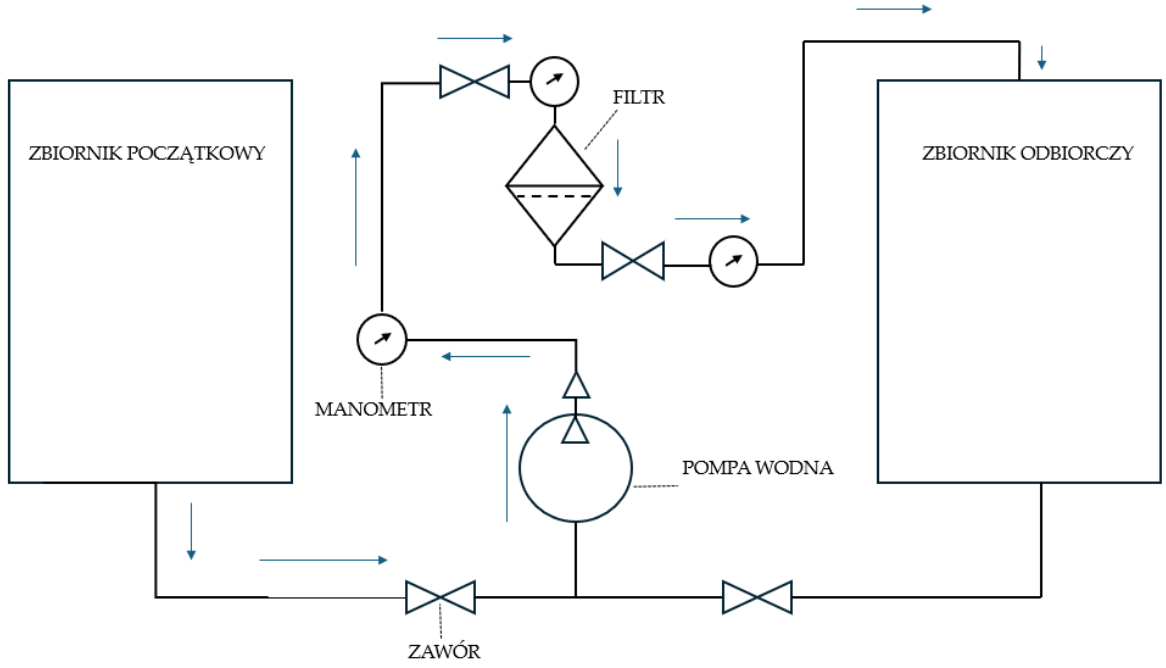
3.3.3. Publikacja 4

Do tego etapu badań (III) wykorzystano ekstrakty roślinne o najwyższej skuteczności przeciwbakteryjnej, które przygotowano zgodnie z procedurą opisaną w publikacji 3, a następnie użyto je do uwodnienia hydrożelu przeznaczonego do dalszych analiz. Zaprojektowany i przygotowany system filtracyjny składał się ze zbiornika początkowego, pompy zapewniającej stały przepływ, zaworów regulujących kierunek i intensywność przepływu, manometrów kontrolujących ciśnienie, filtra przepływowego wypełnionego odpowiednim medium (węgiel aktywny, żywica jonowymienna, hydrożel lub ich mieszanina, w zależności od konfiguracji) oraz zbiornika odbiorczego (Rysunek 7).



Rys. 7. Schemat systemu filtracyjnego.

Do 25 l jałowej wody wprowadzono 1,5 l zawiesin bakteryjnych o gęstości 1×10^8 jtk/ml, uzyskując jednorodne inokulum. Filtrację prowadzono w trzech konfiguracjach złoża (węgiel + żywica, hydrożel, mieszanina), pobierając próbki po 1, 2, 3, 6 i 12 godzinach w celu określenia dynamiki redukcji mikroorganizmów. Walidację systemu filtracyjnego przeprowadzono z użyciem rzeczywistej wody popłucznej pochodzącej z głównego kanału odpływowego zakładu przetwórstwa owoców i warzyw zlokalizowanego w woj. kujawsko-pomorskim.

Efektywność filtracji oceniano na podstawie spadku liczby bakterii w odpływie złoża, wyrażonego jako redukcja $\log_{10}$ jtk/ml. Liczbę jtk/ml określano metodą posiewów seryjnych rozcieńczeń. Uzyskane wyniki poddano obliczeniom statystycznym przy użyciu dwuczynnikowej analizy ANOVA: czynnik A – typ złoża (H, C-IER, H-C-IER), czynnik B – czas poboru (1, 2, 3, 6, 12, 24 h). Różnice istotne statystycznie określano testem Tukeya ($\alpha = 0,05$).

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Tabela 3 Zestawienie podłoży mikrobiologicznych i warunków hodowlanych wykorzystanych we wszystkich etapach badań obejmujących cykl publikacji nr 2 - 4.

Grupa bakterii	Podłoże hodowlane	Warunki inkubacji	Numer publikacji
Escherichia coli i inne Enterobacteriaceae	Agar Endo (Merck)	24 h, 35 ± 0.5 °C	2
Staphylococcus spp.	Chapman-agar (Merck)	48 h, 35 ± 0.5 °C	2
Pseudomonas spp.	Pseudomonas Selective agar + Pseudomonas CN Selective Supplement (Merck)	44 ± 4 h, 25 ± 1 °C	2
Legionella spp.	Legionella BCYE-Agar + Legionella Growth Supplement + Legionella (GVPC) Selective Supplement, (Merck)	10 dni, 36 ± 2 °C	2
Enterococcus spp.	Kanamycin esculin azide agar (Merck)	24 h, 36 °C	2
Salmonella spp.	SS agar (Merck)	24 h, 36 °C	2
Ogólna liczba bakterii	Nutrient Agar I (Merck)	3 dni, 22°C	2, 4
Bakterie testowe –namnażanie i przechowywanie	Tryptic Soy Agar (Merck)	24h, 36 ± 0.5 °C	2, 3, 4
Bakterie testowe – badania lekooporności – badania właściwości przeciw-bakteryjnych ekstraktów roślinnych	Mueller-Hinton Agar (Merck)	24h, 36 ± 0.5 °C	2, 3

Organizacja badań (prac eksperymentalnych i walidacyjnych) prowadzona w ramach niniejszej rozprawy doktorskiej została podzielono na trzy etapy badawcze, które zilustrowano na rysunku 8, z uwzględnieniem publikacji wchodzących w jej skład.

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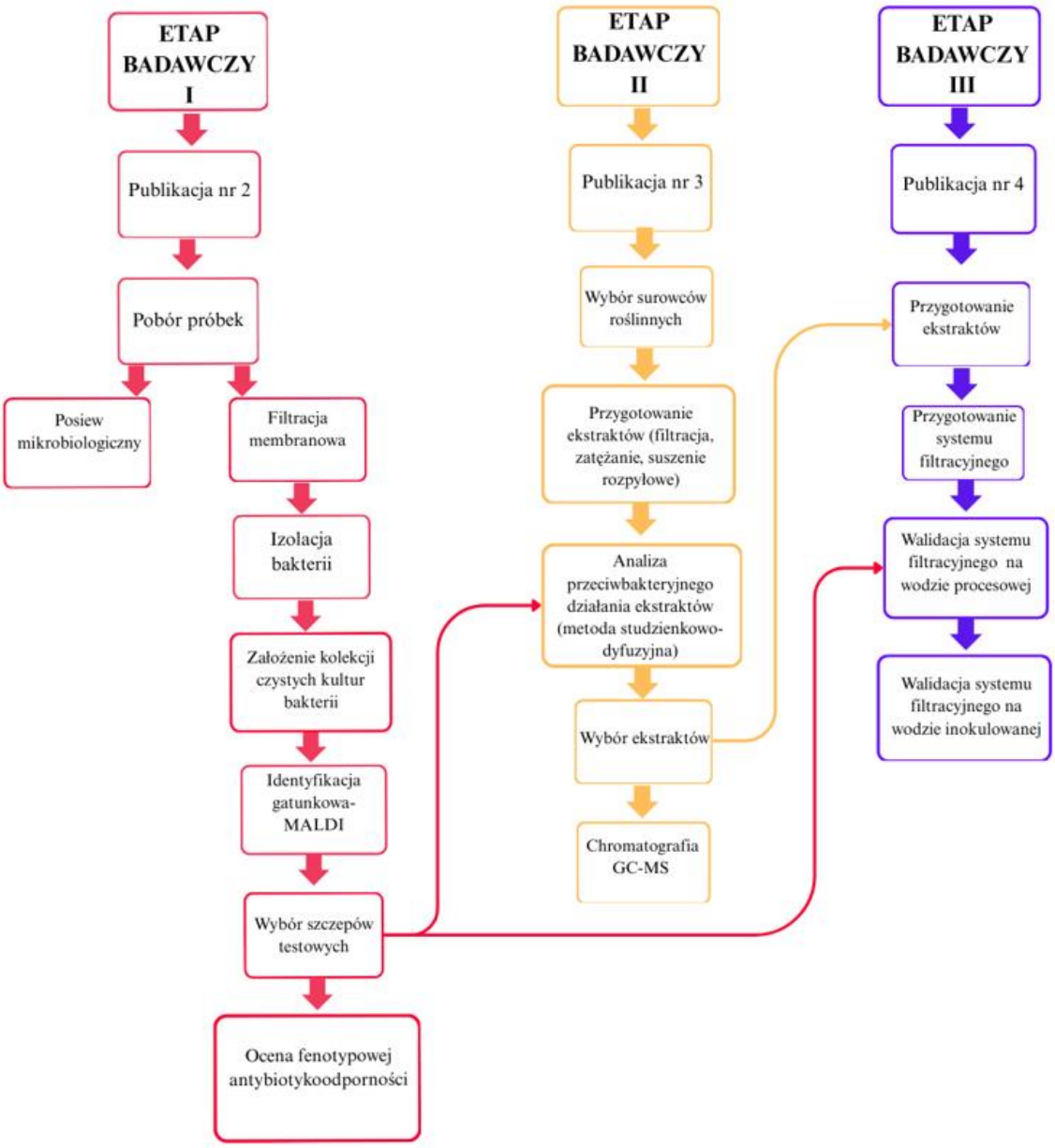


Rys. 8. Etapy przeprowadzonych badań eksperymentalnych i opisanych w cyklu publikacji stanowiących rozprawę doktorską (opracowanie własne).

3.4. OMÓWIENIE PUBLIKACJI STANOWIĄCYCH ROZPRAWĘ DOKTORSKĄ

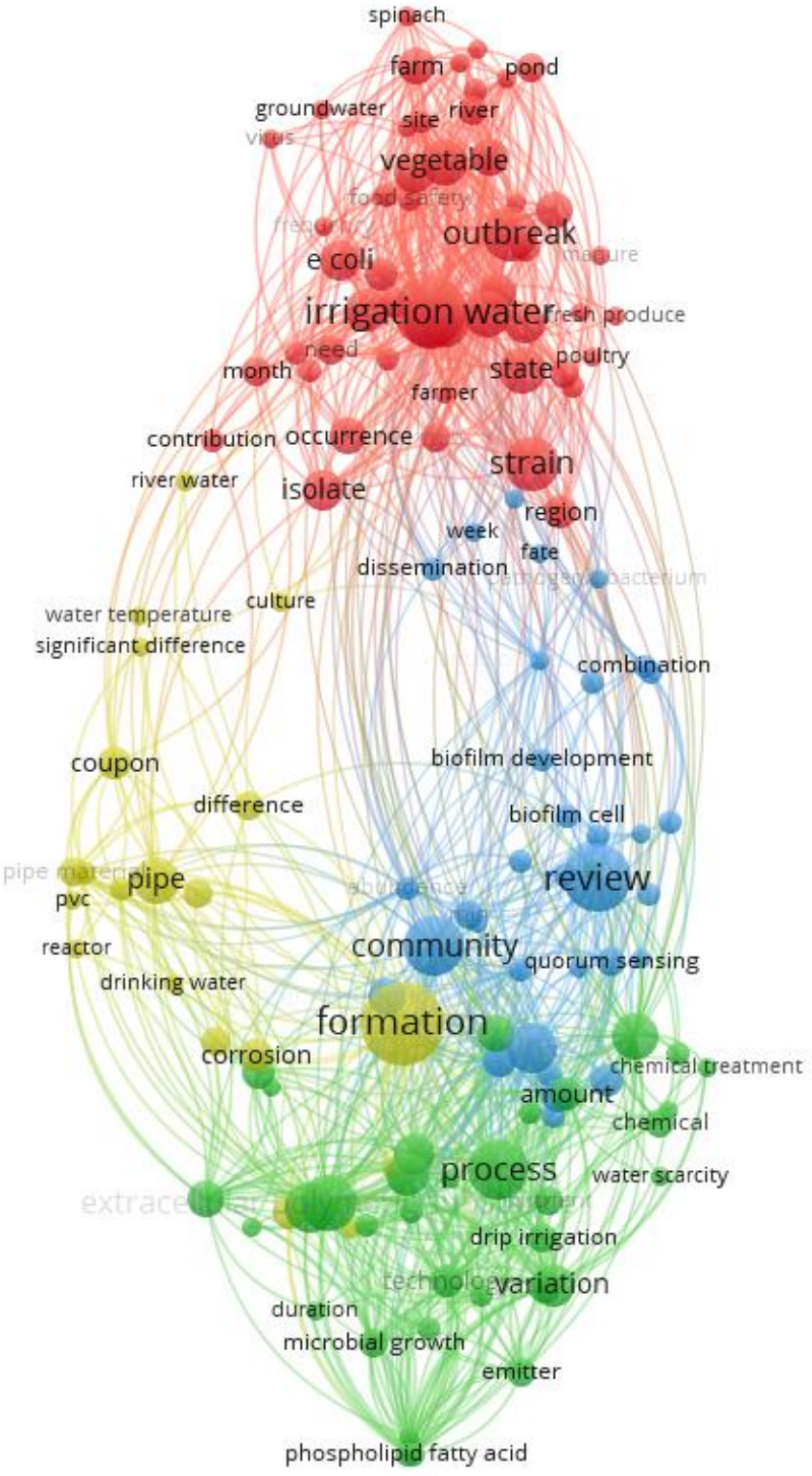
3.4.1. Publikacja 1

Niniejsza praca prezentuje aktualny stan wiedzy na temat zanieczyszczeń mikrobiologicznych w systemach wodnych stosowanych w rolnictwie oraz metod ograniczania rozwoju biofilmu i patogenów. Publikacja przedstawia przeglądową analizę mechanizmów powstawania i dojrzewania biofilmów w rolniczych systemach nawadniania, ich składu mikrobiologicznego oraz czynników środowiskowych i technicznych sprzyjających ich rozwojowi. W pracy, zgodnie z najnowszymi źródłami naukowymi, podkreślono, że biofilmy wpływają negatywnie na hydraulikę i zwiększają straty wody, a zarazem stanowią rezerwuar patogenów roślin, ludzi i zwierząt, co uzasadnia potrzebę ciągłego monitoringu całych systemów irygacyjnych oraz łączenia metod kontroli (chemicznych, fizycznych i biologicznych). Publikacja nr 1 porządkuje i podsumowuje doniesienia o zagrożeniach, źródłach skażenia i strategiach eradykacji, proponując zintegrowane podejście do projektowania, eksploatacji i dezynfekcji instalacji jako podstawę wytycznych zarządzania wodą w rolnictwie.

W oparciu o liczne doniesienia literaturowe w artykule opisano zagrożenia wynikające z wykorzystania skontaminowanej wody w produkcji rolniczej, wskazując je jako jedno z kluczowych źródeł **przedzbiorczej kontaminacji owoców i warzyw**. W świetle aktualnych badań, woda do nawadniania może stanowić rezerwuar patogenów ludzi i roślin (m.in. *E. coli* STEC, *Salmonella spp.*, *Listeria monocytogenes*, *Campylobacter* spp.).

Wykorzystane piśmiennictwo obejmowało publikacje z lat 2000-2023, a źródła pochodziły głównie z baz Scopus, Web of Science i PubMed. Włączano wyniki badań, które zawierały dane ilościowe i jakościowe dotyczące bakterii, charakterystykę biofilmów lub ocenę skuteczności metod dezynfekcji w warunkach laboratoryjnych, in situ bądź półtechnicznych. Artykuł przyjął strukturę tematyczną składającą się z sześciu części: wprowadzenia uwzględniającego uwarunkowania klimatyczne i gospodarcze; charakterystyki biofilmu jako zagrożenia produkcyjnego, szczegółowego opisu etapów formowania biofilmu, omówienia składu mikrobiologicznego biofilmów, przeglądu strategii ich eradykacji oraz podsumowania z rekomendacjami.

Dla zobrazowania struktury tematycznej literatury dotyczącej biofilmów w rolniczych systemach nawadniania przeprowadzono analizę współwystępowania terminów (ang. *co-occurrence analysis*) z użyciem oprogramowania VOSviewer 1.6.20 (2020; Centre for Science and Technology Studies, Leiden University, Holandia). Uzyskana mapa sieciowa (Rysunek 9) prezentuje relacje semantyczne pomiędzy 139 terminami występującymi w tytułach i słowach publikacji, podzielonymi na 4 klastry tematyczne.



Rys. 9. Mapa sieciowa współwystępowania terminów z danych bibliograficznych publikacji nr 1.

3.4.2. Publikacja 2

Etap badawczy I

Uzyskane wyniki z przeprowadzonych badań wykazały, że w próbkach wody popłucznej pobranej z czterech zakładów przetwórstwa owocowo-warzywnego występowały szczepy bakteryjne, reprezentujące szerokie spektrum taksonomiczne. W wodzie po płukaniu ogórków i śliwek stwierdzono obecność między innymi takich gatunków jak S*taphylococcus sciuri* (obecny zarówno w wodzie z ogórków, jak i śliwek) *oraz Micrococcus luteus, Lelliottia amnigena, Enterococcus casseliflavus* i *Comamonas testosteroni*. Natomiast w wodzie pochodzącej z zakładu przetwórstwa cebuli wśród oznaczonych bakterii występowały potencjalnie patogenne *Pseudomonas aeruginosa*, *Enterococcus faecalis* i *Klebsiella pneumoniae*, a także *Enterobacter ludwigii*, *Citrobacter braakii* oraz *Kerstersia gyiorum*. W zakładzie specjalizującym się w przetwórstwie pomidorów, w wodzie popłucznej wykryto m.in. *Pediococcus pentosaceus*, *Klebsiella oxytoca* i *Serratia marcescens*. W próbkach wody pobranych z zakładu przetwórstwa buraków i ogórków stwierdzono obecność m.in. *Proteus vulgaris*, *Providencia alcalifaciens* i *Pseudomonas putida*. **Szczegółowe wyniki dotyczące identyfikacji gatunkowej bakterii zostały zaprezentowane w sekcji: *3.1. Species Identification*.**

Najwyższy poziom kontaminacji bakteryjnej odnotowano w próbce wody po płukaniu buraka ćwikłowego, gdzie ogólna liczba bakterii wyniosła $2{,}54 \times 10^8$ jtk/ml wody. Najniższy poziom zanieczyszczenia bakteryjnego stwierdzono w wodzie po myciu cebuli – $1{,}70 \times 10^5$ jtk/ml wody (**publikacja nr 2, Ryc. 3).**

Dla sześciu wytypowanych i reprezentatywnych dla środowiska wód popłucznych bakterii określono profile lekooporności. W oparciu o przeprowadzone antybiogramy stwierdzono, że *Enterococcus faecalis* wykazał pełną wrażliwość na wszystkie testowane antybiotyki. *Pseudomonas aeruginosa* był wrażliwy m.in. na cefiderokol, meropenem, tobramycynę i amikacynę, natomiast wykazywał pośrednią wrażliwość (I) wobec kilku innych leków, w tym piperacyliny i cefepimu. Szczep *Klebsiella oxytoca* był oporny na cefepim. Natomist *Klebsiella pneumoniae* oraz *Serratia marcescens* wykazywały wrażliwość ze zwiększoną ekspozycją na imipenem. *Proteus vulgaris* okazał się ogólnie wrażliwy, z wyjątkiem klasyfikacji jako I wobec imipenemu. Szczegółowe zestawienie wyników antybiogramów zawiera **sekcja *3.2. Antibiotic Susceptibility,* Tabela 2.**

Podsumowując, badania wykazały zmienność w zanieczyszczeniu mikrobiologicznym wody popłucznej, w zależności od przetwarzanego surowca. Wyniki wskazują również na szerokie zróżnicowanie taksonomiczne bakterii w wodach popłucznych z przetwórstwa owocowo-warzywnego. Wykrycie szczepu *Klebsiella oxytoca* opornego na cefepim wskazuje, że niezbędne jest stałe monitorowanie jakości mikrobiologicznej wód używanych do płukania surowców oraz wdrażanie skutecznych procedur sanitarnych w zakładach przetwórczych.

3.4.3 Publikacja 3

Etap badawczy II

Na podstawie przeprowadzonych badań stwierdzono, że wybrane ekstrakty roślinne charakteryzowały się zróżnicowaną skutecznością przeciwdrobnoustrojową w zależności od badanego szczepu **(sekcja: *3.1. Evaluation of Extract Activity* Tabela 2)**. Spośród 13 testowanych ekstraktów największą efektywność przeciwbakteryjną uzyskano dla ekstraktu z zielonej herbaty (*Camellia sinensis*) i maliny zwyczajnej (*Rubus idaeus*), których strefy zahamowania dochodziły do 8,0 mm wobec *P. aeruginosa* i *E. faecalis*. Ekstrakty z kory dębu (*Quercus robur*), kory brzozy (*Betula pendula)*, kwiatów lipy (*Tilia cordata*) i kory wierzby (*Salix alba*) wykazały również aktywność przeciwdrobnoustrojową, w granicach od 2,0 mm do 6,0 mm. Badania nie potwierdziły działania przeciwdrobnoustrojowego dla sześciu ekstraktów z: fusów kawy (*Coffea* spp.), owoców róży (*Rosa canina*), korzenia mniszka (*Taraxacum officinale*), kory dębu korkowego (*Quercus suber*), ziela werbeny (*Verbena officinalis*) oraz pędów sosny (*Pinus sylvestris*). Również ekstrakt z liści pokrzyw (Urtica dioica) charakteryzował się niską skutecznością w hamowaniu wzrostu *P. aeruginosa* (strefa inhibicji < 1,0 mm). W oparciu o uzyskane wyniki wytypowano najbardziej skuteczne ekstrakty o właściwościach przeciwdrobnoustrojowych (z fusów herbaty, pędów malin, kory dębu, brzozy i wierzby oraz kwiatów lipy drobnolistnej) wobec wszystkich testowanych szczepów bakterii. Następnie przeprowadzono kolejne testy w celu sprawdzenia ich synergistycznego działania **(publikacja nr 3, Tabela 3).**

Największy wzrost strefy zahamowania odnotowano dla kombinacji *Q. robur* + *B. pendula* wobec *P. vulgaris* (125%), w porównaniu do działania pojedynczych ekstraktów. Wzrost strefy inhibicji o 50% uzyskano dla *B. pendula* + *S. alba* wobec *S. marcescens* oraz *T. cordata* + *R. idaeus* wobec *P. aeruginosa*. Znaczny wzrost strefy inhibicji o 33% stwierdzono również dla kombinacji ekstraktów z *T. cordata* + *B. pendula* wobec *K. oxytoca* oraz *Q. robur* + *R. idaeus* wobec *K. pneumoniae*. Ponadto wzmożone działanie wykazano dla połączenia *Q. robur* + *R. idaeus*, a strefa inhibicji wzrosła o 8% wobec *E. faecalis*. Pozostałe kombinacje (zgodnie z **Tabelą 3, publikacja 3)** nie zwiększały średnicy stref zahamowania, co może wynikać z ograniczonej kompatybilności związków czynnych lub braku efektu addytywnego **(publikacja 3, Rys. 3, Tabela 5).**

Dalszym etapem badań była analiza profilu chemicznego ekstraktów metodą GC-MS, mająca na celu identyfikację związków odpowiedzialnych za obserwowane efekty antybakteryjne. Analiza chromatograficzna GC-MS wykazała obecność 203, 223 i 239 pików, odpowiednio dla ekstraktów z herbaty, lipy i malin. Po przeprowadzonej analizie zidentyfikowano 347 związków: 110 związków w ekstrakcie z lipy, 125 w ekstrakcie z malin oraz 112 w ekstrakcie z herbaty. **Szczegółowy wykaz zidentyfikowanych związków, czasu retencji, indeksów retencji oraz TIC (%) dla każdego ekstraktu znajdują się w materiałach uzupełniających (S1) w publikacji nr 3.** Analiza chromatograficzna wykazała, że w ekstrakcie z lipy występowały liczne związki bioaktywne o charakterze potencjalnie bakteriobójczym, m.in. kwas szikimowy, kwas chinowy, inozytol, kwas p-kumarynowy, epikatechina i kwas galusowy. W ekstrakcie z pędów malin stwierdzono obecność kwasu elagowego, kwasu galusowego, kwasu chlorogenowego, kwasu kawowego, jak również kwercetyny. Analiza ekstraktu z herbaty wykazała obecność epikatechiny,

katechiny, galusanu epikatechina, kwasu galusowego, kwasu szikimowego i kwercetyny, które również posiadają potwierdzone działanie przeciwdrobnoustrojowe **(szczegółowe informacje zawarte zostały w publikacji nr 3, Tabela 6).**

Przeprowadzone badania potwierdzają potencjał ekstraktów roślinnych jako źródła związków o aktywności przeciwdrobnoustrojowej, zdolnych zarówno do samodzielnego działania, jak i do wykazywania efektów synergistycznych. Profilowanie GC-MS umożliwiło wskazanie szerokiego spektrum związków, których część wykazuje aktywność biologiczną i stanowi podstawę do dalszych prac nad opracowaniem bioaktywnych materiałów filtracyjnych, ukierunkowanych na poprawę bezpieczeństwa mikrobiologicznego wód popłucznych.

3.4.4. Publikacja 4

Etap badawczy III

Uzyskane wyniki badań wykazały, że efektywność redukcji obciążenia mikrobiologicznego była zależna od zastosowanej konfiguracji materiałów filtracyjnych. Różnice te ujawniały się już w początkowych interwałach i utrzymywały się w czasie, co potwierdziła analiza statystyczna. Najbardziej efektywny okazał się układ zawierający hydrożel, węgiel aktywny i żywicę jonowymienną.

Na podstawie przeprowadzonych badań stwierdzono, że filtr kompozytowy H–C–IER, w pierwszej godzinie filtracji przyczynił się do redukcji liczebność bakterii w wodzie o 2,23 $\log_{10}$, co przełożyło się na spadek liczby bakterii o 99,41%. Analiza próbek pobranych w ciągu trzech kolejnych punktów czasowych, wykazała utrzymanie redukcji powyżej 1,7 $\log_{10}$, co skutkowało spadkiem liczebności bakterii w wodzie o ponad 98,00%. W ostatnim punkcie czasowym efektywność filtracji wyniosła 90,23%. Niższe wartości odnotowano dla konfiguracji C–IER, która ograniczyła liczebność drobnoustrojów o 1,51 $\log_{10}$ w pierwszej godzinie, po czym ustabilizowała się poniżej 1 $\log_{10}$. Uzyskane wyniki wskazują, że w konfiguracji złoża C-IER dochodziło do szybkiego wykorzystania najbardziej reaktywnych miejsc sorpcyjnych. Układ filtracyjny wypełniony hydrożelem (H) zapewniał stałą, lecz niską redukcję (~0,8 $\log_{10}$), co przekładało się na spadek liczby bakterii w testowanej wodzie o ~84 %. Uzyskane wyniki potwierdziły, że dodanie węgla aktywnego i żywicy jonowymiennej nie tylko wprowadziło dodatkowe mechanizmy usuwania (sorpcja, wymiana jonowa), lecz wydłużało także efektywny czas kontaktu fazy wodnej z hydrożelem, poprzez równomierniejsze rozdzielenie strumienia, większą porowatość czynną i eliminację szybkiego przepływu.

Analiza trendów czasowych wykazała, że nachylenie linii regresji dla układu opartego wyłącznie na hydrożelu było niskie (–0,0009), co oznacza stabilny, lecz niski poziom ochrony. W przypadku konfiguracji z węglem aktywnym i żywicą jonowymienną zaobserwowano umiarkowany spadek skuteczności (–0,0177), co odpowiadało częściowemu wysyceniu warstw aktywnych w miarę postępu filtracji. Największą dynamikę spadku odnotowano w filtrze warstwowym z hydrożelem, węglem i żywicą (–0,0487), co mimo najwyższych wartości redukcji bakterii na początku, wskazuje na wyraźne zjawisko wysycenia przy długotrwałej pracy na wodzie silnie obciążonej mikrobiologicznie.

Analiza potwierdziła istotne różnice między układami: konfiguracja z węglem aktywnym i żywicą jonowymienną była mniej skuteczna od filtra warstwowego z udziałem hydrożelu. Dodatkowo, poziomy czynnika czasu wskazują na stopniowe wysycanie złoża i wynikający z tego spadek zdolności przechwytywania mikroorganizmów.

W przypadku próbek wody z zakładu przetwórczego, filtr warstwowy z hydrożelem, węglem aktywnym i żywicą jonowymienną obniżał liczebność bakterii w pierwszych czterech interwałach do $1{,}1 \times 10^2$–$1{,}0 \times 10^3$ jtk/ml, co odpowiadało 1,2–2,0 $\log_{10}$ (około 84–99% redukcji), z maksimum bliskim 2,0 $\log_{10}$ po 1 godzinie (~99%). Od trzeciego punktu czasowego następowała stabilizacja redukcji bakterii na poziomie ~0,8–1,2 $\log_{10}$, czyli około 84–94%, co wskazuje na utrzymującą się, choć malejącą w czasie skuteczność układu. Dodatkowo, próbki wody z zakładu przetwórstwa rolno-spożywczego wykazywały zróżnicowaną morfologię bakteryjną (ziarniaki, pałeczki, gronkowce), charakterystyczną dla wody

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popłucznej z linii mycia warzyw i owoców, co również podkreśla praktyczny charakter walidacji systemu. Wyniki wskazują, że układ H–C–IER działał jako skuteczna bariera mikrobiologiczna w procesie ograniczania kontaminacji mikrobiologicznej wód popłucznych przemysłu rolno-spożywczego, zapewniając poprawę jakości wody. Proponowane rozwiązanie stanowi technologicznie proste uzupełnienie istniejących układów uzdatniania, zwłaszcza tam, gdzie kluczowe jest ograniczenie chlorowania. Z perspektywy eksploatacyjnej oznacza to, że filtr warstwowy powinien pracować w układzie z okresowym płukaniem bądź wymianą warstw aktywnych, najlepiej poprzedzony prostą prefiltracją ograniczającą dopływ zawiesin. Utrzymanie redukcji na poziomie co najmniej 1 $\log_{10}$ w dłuższym horyzoncie czasu, przy jednoczesnym osiąganiu 1,5–2,0 $\log_{10}$ w pierwszych godzinach, można traktować jako realistyczne kryterium operacyjne dla wód popłucznych o wysokim obciążeniu mikrobiologicznym. **Szczegółowe informacje zostały zawarte w publikacji nr 3, sekcje: 3.1. *Results for inoculated water, 3.2. Results for agro-industrial process water*.**

3.5. PODSUMOWANIE I WNIOSKI

Na podstawie uzyskanych wyników sformułowano następujące wnioski:

1. W badanych próbkach wody popłucznej z zakładów owocowo-warzywnych wykazano występowanie zarówno bakterii saprotroficznych, jak i patogenów oportunistycznych.

2. Spośród zidentyfikowanych szczepów, do dalszych badań wytypowano następujące patogeny: *P. aeruginosa*, *P. vulgaris, K. oxytoca*, *K. pneumoniae*, *S. marcescens* i *E. faecalis*. Przeprowadzona analiza wybranych profili fenotypowej antybiotykooporności wykazała wrażliwość testowanych bakterii na użyte antybiotyki, jedynie szczep *K. oxytoca* był oporny na cefepim.

3. Ekstrakty z fusów herbaty (*C. sinensis*), pędów malin (*R. idaeus*), kory dębu (*Q. robur)*, kory brzozy (*B. pendula*), kwiatów lipy (*T. cordata*) oraz kory wierzby (*S. alba)* wykazywały właściwości inhibitujące wzrost testowanych patogenów oportunistycznych izolowanych z wód popłucznych.

4. Zastosowanie kombinacji ekstraktów roślinnych (*B. pendula* + *S. alba*, *T. cordata* + *R. idaeus*, *T. cordata* + *B. pendula*, *Q. robur* + *R. idaeus*) sprzyjało nasileniu efektu hamowania wzrostu drobnoustrojów, co wskazuje na możliwość efektu skojarzeniowego pomiędzy związkami bioaktywnymi.

5. Zastosowanie różnych konfiguracji warstw filtracyjnych (żywica jonowymienna, węgiel aktywny, hydrożel) pozwoliło na identyfikację układów charakteryzujących się najwyższą skutecznością antybakteryjną, co umożliwiło optymalizację procesu filtracji i wskazanie najbardziej obiecujących wariantów do dalszych badań.

6. Dodanie do tradycyjnych komponentów filtracyjnych, takich jak: węgiel aktywny i żywica jonowymienna warstwy hydrożelu nasyconego naturalnymi ekstraktami roślinnymi przyczyniło się do zwiększenia skuteczności redukcji obciążenia bakteryjnego w wodzie popłucznej.

7. Badania pilotażowe potwierdziły możliwość praktycznego wdrożenia rozwiązania filtracyjnego w przemyśle owocowo-warzywnym jako alternatywy dla metod dezynfekcji opartych na związkach chloru.

Uwzględniając założenie pomocnicze i hipotezy pomocnicze stwierdza się:

1. Wytypowane izolaty bakteryjne, pozyskane bezpośrednio z wód popłucznych przemysłu rolno-spożywczego (*P. aeruginosa*, *P. vulgaris, K. oxytoca*, *K. pneumoniae*, *S. marcescens*, *E. faecalis)*, okazały się odpowiednim modelem do prowadzonych badań nad technologią bezchlorowego uzdatniania. Ich wybór był uzasadniony zarówno miejscem izolacji, jak i istotnym znaczeniem mikrobiologicznym w kontekście bezpieczeństwa sanitarnego. Uzyskane wyniki potwierdziły, że szczepy te cechują

się stabilnością hodowlaną oraz pozwalają na ocenę skuteczności zastosowanych składników bioaktywnych pochodzenia roślinnego. **(A1).**

2. Przeprowadzone badania wykazały, że kluczowym czynnikiem ograniczającym liczebność mikroorganizmów wskaźnikowych podczas procesu filtracji były substancje bioaktywne obecne w zastosowanym materiale roślinnym. Analiza porównawcza skuteczności filtracji z użyciem materiału bez ekstraktów roślinnych (C-IER) oraz filtrów wzbogaconych w składniki roślinne (H-C-IER) potwierdziła wyższą redukcję badanych drobnoustrojów w obecności tych związków. Wyniki wskazują, iż mechanizm eliminacji nie był wyłącznie efektem samej bariery fizycznej, lecz wynikał z właściwości przeciwdrobnoustrojowych materiału roślinnego (**H1**).

3. Badania mikrobiologiczne potwierdziły, że zastosowanie połączenia surowców roślinnych, produkujących zróżnicowane związki bioaktywne, pozwalało na skuteczniejszą redukcję liczebności bakterii niż wykorzystanie pojedynczych komponentów. Tym samym wykazano zasadność celowego łączenia surowców – połączenia *B. pendula* + *S. alba*, *T. cordata* + *R. idaeus*, *T. cordata* + *B. pendula*, *Q. robur* + *R. idaeus* zwiększyły strefy zahamowania wzrostu w porównaniu do testowania pojedynczych ekstraktów, co potwierdza słuszność wielokryterialnego doboru materiałów i zasadność stosowania kompozytowych złóż filtracyjnych (**H2**).

4. Przeprowadzone badania mikrobiologiczne wykazały, że filtracja na zastosowanym złożu skutkowała spadkiem liczby bakterii w badanej wodzie. Nie odnotowano przypadków zwiększenia liczebności drobnoustrojów po procesie filtracji, co jednoznacznie potwierdza, że stan mikrobiologiczny wody nie uległ pogorszeniu. Redukcja mikroorganizmów wskaźnikowych stanowi potwierdzenie skuteczności zastosowanego rozwiązania w kontekście bezchlorowego uzdatniania wód popłucznych przemysłu rolno-spożywczego. (**H3**).

Pozytywna weryfikacja wszystkich trzech hipotez pomocniczych potwierdziła hipotezę badawczą o efektywności złoża filtracyjnego opartego na materiałach roślinnych w ograniczaniu zanieczyszczenia bakteryjnego wód popłucznych bez użycia chloru.

Reasumując, przeprowadzone badania dowiodły, że wody popłuczne przemysłu owocowo-warzywnego stanowią istotne źródło zanieczyszczeń mikrobiologicznych, w tym bakterii opornych na antybiotyki i patogenów oportunistycznych. Wykazano, że zastosowanie ekstraktów roślinnych (*C. sinensis*, *R. idaeus*, *Q. robur*, *B. pendula*, *T. cordata, S. alba*) skutecznie ogranicza wzrost drobnoustrojów, przy czym efekt ten był wyraźnie wzmacniany podczas łączenia ekstraktów. Najwyższą skutecznością charakteryzowały się złoża kompozytowe, łączące tradycyjne komponenty filtracyjne (węgiel aktywny, żywica jonowymienna) z hydrożelem nasyconym ekstraktami roślinnymi. Badania przeprowadzone w skali półtechnicznej dowiodły, że takie rozwiązanie zapewnia

skuteczną redukcję liczby mikroorganizmów w wodzie i może stanowić praktyczną alternatywę dla metod dezynfekcji opartych na chlorze.

Pozytywna weryfikacja hipotez pomocniczych potwierdziła hipotezę badawczą, zgodnie z którą złoże filtracyjne zawierające materiały pochodzenia roślinnego stanowi efektywne narzędzie ograniczające zanieczyszczenia mikrobiologiczne w wodach popłucznych przemysłu rolno-spożywczego. Rozwiązanie to wpisuje się w koncepcję zielonych technologii oraz zrównoważonego gospodarowania zasobami wodnymi. Uzyskane wyniki wskazują na możliwość dalszej rozbudowy systemu filtracyjnego poprzez integrację dodatkowych elementów wspomagających, takich jak zbiorniki sedymentacyjne, moduły flotacyjne oraz warstwy sorpcyjne o zróżnicowanej granulacji. Uzupełnienie układu o wstępne procesy klarowania pozwoliłoby na ograniczenie obciążenia materią organiczną i zawiesinami, co mogłoby przełożyć się na wydłużenie czasu efektywnej pracy złoża oraz zwiększenie skuteczności redukcji mikroorganizmów. W dłuższej perspektywie rozwój takich systemów może prowadzić do opracowania wieloetapowych instalacji, które będą mogły znaleźć praktyczne zastosowanie w zakładach przemysłu owocowo-warzywnego jako zintegrowane rozwiązania wspierające gospodarkę wodną w kontekście implementacji zasad gospodarki o obiegu zamkniętym.

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4. STRESZCZENIE

Opracowanie innowacyjnej technologii bezchlorowego uzdatniania wód popłucznych przemysłu rolno-spożywczego z wykorzystaniem składników bioaktywnych pochodzenia roślinnego

mgr inż. Piotr Kanarek

Słowa kluczowe: wody popłuczne**,** przemysł owocowo-warzywny, kontaminacja mikrobiologiczna, uzdatnianie wody, ekstrakty roślinne

Sektor przetwórstwa owocowo-warzywnego, silnie powiązany z rolnictwem, generuje znaczące ilości wód popłucznych po procesach mycia i płukania surowca, charakteryzujących się wysoką kontaminacją mikrobiologiczną. W niniejszej rozprawie oceniono potencjał materiałów pochodzenia roślinnego jako wypełnienia złoża antybakteryjnego w bezchlorowym systemie uzdatniania wód popłucznych, łącząc badania podstawowe z walidacją półtechniczną. Celem badań była kompleksowa weryfikacja hipotezy, stanowiącej, że wykorzystanie materiałów pochodzenia roślinnego w wypełnieniu złoża antybakteryjnego pozwala na skuteczne unieczynnienie lub znaczne ograniczenie liczby bakterii bytujących w wodach popłucznych przemysłu owocowo-warzywnego, co przekłada się na zmniejszenie zastosowania dezynfekcji chlorowej. Badanie obejmowało trzy etapy: (I) izolację i identyfikację szczepów z wód popłucznych czterech zakładów (filtracja membranowa, identyfikacja MALDI-TOF) oraz ocenę fenotypowej lekowrażliwości zgodnie z EUCAST; (II) przygotowanie wodno-etanolowych ekstraktów z 13 surowców roślinnych, oznaczenie ich aktywności antybakteryjnej metodą studzienkowo-dyfuzyjną oraz wytypowanie 6 najskuteczniejszych ekstraktów wraz z profilowaniem fitochemicznym techniką GC-MS; (III) półtechniczną weryfikację trzech zaprojektowanych konfiguracji złoża w wodzie modelowej i popłucznej z monitorowaniem zmian liczebności bakterii. Analiza próbek wód popłucznych z wytypowanych zakładów przetwórstwa owocowo-warzywnego wykazała obecność zróżnicowanych gatunków bakterii, w tym oportunistycznych patogenów o znaczeniu sanitarnym: *Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae, K. oxytoca, Serratia marcescens, Proteus vulgaris*. Analiza lekowrażliwości bakterii potwierdziła obecność szczepu opornego na cefepim. Spośród testowanych ekstraktów najwyższą aktywność przeciwbakteryjną wykazały ekstrakty z herbaty (*Camellia sinensis*) i pędów malin (*Rubus idaeus)* (wobec *P. aeruginosa* i *E. faecalis*), umiarkowaną – ekstrakty z kory dębu (*Quercus robur*), *kory brzozy (Betula pendula),* kwiatu lipy (*Tilia cordata*),

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kory wierzby (*Salix alba*); pozostałe 7 surowców nie wykazało aktywności przeciwdrobnoustrojowej. W wyniku analizy chromatografii gazowej sprzężonej ze spektrometrią mas stwierdzono obecność 347 związków, w tym związków bioaktywnych (m.in. epikatechiny, kwasu szikimowego, chinowego, galusowego oraz kofeiny), których obecność może wskazywać na obserwowane zjawisko inhibicji. Najwyższą redukcję bakterii osiągnięto w filtrze kompozytowym, składającym się z węgla aktywnego, hydrożelu z ekstraktami roślinnymi oraz żywicy jonowymiennej. Przeprowadzone badania pozwalają stwierdzić, że wybrane ekstrakty roślinne skutecznie hamowały wzrost bakterii oportunistycznych z wód popłucznych, a ich immobilizacja w warstwie hydrożelowej wzmacniała działanie układu filtracyjnego. Badania te dostarczają również istotnych informacji o potencjalnym wymiarze aplikacyjnym. Wdrożenie zaprojektowanego złoża filtracyjnego, opartego na bioaktywnych ekstraktach immobilizowanych w hydrożelu i połączonych z warstwami węgla aktywnego oraz żywicy jonowymiennej, może stanowić skuteczną, bezchlorową alternatywę dla dominujących obecnie chemicznych metod oczyszczania wody w środowisku produkcyjnym zakładów rolno-spożywczych.

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5. ABSTRACT

Development of an innovative chlorine-free technology for the treatment of wash water from the agri-food industry using bioactive plant-based components

Piotr Kanarek, MSc

Key words: wash water, fruit and vegetable industry, microbiological contamination, water treatment, plant extracts

The fruit- and vegetable-processing sector, closely linked to agriculture, generates substantial volumes of wash and rinse effluents that exhibit high levels of microbiological contamination. This dissertation evaluates the potential of plant-derived materials as antibacterial bed fillers in a chlorine-free wash water treatment system, combining fundamental studies with semi-technical validation. The aim of the research was a comprehensive verification of the hypothesis that the use of plant-derived materials in antibacterial filter beds enables effective inactivation or a substantial reduction of bacterial populations in wash water from the fruit and vegetable industry, thereby decreasing the need for chlorine-based disinfection. The study consisted of three stages: (I) isolation and identification of bacterial strains from wash water collected in four processing plants (membrane filtration, MALDI-TOF identification) and assessment of phenotypic antimicrobial susceptibility according to EUCAST guidelines; (II) preparation of aqueous-ethanolic extracts from 13 plant materials, evaluation of antibacterial activity using the well-diffusion method, analysis of synergistic effects of selected pairs, and phytochemical profiling by GC-MS; (III) semi-technical verification of three designed bed configurations in both model and process water, with monitoring of changes in bacterial counts. Analysis of wash-water effluent samples from the selected fruit- and vegetable-processing plants revealed a heterogeneous spectrum of bacterial species, including opportunistic pathogens of sanitary relevance: *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *K. oxytoca*, *Serratia marcescens*, and *Proteus vulgaris.* Antimicrobial susceptibility testing revealed the presence of a strain resistant to cefepime. Among the tested extracts, the highest antibacterial activity was observed for tea (*Camellia sinensis*) and raspberry shoots (*Rubus idaeus*) (against *P*. *aeruginosa* and *E*. *faecalis*). Moderate activity was found in extracts from oak bark (*Quercus robur*), birch bark (*Betula pendula*), linden flowers (*Tilia cordata*),

and willow bark (*Salix alba*). The remaining seven raw materials showed no antimicrobial effect. Gas chromatography–mass spectrometry confirmed the presence of 347 compounds, including bioactive molecules (e.g., epicatechin, shikimic acid, quinic acid, gallic acid, and caffeine), which were associated with the observed inhibition. The highest bacterial reduction was obtained with a composite filter consisting of activated carbon, a hydrogel with immobilized plant extracts, and ion-exchange resin. The conducted research demonstrates that selected plant extracts effectively inhibited the growth of opportunistic bacteria isolated from wash water, and their immobilization within the hydrogel layer significantly enhanced filter performance. These findings also provide important insights into potential practical applications. The implementation of the designed filtration bed, based on bioactive extracts immobilized in hydrogel and combined with activated carbon and ion-exchange resin layers, may serve as an effective chlorine-free alternative to the currently dominant chemical methods of water treatment in agri-food production facilities.

6. ŻYCIORYS NAUKOWY I WYKAZ POZOSTAŁYCH OSIĄGNIĘĆ NAUKOWYCH

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1. **Kanarek P**., Breza-Boruta B., Bauza-Kaszewska J., 2021. Evaluation of the hygienisation effectiveness of municipal waste composting process. Acta Sci. Pol. Agric. 20(2), 51-60. (20 pkt MNiSW).
2. Breza-Boruta B., **Kanarek P**., 2021. Effect of disinfectants and EM preparations on the growth inhibition of Lecanicillium fungicola occurring in cultivation of button mushroom (Agaricus bisporus). Acta Sci. Pol. Agric. 20(2), 61-70. (20 pkt MNiSW).
3. Kotwica K., Breza-Boruta B., Bauza-Kaszewska J., **Kanarek P**., Jaskulska I., Jaskulski D., 2021. The cumulative effect of various tillage systems and stubble management on the biological and chemical properties of soil in winter wheat monoculture. Agronomy 11(9), 1726, 1-16. (IF: 3.949, 100 pkt MNiSW).
4. **Kanarek P.,** Bogiel T., Breza-Boruta B., 2022. Legionellosis risk—an overview of Legionella spp. habitats in Europe. Environ. Sci. Pollut. Res. 29, 1-11. (IF: 5.800 , 100 pkt MNiSW).
5. **Kanarek P**., Breza-Boruta B., Bauza-Kaszewska J., Lamparski R., 2022. Application of straw and biopreparations as a sustainable method for increasing the organic carbon content and chemical, physical, and biological soil properties in spring barley culture. Energies 15(19), 6903, 1-17. (IF: 3.200, 140 pkt MNiSW).
6. Bogiel T., Mikucka A., **Kanarek P**., 2022. Agarose gel electrophoresis-based RAPD-PCR—an optimization of the conditions to rapidly detect similarity of the alert

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pathogens for the purpose of epidemiological studies. Gels 8(12), 760, 1-8. (IF: 4.600, 20 pkt MNiSW).

7. **Kanarek P**., Breza-Boruta B., Pawłowski M., Kobierski M., 2023. Evaluation of microbiological and chemical properties of soils as a result of anthropogenic denudation. Agriculture 13(12), 2247, 1-17. (IF: 3.300, 140 pkt MNiSW).
8. Bogiel T., Depka D., Kruszewski S., Rutkowska A., **Kanarek P.,** Rzepka M., Leitao J.H., Deptuła A., Gospodarek-Komkowska E., 2023. Comparison of virulence-factor-encoding genes and genotype distribution amongst clinical Pseudomonas aeruginosa strains. Int. J. Mol. Sci. 24(2), 1269, 1-13. (IF: 4.900, 140 pkt MNiSW, współpraca międzynarodowa).
9. Bogiel T., Dura A., Woźniak M., Mikucka A., **Kanarek P**., 2024. Usefulness of capillary gel electrophoresis-based PCR for detection of Clostridioides difficile strains with hypervirulent ribotypes. Gels 10(5), 343, 1-16. (IF: 5.300 , 20 pkt MNiSW).
10. Bogiel T., Kwiecińska P., Górniak R., **Kanarek P.,** Mikucka A., 2024. The use of gel electrophoresis to separate multiplex polymerase chain reaction amplicons allows for the easy identification and assessment of the spread of toxigenic Clostridioides difficile strains. Gels 10(12), 818, 1-16. (IF: 5.300 , 20 pkt MNiSW).
11. **Kanarek P.,** Breza-Boruta B., Poćwiardowski W., Szulc J., 2024. Sustainable water use in a fruit processing plant: evaluation of microbiological and physicochemical properties of wash water after application of a modular water recovery system. Sustainability 16(5), 2181, 1-14. (IF: 3.300 , 100 pkt MNiSW).
12. Breza-Boruta B., **Kanarek P**., Paluszak Z., Kaczorowska A.-K., Gryń G., 2024. Sustainable livestock production: screening analysis and pilot implementation of a biofilm in piggery biofilters for mitigation of ammonia and hydrogen sulfide emissions. Agriculture 14(6), 906, 1-14. (IF: 3.600 , 100 pkt MNiSW).
13. Bogiel T., Górniak T., Ambroziak W., Zieliński S., Zieja D.A., **Kanarek P**., 2024. High-resolution melting PCR as a fast and simple molecular biology-based method for the identification of hypervirulent Clostridioides difficile strains directly in stool samples. Microorganisms 12(11), 2228, 1-13. (IF: 4.200 , 40 pkt MNiSW).
14. Paluszak Z., **Kanarek P**., Gryń G., Breza-Boruta B., 2024. Deodorizing bacterial consortium: community analysis of biofilms and leachate water collected from an air biofiltration system in a piggery. Environ. Sci. Pollut. Res. 31, 18993-19001. (100 pkt MNiSW).

Szczególnie cenię sobie publikację przeglądową *Legionellosis risk—an overview of Legionella spp. habitats in Europe*, opublikowaną w Environmental Science and Pollution Research (Springer), w której zebrano i zestawiono doniesienia na temat występowania pałeczek z rodzaju *Legionella* na terenie Europy. Artykuł ten był cytowany 34 razy i plasuje się w 82. percentylu (miejsce 78 005) spośród 446 525 indeksowanych prac o podobnej dacie publikacji.

Konferencje naukowe

Dorobek naukowy obejmuje aktywny udział w krajowych i międzynarodowych konferencjach naukowych w formie referatów oraz doniesień posterowych. Tematyka wystąpień dotyczyła przede wszystkim zagadnień mikrobiologii środowiskowej, bezpieczeństwa żywności, oceny zagrożeń mikrobiologicznych w wodach użytkowych i przemysłowych oraz potencjału przeciwdrobnoustrojowego związków naturalnych (Tabela 4).

Tabela 4. Zestawienie dorobku konferencyjnego.

Rodzaj konferencji / Rodzaj wystąpienia	**Opis**
Krajowa/ prezentacja ustna	**Kanarek P**., Breza-Boruta B., Bogiel T., 2025. Charakterystyka liczebności i oporności *Enterococcus* spp. w wodach kanału Bydgoskiego i stawu miejskiego osiedla Prądy w kontekście lokalnej antropopresji. *XII Ogólnopolska Konferencja Hydromikrobiologiczna HYDROMICRO,* Bydgoszcz 25-27.06.2025
Sympozjum międzynarodowe/ poster	Breza-Boruta B., **Kanarek P**., Bauza-Kaszewska J., 2025 Biofilm bakteryjny i jego wpływ na prawidłowe funkcjonowanie systemów do nawadniania upraw rolnych, *XXVI Sympozjum Nawadniania Roślin,* 10-12.06.2025
Międzynarodowa / poster	**Kanarek P**., Breza-Boruta B., Bogiel T., 2024. Microbiological contamination of wash water in the vegetable industry: a case study of fresh-cut processing plant in Poland. *RETASTE: Rethink Food Resources, Losses, and Waste, 4th International Conference*, Heraklion, Grecja, 25–27.09.2024.
Krajowa/ prezentacja ustna	**Kanarek P**., Breza-Boruta B., Bogiel T., 2024. Ocena przeciwdrobnoustrojowego potencjału ekstraktów roślinnych wobec patogenów wyizolowanych z wód przemysłowych –wyniki badań screeningowych. *56. Ogólnopolska Konferencja Mikrobiologiczna: Mikrobiologiczne wyzwania – rozwiązania dla środowiska i zdrowia,* Fojutowo, 11–13.09. 2024.
Międzynarodowa / prezentacja ustna	**Kanarek P**., Breza-Boruta B., 2023. Occurrence of ESKAPE pathogens in wash waters of the agri-food industry in view of the implementation of a closed loop economy. *Closed cycles and the Circular Society 2023: The power of ecological engineering* of the International Ecological Engineering Society (IEES), Chania, Grecja, 1–5.10.2023.
Krajowa/ poster	Breza-Boruta B., Bauza-Kaszewska J., **Kanarek P**., 2023. Ocena skuteczności higienizacyjnej systemu uzdatniania wody wykorzystywanej w zakładach przetwórstwa rolno-spożywczego, Fojutowo, 12-15 czerwca 2023.

Krajowa/ prezentacja ustna	**Kanarek P.,** Breza-Boruta B., Wiśniewska W., 2023. Analiza porównawcza wybranych składników bioaktywnych i liczebności bakterii LAB w wybranej żywności funkcjonalnej. *I Ogólnopolska Konferencja Naukowa „Żywność i żywienie w pigułce”*, Gdański Uniwersytet Medyczny, Gdańsk, 22.04.2023.
Międzynarodowa / poster	**Kanarek P.,** Breza-Boruta B., Poćwiardowski W., Ligocka A., Bauza-Kaszewska J., 2022. A study of the effects of silver ions and hydrogen peroxide on selected waterborne pathogens and the possibility of using these disinfectants for sanitizing swimming pool water. *3rd International Conference Strategies toward Green Deal Implementation – Water, Raw Materials & Energy*, 5–7.12.2022.
Międzynarodowa / poster	**Kanarek P**., Bogiel T., Breza-Boruta B., Maciuszek A., 2022. Anthropogenic water systems as a source of *Legionella* bacteria. *5th International Conference Environmental Engineering and Design*, Zielona Góra, 13–14.10.2022.
Międzynarodowa / poster	Breza-Boruta B., Macisuzek A., **Kanarek P**., Waste treatment facility as a potential source of microbial emissions to the environment. *5th International Conference Environmental Engineering and Design*, Zielona Góra, 13–14.10.2022.
Międzynarodowa / prezentacja ustna	**Kanarek P.,** Breza-Boruta B., 2022. Microbiological assessment of the agro-food processing plant’s wash water, in the context of safety and recyclability. *Second Virtual International Conference “Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality”*, Toruń, 15–16.09.2022.
Krajowa / prezentacja ustna	**Kanarek P.,** Breza-Boruta B., 2022. Badania nad opracowaniem składu konsorcjum mikrobiologicznego o właściwościach celulolitycznych i ksylanolitycznych do szybkiej mineralizacji słomy na bazie promieniowców pozyskanych metodą skriningu. *II Konferencja Młodych Naukowców, Instytut Włókien Naturalnych i Roślin Zielarskich – PIB*, Poznań, 2022.

Nagrody i wyróżnienia:

- Wyróżnienie w konkursie na najlepsze wystąpienie referatowe: **Kanarek P**., Breza-Boruta B., Bogiel T., 2025. Charakterystyka liczebności i oporności *Enterococcus* spp. w wodach kanału Bydgoskiego i stawu miejskiego osiedla Prądy w kontekście lokalnej antropopresji. *XII Ogólnopolska Konferencja Hydromikrobiologiczna HYDROMICRO,* Bydgoszcz 25-27.06.2025
- Wyróżnienie w konkursie na najlepsze wystąpienie w sesji komunikatowej I: **Kanarek P.,** Breza-Boruta B., Wiśniewska W., 2023. Analiza porównawcza wybranych składników bioaktywnych i liczebności bakterii LAB w wybranej żywności funkcjonalnej. *I Ogólnopolska Konferencja Naukowa „Żywność i żywienie w pigułce”*, Gdański Uniwersytet Medyczny, Gdańsk, 22.04.2023.
- Nagroda JM Rektora Politechniki Bydgoskiej za wybitne osiągnięcia naukowe i działalność naukową, 2021 r.

Działalność organizacyjna:

1. Członek Komitetu Organizacyjnego: I SEMINARIUM PRZYRODNICZYCH KÓŁ NAUKOWYCH” WEKTOR NAUKI”, 2 grudnia 2022, Bydgoszcz.
2. Członek Zespołu Organizacyjnego: Inżynieralia Politechniki Bydgoskiej 2022 r.
3. Członek Komitetu Organizacyjnego: 56. Ogólnopolska Konferencja Mikrobiologiczna „Mikrobiologiczne Wyzwania- Rozwiązania dla Środowiska i Zdrowia”, 11-13 września 2025, Fojutowo.

Recenzja:

1. *Infection Ecology & Epidemiology* (ID recenzenta: qXxzwi5N).

Szkolenia i webinary:

1. Akademia Bezpieczeństwa Żywności organizowane przez Argenta, 9–10.03.2022 r.
2. Spotkanie naukowo-szkoleniowe Polskiego Towarzystwa Mikrobiologicznego, oddział w Bydgoszczy, 30.03.2022
3. Warsztaty „Spektrometria mas – nowoczesność w każdym laboratorium”, Poznań, 3.06.2022 r.
4. VII Ogólnopolska konferencja Naukowo-Szkoleniowa “STARE I NOWE PATOGENY- Aktualne problemy”, Uniwersytet Medyczny w Lublinie, 15-16 maja 2021 r.
5. VIII Ogólnopolska Konferencja Naukowo-Szkoleniowa "STARE I NOWE PATOGENY – Aktualne problemy", Uniwersytet Medyczny w Lublinie, dn. 17–18.02.2023 r
6. „Sekrety techniki ELISA”, MERCK, 12.10.2022 r.
7. XI Konferencja Online – Ogólnopolskie spotkania Mikrobiologów i Epidemiologów, 11 września, 2023
8. Szkolenie online dla naukowców: *Transfer Wiedzy W Zakresie Realizacji GOZ w Sektorze Wodno-Ściekowym* – Instytut Gospodarki Surowcami Mineralnymi i Energią PAN, 26.03.2024 r.
9. IX Ogólnopolska Konferencja Naukowo-Szkoleniowa *„STARE I NOWE PATOGENY – Aktualne problemy”*, Uniwersytet Medyczny w Lublinie, 16–17.02.2024 r.
10. Webinarium naukowo-szkoleniowe zorganizowane przez Polskie Towarzystwo Mikrobiologów Oddział w Bydgoszczy oraz firmę Argenta, 17.11.2023 r.

11. XII Konferencja Online – Ogólnopolskie Spotkania Mikrobiologów i Epidemiologów, 6.11.2023 r.
12. Posiedzenie naukowo-szkoleniowe PTM, oddział terenowy Bydgoszcz, 21.11.2024 r.
13. Webinarium naukowo-szkoleniowe, PTM oddział terenowy Bydgoszcz; 23.11.2022 r.
14. TUV NORD Polska; Analiza ryzyka i zarządzanie ryzykiem - wyroby medyczne na bazie normy ISO 14971:2019; 9 lipca 2024;
15. Mikrobiologia farmaceutyczna- nowoczesne metody oznaczania endotoksyn i pirogenów, Warszawa 18.04.2024

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7. ZAŁĄCZNIKI

7.1. Kopie artykułów naukowych stanowiących cykl publikacji rozprawy doktorskiej
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7.3. Oświadczenia Współautorów artykułów naukowych
7.4. Kopie potwierdzające zgłoszenie patentowe i wzór użytkowy
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Review Article

Microbial composition and formation of biofilms in agricultural irrigation systems- a review

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ABSTRACT

Microbial contamination of water, as well as the development of biofilm in irrigation systems, is one of the factors contributing to water losses, leading to a decrease in the optimization of the irrigation process and, consequently, a decrease in plant production. Water shortages are a highly undesirable phenomenon, particularly within the context of anthropogenic climate changes and the constraint of potable water resources. Biofilm can also be a reservoir of pathogenic microorganisms for human health, animal welfare, and plant production. Contaminated water used to irrigate vegetables that do not require heat treatment can lead to pathogen propagation, causing disease outbreaks. Therefore, it is essential to understand the dynamics of biofilm development and its underlying mechanisms, as well as its relation to water quality, to develop strategies that could help reduce or prevent biofilm formation and its negative effects. The analysis of factors promoting the development and composition of biofilm in irrigation systems allows the implementation of the most effective preventive methods, which translate into the optimization of the irrigation process and plant production. This paper aims to analyze reports related to the formation and microbial composition of biofilms occurring in agricultural irrigation systems, as well as to present the risks associated with biofilm formation and methods for its eradication. This review summarizes reports related to the various factors influencing biofilm formation and irrigation water quality, which may be a prelude to a comprehensive assessment and formulation of guidelines related to the management of water-based irrigation systems to improve biosafety.

1. Introduction

Anthropogenic climate change, manifested by weather extremes, has negative impacts on people, the environment, agricultural production, and livestock (Burrell et al., 2020; Ortiz-Bobea et al., 2020). Furthermore, progressive population growth (estimated at 9.7 billion people by 2050) necessitates productivity investment in agriculture and livestock production. It is a critical concern since climate change is significantly disrupting food production processes, not only through degradation, desertification, and soil aridity but also by affecting regional water relations (e.g., via prolonged periods of drought and high temperatures), thus making adequate crop irrigation difficult (Arora, 2019). These factors are currently affecting the limitations faced by agriculture, especially since it is one of

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the most water-intensive sectors of the economy. A detailed understanding of the distribution of water consumption and losses will allow the implementation of sustainable water management strategies in agriculture (Mekonnen and Gerbens-Leenes, 2020; Novoa et al., 2019) One approach is the introduction of advanced irrigation models, together with an evaluation of their efficiency and flexibility. Adapting irrigation to soil type and plant species can serve as an effective tool for optimizing the process. (Ostad-Ali-Askari, 2022; Shayannejad et al., 2022). Another efficient method is to utilize deficit irrigation, which attempts to reduce water loss and deliver water to the plants during the most crucial times of the crop. (Ostad-Ali-Askari et al., 2017).

Microbial contamination of water and biofilm development in irrigation systems represent factors influencing water losses, resulting in reduced optimization of the irrigation process and, consequently, a decrease in crop production. In addition to this, biofilm can be a reservoir of pathogenic microorganisms, posing threats to public health by causing outbreaks of foodborne illness. All these factors make it necessary to monitor irrigation systems. Also, conducting a detailed assessment of the factors promoting biofilm formation can allow the implementation of predictive and preventive tools that will affect the mitigation of water and production losses in agriculture and horticulture.

To date, there are few reviews demonstrating the multifaceted microbiological risks to irrigation water. This work holistically presents water contamination originating from the water source as well as the link between its source and microbial propagation through biofilms. The compilation of diverse examples of scientific investigations of irrigation water contamination can provide a prelude to a comprehensive assessment and formulation of guidelines related to the management of irrigation water systems to improve biosecurity. This paper aims to analyze reports connected with the formation and microbial composition of biofilms in agricultural irrigation systems. The study also presents the hazards associated with microbial contamination of irrigation waters and the methods for biofilm eradication.

2. Biofilm as a threat to agricultural production

Crop irrigation utilizes water from diverse sources, such as groundwater, surface water, tap water, treated wastewater, and tank water. The water should be free of agents that negatively affect agricultural production and the environment (such as heavy metals, pesticides, pathogens, protozoa, or parasite eggs) (Rusiñol et al., 2020). Challenges related to microbiological water safety include preventing the spread of plant, human, and animal pathogens. The utilization of treated wastewater aligns with rational water resource management; however, it may carry the risk of pathogen propagation. Hence, continuous monitoring of microbiological risk and the implementation of barriers that restrict contact between treated wastewater and edible plant parts are recommended. Overhead spraying and furrow irrigation are therefore discouraged before harvesting. Additionally, a crucial production aspect involves the preliminary cleaning of plant materials (Ofori et al., 2021)

The occurrence of phytopathogens is often associated with the usage of reclaimed irrigation water, which promotes a kind of “circular cycle” of phytopathogens, leading to crop diseases (Redekar et al., 2019; Stewart-Wade, 2011). The use of surface water for crop irrigation also contributes to the transfer of phytopathogens. The survival of plant pathogens in surface water depends on diverse factors, such as the chemical composition of the water, temperature, the presence of antagonistic microflora, the amount of suspended solids (composed of dead organic matter)”, the thickness of the bottom sediment, the season, and the presence of underwater flora (which acts as a periodic reservoir of pathogens). Irrigation systems (especially for irrigation channels) supplied with surface water are also an attractive niche for fungus-like organisms of the genera *Phytophthora* and *Pythium*, which, through the production of zoospores, can rapidly lead to phytophthorosis of numerous economically important plants (e.g., *Solanaceae, Cucurbitaceae, Fabaceae families*) (Jones et al., 2014; Lamichhane and Bartoli, 2015; Lemanczyk and Lisiecki, 2015).

On the other hand, irrigation water may function as a reservoir for human pathogens (Table 1). The first peer-reviewed report

Table 1
Selected reports on pre-harvest contamination of irrigation water.

Pathogen	Type of water	Vegetable	Country	Authors*
Salmonella spp.	irrigation pond	tomato	USA	(Greene et al., 2008)
	irrigation canal	n.s.	Italy	(Cito et al., 2016)
	nonpotable water	rucola lettuce, mixed salad	Italy	(Nygård et al., 2008)
	river water, dam water	spearmint, lettuce	Jordan	(Tarazi et al., 2021)
	river water	lettuce, broccoli, cabbage	Colombia	(Henao-Herreño et al., 2017)
Campylobacter spp.	nonpotable water	rucola	Italy	(Nygård et al., 2008)
	irrigation ponds	n.s.	USA	(Gu et al., 2013)
Listeria spp.	surface water after rainfall	tomato green amaranth	Nigeria	(Agboola and Bisi-Johnson, 2023)
	ponds and river	spinach	Nigeria	(Mawak et al., 2009)
Escherichia coli	groundwater interacting with surface water	spinach	USA	(Gelting et al., 2011)
	irrigation canal	romaine lettuce	USA	(Bottichio et al., 2020)
	groundwater	lettuce tomato spinach	Portugal	(Araújo et al., 2016)

* Selection of studies was based on: global representation criterion to ensure a comprehensive perspective, selection of a broad spectrum of vegetables, reference to 4 pathogens of public health concern; n.s not specified.

demonstrating the impact of biofilms on the microbiological quality of water was an investigation conducted by Pachepsky et al. The authors postulate that the microbiological quality of water should be monitored not only at intake but also during transport in the irrigation system (Pachepsky et al., 2012). Contamination of water with fecal indicator bacteria - especially Shiga toxin-producing *E. coli*, poses a direct threat to human health and is a serious challenge in vegetable and fruit production. Vegetables are commonly consumed raw, allowing the pathogen to spread. The 2006 outbreak in the USA, linked to fresh spinach, exemplifies the importance of STEC. This incident caused nearly 200 confirmed illnesses, hospitalized 100 people, and led to five deaths (Wendel et al., 2009; Kintz et al., 2019). Contamination of raw material (unprocessed fruits and vegetables) with bacteria such as *Listeria monocytogenes, Salmonella* spp., *Campylobacter* spp., and *Shigella* spp., can lead to foodborne illness outbreaks and pose public health risks (Gurtler and Gibson, 2022; Mcheik et al., 2018). The type of irrigation technology is significant in this aspect; drip and furrow irrigation usage are considered safer than sprinklers. The use of sprinklers leads to the direct application of pathogens to the edible parts of plants and their internalization (Alegbeleye et al., 2018). The internalization of human enteric pathogens within plant roots is a significant pathway for plant contamination. This process is influenced by several factors, including the type of plant, its age, environmental stresses, physical damage, developmental stage, and the specific strain of bacteria involved (Hirneisen et al., 2012).

Pre-harvest contamination of vegetables by contaminated water poses a significant challenge to crop production (Table 1). Investigations associated with outbreaks of pathogens like *E. coli, Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* are linked to the consumption of unprocessed vegetables such as tomatoes, rucola, lettuce, broccoli, cabbage, and spinach. The issue is not limited to developing countries; it also presents a challenge to highly developed economies where agricultural production is intensified.

Also, enteric viruses: Norovirus, Rotavirus A, adenoviruses, enteroviruses, astroviruses contained in irrigation water can pose a significant threat as etiological agents of gastroenteritis, meningitis, and hepatitis (Emilse et al., 2021; Cheong et al., 2009). Serious risks also include the contamination of irrigation water with helminth eggs. This problem is particularly relevant in developing countries. Among the parasites contaminating irrigation water with eggs are *Ascaris lumbricoides, Trichuris trichiura, Anchylostoma* spp., *Hymenolepis nana, Strongyloides stercoralis, Taenia* spp., and *Enterobius vermicularis* (Campos et al., 2018).

The presence of bacteria in irrigation water is not only related to their existence in a planktonic state (Fig. 1). A biofilm, defined as an organized spatial structure of microorganisms suspended in a self-produced matrix of extracellular polymeric substances (EPS), represents a widespread and effective strategy for microbial survival (Yin et al., 2019). The success of this evolutionary strategy is primarily related to the biofilm structure's high resistance to environmental stressors (Garrett et al., 2008). The constituted biofilm demonstrates resistance to UV radiation, elevated temperature, periodic disinfection, high salinity, decreased nutrient availability, high pressure, as well as the effects of antibiotics. Moreover, biofilms are environments for the inter-bacterial transfer of antibiotic resistance and virulence genes, which effectively hinder the eradication of these pathogens (Abebe, 2020).

Biofilms, in addition to being a reservoir of potentially harmful microorganisms, also affect flow restriction through their growth, thus blocking water transport (Zhang et al., 2021). Biological clogging in water systems is often associated with a concentration of organic matter suspended in the water, a source of nitrogen and phosphorus for biofilm-forming microorganisms (Petit et al., 2022).

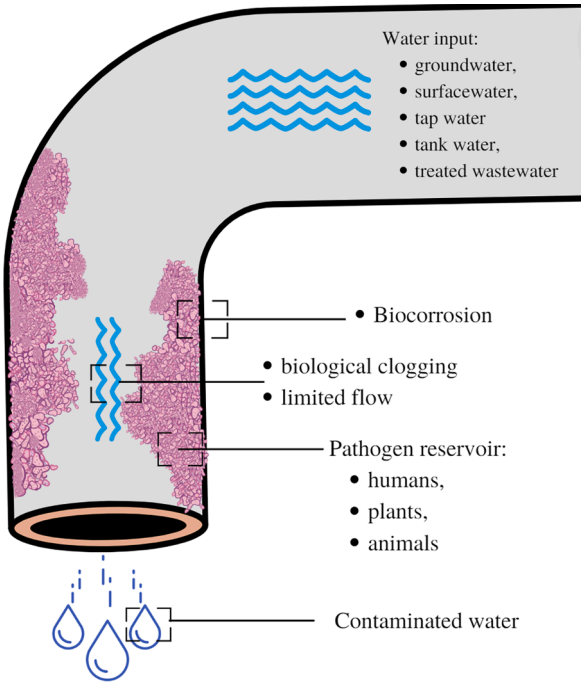

Fig. 1. Biofilm hazards in irrigation systems (own source).

Biological clogging is particularly problematic in a drip system, impeding the precise application of water and nutrients, resulting in losses in crop production. A frequently used treatment is increasing the flow level to detach the biofilm structure. However, it is noteworthy that the biofilm can reattach to the pipe surface and start regrowth again. Thus, it is vital to select appropriate hydrodynamic conditions (Wang et al., 2020).

Biofilms also play a significant role in microbiologically influenced corrosion (MIC) of water distribution systems through the direct excretion of corrosive metabolites and by capturing metal electrons in the pipe-building materials of water transport systems for cellular respiration. The second type of MIC is associated with the secretion of acids and enzymes (Jia et al., 2018; Loto, 2017). Particularly exposed are surfaces directly attached to the biofilm, as corrosive agents (e.g, H_2S, NH_3, or PH_3) accumulate at the material-biofilm interface, which can consequently lead to the destruction of the water distribution system through leakage. Therefore, biomonitoring of the entire system is essential, e.g., using the measurement of ATP concentration in the water as well as the selection of suitable pipe material in the irrigation system design (Dou et al., 2021; Ogawa et al., 2020; Pan et al., 2021). It is noteworthy that MIC is not only limited to metallic surfaces, such as iron, steel, copper, or titanium but also affects non-metallic surfaces, such as concrete (Li et al., 2013).

3. Biofilm formation in irrigation systems

Biofilm formation is based on several steps: in the initial stage of the process, planktonic cells reversibly attach to the surface, followed by a phase of irreversible attachment to the surface and the formation of microcolonies. The next step involves biofilm maturation, associated with the production of EPS structure by microorganisms. In time, the constituted biofilm transitions to the dispersion phase, leading to the release of bacterial cells, and the cycle begins again. (Renner and Weibel, 2011; Toyofuku et al., 2016). The potential for surface-attaching cells to start biofilm mass formation is regulated by quorum sensing (Qs) mechanisms, which is the ability to secrete and receive small, specific signaling molecules. The Qs mechanism is related to the extracellular secretion of auto-inducers (AIs) by bacteria, the concentration of which is directly proportional to cell density. Exceeding a threshold level of AIs in a microcolony induces downstream gene expression, affecting the initiation of EPS matrix production (Sahreen et al., 2022; Saxena et al., 2019). AIs-mediated communication in QS not only allows to produce a mechanical barrier such as the EPS matrix but also stimulates bacteria to express virulence factors, bioluminescence, acquire antibiotic resistance traits, or initiate the sporulation process. All these factors reinforce the evolutionary survival strategy of microorganisms in harsh environments (Waters and Bassler, 2005). The first factor influencing biofilm formation in irrigation systems is the water source, which not only carries potential microbial inoculum but also, through biological, chemical, and physical characteristics, promotes or reduces the possibility of starting biofilm formation processes (Raudales et al., 2014). Most scientific reports are related to the microbiological assessment of water recovered from wastewater in irrigation systems. The reuse of water from this source is in line with the principles of rational water resources management, however, it carries risks due to the rapid growth of biofilm in the water distribution system. This is due to the fact that water recovered from wastewater contains a higher amount of nutrients, mineral salts, microorganisms (including pathogenic ones), as well as a suspended fraction (Li et al., 2013; Wang et al., 2022; Zhou et al., 2016). In addition, the use of reclaimed water is associated with the transfer of antibiotic resistance genes through the biofilm and the resulting increase in antibiotic-resistant bacteria. One reason for this is that the infrastructure of conventional wastewater treatment plants is not fully adapted to fully eliminate antibiotics, antibiotic-resistant strains, and antibiotic-resistant genes from water (Brienza et al., 2022; Fahrenfeld et al., 2013). Also, the use of surface water may carry the risk of increasing the number of antibiotic-resistant strains inhabiting biofilms in irrigation systems. A study by Qian et al. (2017) found that introducing a temperature cycle of 20-50°C into the system had a negative effect on biofilm growth processes. However, the high and low water temperatures can be a stressor for plants, causing a decrease in yields. Water temperature in irrigation systems is most often related to environmental temperature and exhibits variability depending on the season and geographic location, making it difficult to develop a universal model, especially under uncontrolled conditions (Liu et al., 2016). Environmental conditions in irrigation systems not related to water quality and origin also significantly affect the dynamics of biofilm formation (Fig. 2). Managing the hydrodynamic conditions of the irrigation system significantly affects the shape, growth rate, and concentration of cells in the biofilm (Krsmanovic et al., 2021). Flow in a water distribution system can multifacetedly affect the process

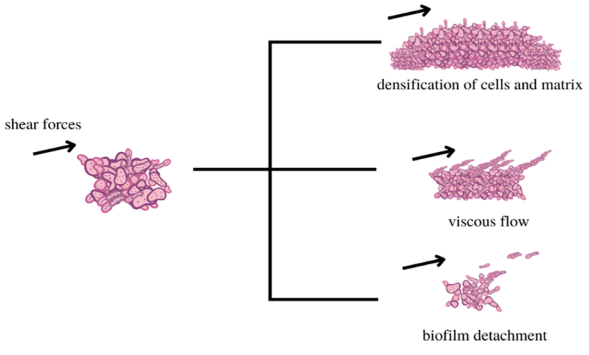

Fig. 2. Differential effect of shear forces on biofilm during flow (own source).

of biofilm formation and degradation. Shear forces in flowing streams lead to increased biofilm stress, which stimulates the expression of genes related to EPS-building polysaccharides, affecting structure formation (Weaver et al., 2012). Bacterial cells dynamically respond to flow changes by adjusting EPS production to shear intensity (Menniti et al., 2009). High fluid flow results in thinner biofilms with higher cell concentrations in condensed polysaccharide-protein matrices, akin to structure "hardening" under adverse conditions (Araújo et al., 2016; Conrad and Poling-Skutvik, 2018). Another response to shear impact is biofilm's viscous flow, behaving as a viscoelastic fluid and adapting its shape in wave patterns following flow direction (Gloag et al., 2018; Nguyen et al., 2021). Lastly, shear forces can detach biofilm portions, allowing migration in water, possibly reattaching in other system sections (Krsmanovic et al., 2021). A detailed understanding of the issues involved in the mechanics of biofilm under varying flow conditions can lead to the development of predictive and preventive models that will help reduce losses in irrigation systems and agricultural production.

4. Microbial composition of biofilm in irrigation systems

Studies of biofilm dynamics and quality composition mostly focus on mono-species biofilms, which are rare in the environment. A biofilm might be a conglomerate formed by diverse species of bacteria, fungi, protozoa, and algae. The interactions taking place in the matrix successfully enhance the structure's resistance to diverse adverse environmental conditions. The growth of multi-species biofilms is promoted through interactions such as coaggregation, co-metabolism, interspecies protection, and lateral gene transfer (Reuben et al., 2019; Sanchez-Vizuete et al., 2015). Bacteria in a biofilm can also exhibit mutual antagonism, hindering the intercalation of other species or disrupting the production of EPS itself.

Most of the reports related to the microbial community in biofilms in water distribution systems have been associated with drinking water distribution systems, due to public health safety concerns. Nevertheless, microbial monitoring of agricultural irrigation systems is essential to maintain agricultural productivity and food safety (Table 2). Frequently, the composition of biofilms is related to the presence of environmental microorganisms naturally occurring in the primary water reservoir (such as surface water) (Pachepsky et al., 2012). A study by Song et al. showed that the most abundant phylum colonizing biofilms was *Proteobacteria*, followed by *Acidobacteria, Bacteroidetes, Actinobacteria*, and *Chloroflex*i (Song et al., 2021). The study of biofilm formation processes in the recovered irrigation water also showed the dominant contribution of Proteobacteria against successively *Actinobacteria, Chloroflexi*, and *Cyanobacteria*. The use of molecular methods has led to the differentiation of species belonging to families: *Bacillaceae, Legionellaceae, Leptolyngbyaceae*, and *Pseudanabaenaceae*, among others (Wang et al., 2022). Studies conducted on the fluctuation of microbial communities in biofilms of irrigation systems supplied with reclaimed water found the presence of the following classes of bacteria: *Bacilli, Cyanobacteria, Alphaproteobacteria, Acidobacteria, Gammaproteobacteria, Clostridia, Betaproteobacteria, Actinobacteria, Chloroflexia, Caldilineae*, and *Thermomicrobia*. The average presence of *Bacilli* at 45.9% in the biofilms studied was associated with the ability to produce endospores and, thus higher rate of species survival (Hou et al., 2020). On the other hand, a study conducted on the biofilm community in groundwater and surface water distribution systems showed that after 9 months, the dominant group was *Mycobacterium* species, which are known to demonstrate high tolerance to temperature changes, periodic disinfection, and low nutrient supply (Revetta et al., 2016).

Studies of biofilms in irrigation systems have also indicated that they could be colonized by total coliforms and fecal coliforms, including those with increased antibiotic resistance. The authors conclude that the source of water contamination could originate from: the field surrounding the river, from wild animals, as well as from upstream located camping (Blaustein et al., 2015). *E. coli* bacteria also exhibit a greater susceptibility to biofilm formation in new aluminum pipes compared to in-service pipes. This may be related to ongoing in-service corrosion, leading to the release of aluminum, which showed a biocidal effect against the bacteria (Shelton et al., 2013). The presence of *Pseudomonas* spp. bacteria in irrigation systems is a result of the widespread ubiquity of these microorganisms. The main representative of the genus, i.e., *P. aeruginosa*, is the most significant factor among Gram-negative bacteria,

Table 2
The microbial composition of biofilm (major bacterial groups) in irrigation systems described in the different studies.

Type of water used	Identified bacteria	Authors (source)
reclaimed water	Phylum: *Proteobacteria, Acidobacteria, Bacteroidetes, Actinobacteria,* Chloroflexi	Song et al., 2021
	Phylum: *Proteobacteria, Actinobacteria, Chloroflexi, Cyanobacteria* (Family: *Bacillaceae, Legionellaceae, Leptolyngbyaceae, Pseudanabaenaceae*)	Wang et al., 2022
	Class: *Bacilli, Cyanobacteria, Alphaproteobacteria, Acidobacteria, Gammaproteobacteria, Clostridia, Betaproteobacteria, Actinobacteria, Chloroflexia, Caldilineae, Thermomicrobia.*	Hou et al., 2020
	Pseudomonas spp.	Zhou et al., 2017
	Salmonella spp.	Santiago et al., 2018
	Legionella spp.	Lequette et al., 2019
groundwater and surface water	*Mycobacterium* spp., *Limnobacter* spp., *Pseudomonas* spp., *Sphingopyxis* spp., *Blastomonas* spp., *Gluconacetobacter* spp., *Ammoniphilus* spp.	Revetta et al., 2016
	Fecal coliforms, total coliforms	Blaustein et al., 2015
	Escherichia. coli, fecal coliforms	Shelton et al., 2013
	Campylobacter jejuni	Gu et al., 2013

affecting the clogging of water emitters due to its rapid biomass decomposition and biofilm growth (Zhou et al., 2017). Irrigation systems using reclaimed water are exposed to *Salmonella* bacteria, which may be present in the water even after UV disinfection. *Salmonella* spp. can intercalate into the biofilm, thus contributing to a long-term presence in irrigation systems and leading to the contamination of fresh vegetables (Santiago et al., 2018). Studies related to the presence of *Legionella* bacteria most often refer to the infrastructure of heated water distribution systems; however, a study by Lequette et al. found *Legionella* spp. in all biofilms taken from irrigation pipes and in 14 of 17 biofilms formed in drippers. It is worth bearing in mind, for example, that the use of sprinkler technology can contribute to the transmission of *Legionella pneumophila* through the generation of bioaerosol, a carrier of this pathogen (Lequette et al., 2019; Pepper and Gerba, 2018). The presence of *Campylobacter jejuni* in irrigation ponds shows fluctuating seasonality; being able to survive in the form of mono- and multispecies biofilms (Elgamoudi and Korolik, 2021; Gu et al., 2013).

It is noteworthy that it is difficult to develop a single model for the qualitative composition of biofilms in irrigation systems due to factors such as geographic location (climatic conditions), type of irrigation (furrow, drip, sprinkler), materials used in the construction of the irrigation system, age of the system, intensity of operation, origin of water, fertilizer addition, and system disinfection techniques. Each of the factors listed can promote or degrade the presence of particular microorganisms in the biofilm community. Microbiological monitoring, especially when reclaimed water is used, should focus on indicator bacteria (faecal bacteria), the presence of which may indicate a disturbance in the water treatment process. In addition, testing the water for *Pseudomonas* spp. may be a useful tool in assessing susceptibility to biofilm development, due to the organic fraction loading of the water.

5. Biofilm eradication strategies

Biofilm eradication is a significant challenge in irrigation water distribution systems, process systems, and drinking water distribution systems. Nowadays, a diversity of chemical, physical, and biological methods is in use worldwide (Table 3). Chemical methods include the use of sodium hypochlorite, calcium hypochlorite, and hydrogen peroxide; the use of both classical sodium hypochlorite and oxidative methods based on hydrogen peroxide effectively prevent biofilm growth in irrigation systems (Green et al., 2018; Song et al., 2017). In addition to its biocidal effect, the presence of chlorine in the water distribution system affects functional groups on the surface of bacteria, preventing permanent attachment to the substrate by breaking the hydrogen, polymer, and hydrophobic bonds of bacteria to the substrate (Xue and Seo, 2013). Although the use of chlorination is still widespread, it is an invasive measure with significant environmental impacts. One of the green alternatives is, (in addition to hydrogen peroxide), the use of peracetic acid (Carrasco and Urrestarazu, 2010). A study by Akinbobola et al. showed the tolerance of *P. aeruginosa* biofilms to high concentrations of peracetic acid, which is in opposition to the study by Chino et al., which showed the efficacy of peracetic acid against *P. aeruginosa* and *S. aureus* biofilms. This demonstrates the need for more research in different models (Akinbobola et al., 2017; Chino et al., 2017).

An additional option is the use of physical methods of biofilm eradication. These include ultrasound, an electrical field, plasma, a magnetic field, and irradiation. Methods at the current level can be used to support the main disinfection process (Liu et al., 2022). Ultrasonic sterilization is a safe method for biofilm degradation. Moreover, combining it with chemical methods shows an enhanced, synergistic effect against biofilms formed by pathogens such as *S. aureus* and *Salmonella* spp. (Shao et al., 2020; J. Zhang et al., 2021). The use of a pulsed electric field, along with the addition of weak organic acids, can also become an effective treatment, even against biofilms (Novickij et al., 2019). The use of irradiation allows the activation of radicals, damaging the biological structures of cells. Irradiation has been shown to effectively reduce cell populations, both in the planktonic state and in biofilms. Even though physical methods seem quite promising, they are currently mostly used as an adjunct to classical disinfection.

Non-invasive biological methods include the use of enzymes, phages, and antagonistic microorganisms (Simões et al., 2010). Combined with detergents, enzymes can play a key role in biofilm mitigation by dissolving EPS; however, the existing economic limitation (excessive cost of enzymes) does not allow for full-scale application (Richert and Dabrowska, 2021; Simões et al., 2010). Studies conducted to date show an increased interest in phages that actively penetrate and destroy biofilm structures. Current research focuses on lytic phages, which lack integrase and other enzymes involved in horizontal gene transfer (Domingo-Calap and Delgado-Martínez, 2018; Tinoco et al., 2016).

Even though non-traditional chemical methods appear to be an attractive alternative, it is important to consider their performance

Table 3
Selected methods of biofilm eradication.

		Method	Authors
Type of disinfection	chemical	Chlorination*	(Song et al., 2017) (Lequette et al., 2019) (Green et al., 2018)
		hydrogen peroxide*	(Green et al., 2018) (Japhet et al., 2022)
		peracetic acid*	(Chino et al., 2017) (Akinbobola et al., 2017)
	physical	Ultrasound	(Shao et al., 2020)
		pulsed electrical field	(Novickij et al., 2019)
		Irradiation	(Niemira, 2007)
	biological	Phages	(Sun et al., 2022)
		Enzymes	(Liu et al., 2014)

* methods used at full scale

at the scale of the entire irrigation water distribution system. Some of the research is based on the laboratory scale, and their implementation to the technical scale should be preceded by a careful efficiency and economic analysis.

Studies demonstrate that the type of material used in an irrigation system affects the dynamics of biofilm formation. There is a strong link between the type of pipe material in water distribution systems and the dynamics of biofilm growth (Trinh et al., 2020). Among the materials used to create water distribution systems are, for example, ductile iron, stainless steel, or synthetic materials (polyethylene, polypropylene, and polyvinyl chloride) (Yan et al., 2022). Choosing the appropriate material in the water distribution system construction results in system longevity and efficiency by reducing biofilm growth. There are numerous reports on biofilm dynamics in diverse water systems. In metal-based systems, the use of a copper pipe results in the lowest potential for biofilm formation, in contrast to stainless steel and zinc-coated steel. The pipe material also influences the biofilm formation rate and the quality of the EPS composition. The use of copper pipes has the most rapid effect on disrupting the bacterial redox potential, leading to fluctuations in the metabolic activity of microbes (Wang et al., 2020; Yu et al., 2010). Also, pipes made of synthetic materials are not free from potential biofilm formation, with high-density polyethylene (HDPE) showing greater susceptibility than polyvinyl chloride (PVC) (Rożej et al., 2015). Plastic-based water distribution systems show less bacterial diversity and slower biofilm biomass growth, compared to steel pipes (Jang et al., 2011). Cemented water channels in open irrigation systems are also exposed to rapid biofilm growth. It is caused by the porous surface structure, the constant influx of nutrients from surface water, and the presence of algae and protozoa, which stimulate complex trophic relationships in biofilms (M. Zhang et al., 2021).

Despite the variety of methods used in biofilm eradication, it is necessary to match the method to the type of system and consider the specific conditions of the environment in which it is used.

6. Summary

The impact of diverse factors on the formation and microbial composition of biofilms in agricultural irrigation systems is the subject of extensive research worldwide. Changes associated with anthropogenic climate change, water footprint reduction, and sustainable agricultural production, along with efforts to reduce the pathogenic impact of biofilm on public health, make it necessary to continuously monitor water distribution systems. This work presents an overview of selected reports related to the threat, growth-promoting factors, qualitative biofilm composition, as well as methods for its eradication.

An integrated approach to managing the biosecurity of water irrigation systems is crucial to reducing the spread and growth of pathogens. The assessment of an irrigation water distribution system should start with the water source used, which is the first factor influencing the presence of microorganisms. The type of irrigation may also promote microbial presence and biofilm formation. It is also important to select materials with low porosity and the potential to counteract biofilm development at the design stage. Periodic sampling of water from various stages of distribution for the presence of waterborne pathogens is also a useful preventive tool for early detection of risk. In conclusion, reducing the impact of biofilm in agricultural water distribution systems can already occur at the stage of system design and material selection. Subsequently, the water source selection may allow the prediction of changes occurring in the system, and monitoring of changes can be based on modern molecular techniques. In addition, it is postulated that classical chemical disinfection should be supported by additional physical and chemical methods that can enhance the synergistic effect. All this can contribute to effective biofilm control in irrigation systems. Given the above, there is a necessity to increase research on biofilms in irrigation systems, which can contribute to the development of decision-making pathways in the management of agricultural water distribution systems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

Authors state that the research was conducted according to ethical standards.

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In the Depths of Wash Water: Isolation of Opportunistic Bacteria from Fresh-Cut Processing Plants

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Abstract: The fruit and vegetable industry in post-harvest processing plants is characterized by a substantial consumption of water resources. Wash waters may serve as an environment for the periodic or permanent habitation of microorganisms, particularly if biofilm forms on the inner walls of tanks and flushing channels. Despite the implementation of integrated food safety monitoring systems in numerous countries, foodborne pathogens remain a global public health and food safety concern, particularly for minimally processed food products such as vegetables and fruits. This necessitates the importance of studies that will explore wash water quality to safeguard minimally processed food against foodborne pathogen contamination. Therefore, the current study aimed to isolate and identify bacteria contaminating the wash waters of four fresh-cut processing plants (Poland) and to evaluate the phenotypic antibiotic resistance profiles in selected species. Bacteria were isolated using membrane filtration and identified through mass spectrometry, followed by antibiotic susceptibility testing according to EUCAST guidelines. The results revealed that the level of contamination with total aerobic bacteria in the water ranged from 1.30×10^6 cfu/mL to 2.54×10^8 cfu/mL. Among the isolates, opportunistic pathogens including *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus vulgaris* strains were identified. An especially noteworthy result was the identification of cefepime-resistant *K. oxytoca* isolates. These findings highlight the importance of monitoring the microbial microflora in minimally processed foods and the need for appropriate sanitary control procedures to minimize the risk of pathogen contamination, ensuring that products remain safe and of high quality throughout the supply chain.

Keywords: water-borne pathogens; antibiotic susceptibility; fresh-cut processing plants; wash waters; agri-food processing

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1. Introduction

The fruit and vegetable industry in post-harvest processing plants is characterized by the substantial consumption of water resources. These resources are primarily utilized for pre-treating raw materials during washing and rinsing processes [1]. Utilizing tap water from the distribution network constitutes one of the early stages of vegetable and fruit processing. Its primary objective is to initiate the purification of the raw material from soil particles, pesticides, and undesirable organic matter fragments [2]. Mechanical treatment of fruit and vegetables with the use of turbulent water flow may not be sufficient to reduce microbial contamination levels [3]. Chlorine compounds, due to their widespread availability and ease of application, are the most widely used disinfectants in the reduction of microbial contamination of wash water. However, chlorine is known to react with suspended organic matter, leading to the creation of harmful by-products, including those with carcinogenic properties [4]. Other non-invasive disinfection methods

may include ozonation, nanofiltration of water, and the use of peracetic acid [5]. However, currently, some facilities still do not implement disinfection procedures in water used for washing fruits and vegetables. This approach can promote pathogen contamination during production or processing of food products and pose a public health and food safety risk [6,7].

There are two types of microbial contamination in vegetables and fruits: pre-harvest contamination and post-harvest contamination (Figure 1). Pre-harvest contamination is most commonly associated with agricultural practices, including fertilization and irrigation [8,9]. One of the significant factors contributing to pathogen contamination is the source of irrigation water (e.g., surface water, groundwater, treated sewage, reservoir water), as confirmed by numerous investigations associated with disease outbreaks caused by foodborne pathogens (such as salmonellosis, listeriosis, and campylobacteriosis) [10–12]. Another potential source of contamination is improperly managed irrigation water distribution systems, which create a favorable niche for the development of biofilm, an important source of secondary water contamination [13]. The application of manure, although a common agricultural practice, may also entail risks associated with increased exposure of vegetables to pathogens. Therefore, it is crucial to adhere to proper organic fertilizer application practices, implement a pre-harvest quarantine period, and utilize known sources of supply [14,15]. Another factor that may be a part of pre-harvest contamination is direct zoonotic contamination, resulting from both intensive and extensive livestock production facilities in the vicinity of the crop site. Also, the presence of wild animals and crop pests (e.g., birds, rodents, and insects) can contribute to the transmission of microorganisms to the raw material. The close proximity of different entities is also noteworthy—for instance, the positioning of composting plants, where inadequately managed leachate water can serve as a source of bacterial spread [8,16].

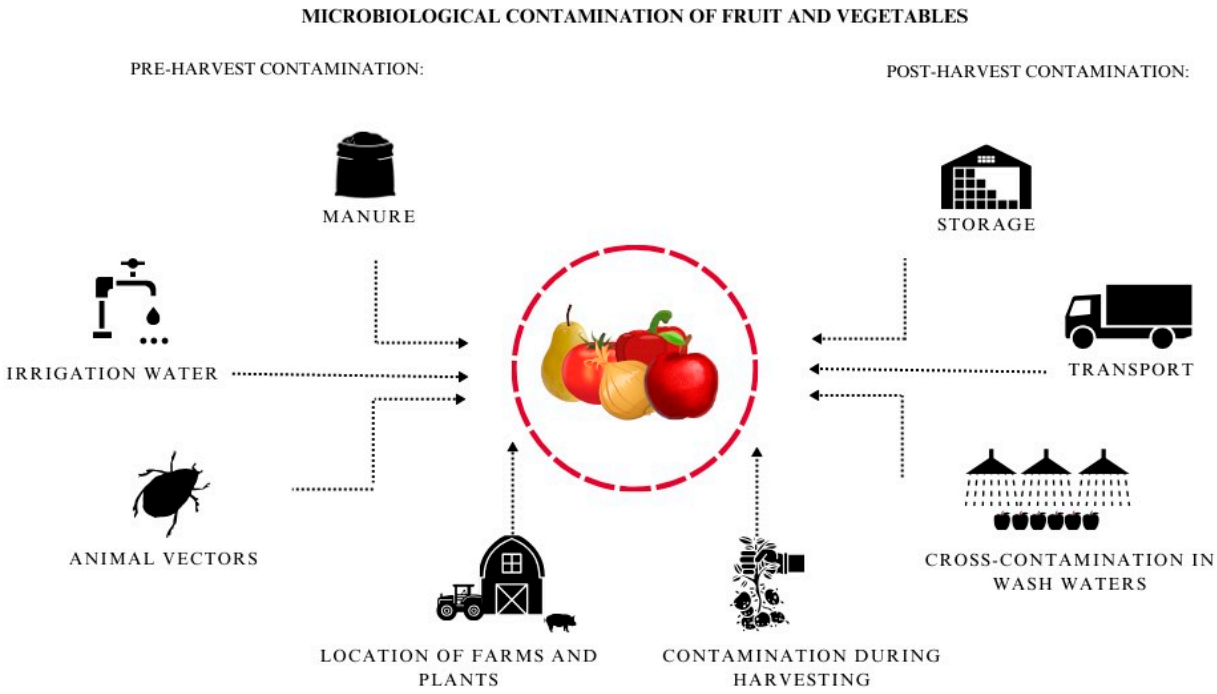

Figure 1. Summary of factors influencing possible pre-release contamination of fruit and vegetables.

Fruit and vegetables may be affected by microbial exposure in post-harvest contamination already in the harvest stage, e.g., through inappropriate hygiene practices of field workers or inadequate maintenance and preservation of tools and machinery used during the harvesting of the raw material [17,18]. An important aspect requiring careful supervision is the transportation of vegetables and fruits. This includes ensuring the cleanliness of transportation containers and adopting transportation methods that minimize exposure

to external factors [19]. Improper pre-processing storage conditions for raw materials (such as lack of warehouse disinfection before storage, excessively high or low temperature, humidity, or ventilation) can induce the development of storage diseases, primarily associated with the activity of filamentous fungi [20]. The final threat contributing to cross-contamination is the washing process of fruits and vegetables without water exchange (or its disinfection between batches). In this case, the washing water can serve as a habitat for microorganisms, either periodically or permanently, especially if biofilm is formed on the inner walls of tanks and rinsing channels.

Currently, there is still a significant risk of foodborne pathogens spreading, particularly for minimally processed food, despite the implementation of integrated food safety monitoring systems in numerous countries [21]. Microbial foodborne diseases result from the direct ingestion of bacteria-contaminated food products, subsequent growth of the microorganisms, and the secretion of toxins that affect physiological host functioning. Another method of exposure is the consumption of food already contaminated with bacterial endotoxins and exotoxins [22,23]. Typical symptoms of foodborne illnesses include abdominal pain, fever, diarrhea, vomiting, nausea, and, in more severe cases, systemic bacteremia and consequent death [24,25].

The development of increasing antibiotic resistance (AMR) and multidrug resistance in bacteria has also been recognized in recent years. Most commonly, these cases are associated with clinical strains posing risks to patients exposed to nosocomial infections. Nowadays, the importance of environmental hot spots in the transmission of antibiotic resistance genes is increasingly emphasized [26]. Enlarged environmental pressure on bacteria, including those with antimicrobial resistance genes, resulting from the supply of antibiotics to the environment, promotes the development (mutational and then vertical) or acquisition (via horizontal gene transfer) of antimicrobial resistance genes. In this case, AMR strains' development in water or soil may result in secondary exposure to the anthropogenic environment, acting as a specific "feedback loop" [27]. The increased transmission of resistance genes and their spread among pathogens that pose a public health risk is particularly alarming. For example, ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) may demonstrate high resistance to multiple classes of antibiotics, including both first-line and last-resort options, which can undermine the effectiveness of treatments and impact patient health [28]. Current studies on the antibiotic resistance of strains mainly focus on two fields. The first area involves clinical research, mainly concerned with the resistance of microorganisms in hospital environments. The second research area concerns high-risk points, such as wastewater treatment plants, which are critical links between anthropogenic and natural environments [29–31].

There is limited research available on the isolation and phenotypic analysis of antibiotic-resistant microorganisms in washing waters derived from the fruit and vegetable processing sector.

Thus, the objective of this study was to investigate, isolate, and identify bacteria found in the wash waters of four fresh-cut processing plants located in Poland and to evaluate the phenotypic antibiotic resistance profiles in selected species.

2. Materials and Methods

2.1. Characteristics of Fresh-Cut Processing Plants

Wash water samples were collected in the full harvesting season, early autumn (September 2022). Post-harvest processing plants are situated in the Kuyavian–Pomeranian (plants: A, B, C) and Greater Poland (plant D) Voivodships (Figure 2) in Poland. These regions are characterized by a highly developed agriculture and food processing industry. The plants process a wide range of fruits and vegetables, both for the domestic market and for export purposes. The plants specialize in ready-to-eat, packaged fruits and vegetables, salads, concentrates, and frozen foods (Table 1). During the interview conducted before sampling, it was noted that no disinfection methods are used during washing at the tested

facilities, which may have influenced the contamination of the wash water. All facilities included in this study used tap water, which is regularly tested by sanitary inspection to ensure it meets quality standards.

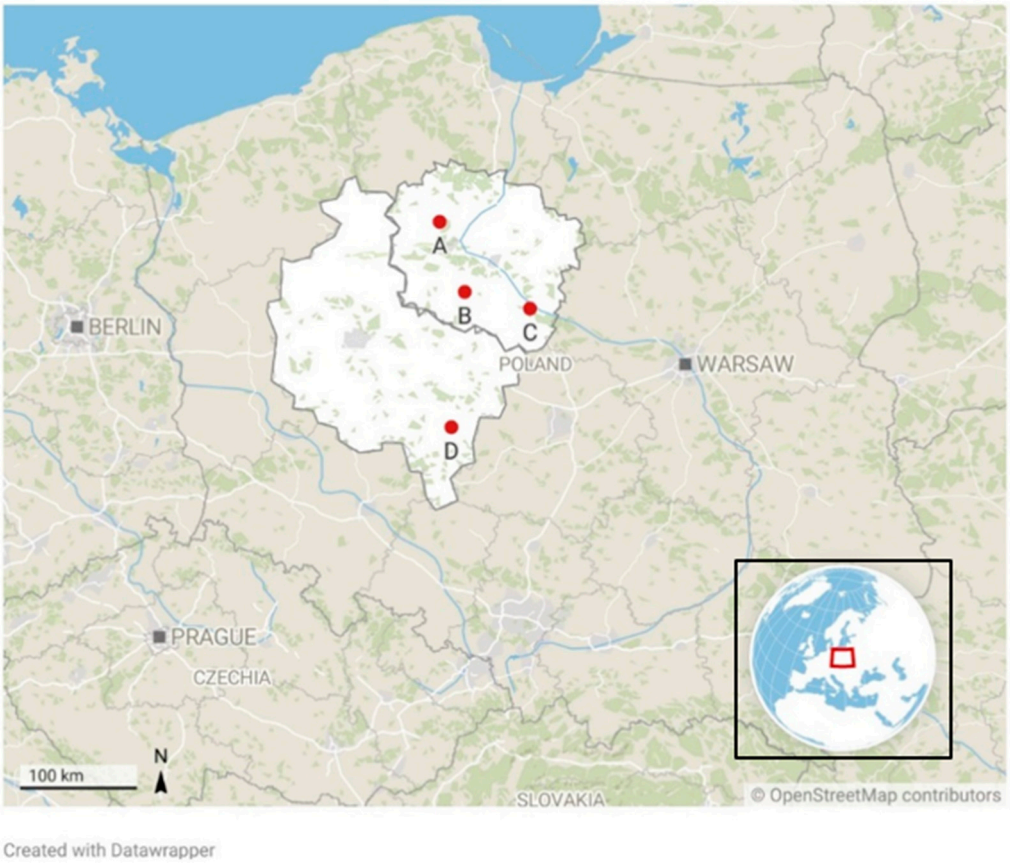

Figure 2. Location of fresh-cut processing plants.

Table 1. Summary of vegetables and fruit types processed in the investigated units.

Plant	Type of Processed Fruit/Vegetable	Type of Final Product
A	strawberries, raspberries, cherries, currants, rhubarb, plums, apples, tomatoes, cucumbers, leeks, broccoli, cauliflower	ready-to-eat fruits and vegetables, frozen products
B	onions	peeled onion, onion rings
C	tomatoes, apples	ready-to-eat fruits and vegetables, apple and tomato concentrate
D	beetroots, cucumbers, onions	ready-to-eat fruits and vegetables, pickled cucumbers, vegetable salads

Abbreviations: A, B, C, D—locations of fresh-cut processing plants.

2.2. Wash Water Sampling

Water samples for microbiological analyses were collected under the Polish Standard (PN-EN ISO 19458:2007) [32]. Water samples were taken 3 times, at intervals of 1 h, during the washing of the raw material in the washing tank. Three liters of water were collected from each plant for each analyzed raw material during a single sampling event, using 1 L glass bottles (Chemland, Poland) that had been steam-pre-sterilized. Sampling was conducted under strict sanitary conditions, including the use of facemasks, gloves, disinfection of bottle caps, and storage of samples in insulated transport containers until delivery for analysis. Following collection, the samples were transported to the laboratory of the Department of Microbiology and Food Technology located within the investigated

area (Bydgoszcz, Poland). Then, the samples for species identification were pooled to increase environmental representativeness.

2.3. Species Identification

After pooling, 3 L of baseline sample was obtained from each washed raw material. Subsequently, microbiological testing of the water was conducted through membrane filtration (filter diameter: 0.22 μm) employing a 3-station filtration unit (Sartorius, Göttingen, Germany). For each bacterial group tested, 100 mL of water was filtered in duplicate. After filtration, the filters were placed on a dedicated microbiological medium. For the total bacterial count test, surface plating was performed by pipetting 1 mL of water onto the medium and spreading it with a spatula. The following groups of bacteria were isolated on dedicated culture media: *Escherichia coli* (and the remaining *Enterobacteriaceae* family) (medium: Agar Endo, Merck; incubation: 24 h at 35 ± 0.5 °C), *Staphylococcus* spp. (Chapman-agar, Merck; incubation: 48 h at 35 °C), *Pseudomonas* spp. (Pseudomonas Selective agar with Pseudomonas CN Selective Supplement, Merck; incubation: 44 ± 4 h at 25 ± 1 °C), *Legionella* spp. (Legionella BCYE-Agar with Legionella Growth Supplement, and Legionella (GVPC) Selective Supplement, Merck; incubation: 10 days at 36 ± 2 °C), *Enterococcus* spp. (Kanamycin esculin azide agar, Merck; incubation: 24 h at 36 °C), and *Salmonella* spp. (SS agar, Merck; 24 h at 36 °C), total aerobic bacterial count (Standard Agar I, Merck). After the incubation period, colonies specific to certain groups of microorganisms were counted to determine colony-forming units (cfu) per 100 mL (and cfu per ml for total aerobic bacterial count). Pure bacterial cultures were then cultured, and species identification was performed by mass spectrometry via MALDI Biotyper apparatus (Bruker Daltonik GmbH, Bremen, Germany) with CE and IVD certification (according to Directive 98/79/EC).

2.4. Evaluation of Phenotypic Antibiotic Susceptibility of Selected Strains

Antimicrobial susceptibility testing of the selected species was performed and interpreted by the standard disc-diffusion method, according to the European Committee on Antimicrobial Susceptibility Testing guidelines [33]. The following antibiotics (individual antibiotics applied for dedicated strains, according to the guidelines) were used in 2 replications: piperacillin (30 μg), ceftazidime (10 μg), cefiderocol (30 μg), imipenem (10 μg), meropenem (10 μg), tobramycin (10 μg), levofloxacin (5 μg), ciprofloxacin (5 μg), ticarcillin-clavulanic acid (75–10 μg), cefepime (30 μg), amikacin (30 μg), gentamicin (10 μg), moxifloxacin (5 μg), amoxicillin-clavulanic acid (20–10 μg), trimethoprim (5 μg), ampicillin (2 μg), tigecycline (15 μg), linezolid (10 μg), vancomycin (5 μg). Inhibition zone diameters (mm) of each antimicrobial disc were measured and averaged based on repetitions. The isolates were classified as resistant (R), susceptible, increased exposure (I), and susceptible (S). The criteria for selecting species for the antibiotic susceptibility assessment were based on the selection of opportunistic pathogens posing potential threats to public health safety. The criteria for antibiotic selection were based on selecting representativeness for a broad spectrum of antibiotic classes.

3. Results

3.1. Species Identification

The results demonstrated no growth on the *Legionella* spp. selective medium or *Salmonella–Shigella* Agar. Morphologically distinct colonies observed on other media were subcultured onto fresh media for further species identification. The results demonstrated the presence of both susceptible and resistant bacterial species. The isolated microorganisms belonged to diverse bacterial genera. Identification using mass spectrometry demonstrated the isolation of diverse species representing families: *Staphylococcaceae*, *Lactobacillaceae*, *Micrococcaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Pseudomonadaceae*, *Alcaligenaceae*, *Flavobacteriaceae*, *Comamonadaceae*, and *Morganellaceae* (Table 2). A variety of bacterial species, including *Staphylococcus sciuri*, *Micrococcus luteus*, *Lelliottia amnigena*, and *Enterococcus cas-*

seliflavus, were present in the wash water samples collected from location A. *Staphylococcus sciuri* bacteria were isolated in both cucumber and plum washing water samples. At the onion processing plant facility (B), differentiated bacteria, including indicators of fecal contamination, were also found. Location C demonstrated, among others, *Pediococcus pentosaceus*, *Enterobacter ludwigii*, and *Micrococcus luteus*, as well as bacteria requiring increased preventive control. Water samples from plant D identified bacteria such as *Lelliottia amnigena*, *Pseudomonas putida*, *Staphylococcus equorum*, *Proteus vulgaris*, *Empedobacter falsenii*, and *Providencia alcalifaciens*. The detected bacteria also included opportunistic pathogens of clinical importance (*Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus vulgaris*).

Table 2. Results of bacterial species identification.

No.	Location	Raw Material Type	Species
1	A	cucumber	*Staphylococcus sciuri*
2	A	cucumber	*Micrococcus luteus*
3	A	plum	*Staphylococcus sciuri*
4	A	cucumber	*Lelliottia amnigena*
5	A	cucumber	*Enterococcus casseliflavus*
6	A	cucumber	*Comamonas testosteroni*
7	B	onion	*Enterobacter ludwigii*
8	B	onion	*Kerstersia gyiorum*
9	B	onion	*Citrobacter braakii*
10	B	onion	*Pseudomonas aeruginosa*
11	B	onion	*Enterococcus faecalis*
12	B	onion	*Klebsiella pneumoniae*
13	C	tomato	*Pediococcus pentosaceus*
14	C	tomato	*Enterobacter ludwigii*
15	C	tomato	*Micrococcus luteus*
16	C	tomato	*Klebsiella oxytoca*
17	C	tomato	*Pseudomonas protegens*
18	C	tomato	*Serratia marcescens*
19	D	cucumber	*Lelliottia amnigena*
20	D	cucumber	*Pseudomonas putida*
21	D	beetroot	*Proteus vulgaris*
22	D	cucumber	*Staphylococcus equorum*
23	D	onion	*Empedobacter falsenii*
24	D	cucumber	*Pseudomonas aeruginosa*
25	D	cucumber	*Providencia alcalifaciens*

Abbreviations: A, B, C, D—locations of fresh-cut processing plants.

The analysis results of bacterial contamination levels in the washing waters varied (Figure 3). The highest level of contamination reached 2.54×10^8 cfu/mL (sample taken after flushing of beetroot; location D). The lowest level of contamination was 1.70×10^5 cfu/mL at location B (sample collected after onion wash).

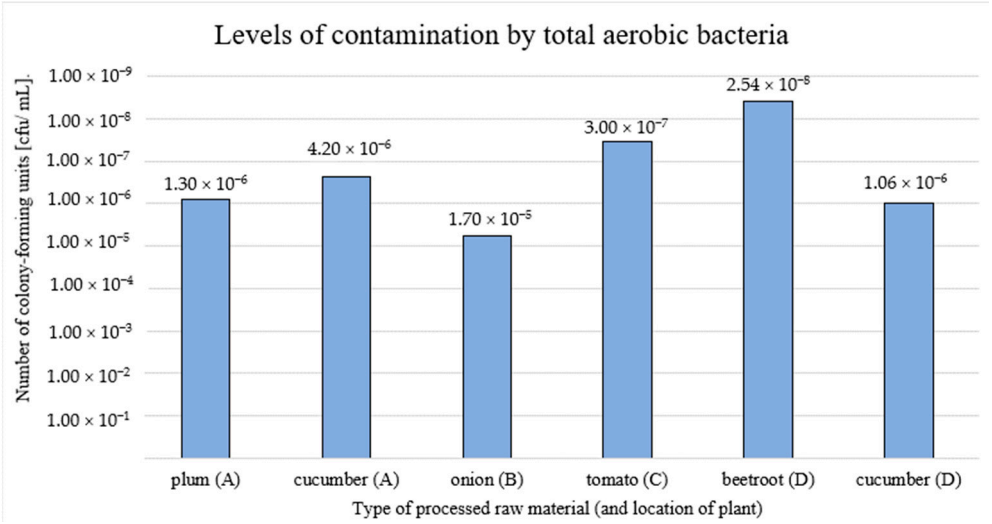

Figure 3. Levels of microbiological contamination of water after fruit and vegetable wash processes (A, B, C, D—locations of fresh-cut processing plants).

3.2. Antibiotic Susceptibility

Six bacterial strains obtained from the selected samples were tested: *Enterococcus faecalis* (isolation: site B; type of washed raw material: onions), *Pseudomonas aeruginosa* (isolation: site D; type of washed raw material: cucumbers), *Klebsiella oxytoca* (isolation: site C; type of washed raw material: tomatoes), *Klebsiella pneumoniae* (isolation: site B; type of washed raw material: onions), *Serratia marcescens* (isolation: site C; type of washed raw material: tomatoes), and *Proteus vulgaris* (isolation: site D; type of washed raw material: beetroots) (Table 3).

Table 3. Assessment of antibiotic susceptibility profiles of the investigated bacteria.

Type of Antibiotic		Antibiotic Susceptibility Profiles of Bacteria					
Class	**Antibiotic**	***K. oxytoca***	***K. pneumoniae***	***S. marcescens***	***P. vulgaris***	***P. aeruginosa***	***E. faecalis***
Penicillins	piperacillin	S	S	S	S	I	n. a.
Penicillins	amoxicillin-clavulanic acid	S	S	n. a.	S	n. a.	n. a.
Penicillins	ticarcillin-clavulanic acid	S	S	S	S	I	S
Penicillins	ampicillin	n. a.	n. a.	n. a.	n. a.	n. a.	S
Cephalosporins	cefepime	R	S	S	S	I	n. a.
Cephalosporins	cefiderocol	S	S	S	S	S	n. a.
Cephalosporins	ceftazidime	S	S	S	S	I	n. a.
Carbapenems	meropenem	S	S	S	S	S	n. a.
Carbapenems	imipenem	I	I	I	I	I	I
Fluoroquinolones	ciprofloxacin	S	S	S	S	I	S
Fluoroquinolones	moxifloxacin	S	S	n. a.	S	n. a	*
Fluoroquinolones	levofloxacin	S	S	S	S	I	S
Aminoglycosides	gentamicin	S	S	S	S	n. a.	n. a.
Aminoglycosides	amikacin	S	S	S	S	S	n. a.
Aminoglycosides	tobramycin	S	S	I	S	S	n. a.
Chemotherapeutics	trimethoprim	S	S	S	S	n. a.	n. a.
Chemotherapeutics	trimethoprim	n. a.	n. a.	n. a.	n. a.	n. a.	S
Glycopeptides	vancomycin	n. a.	n. a.	n. a.	n. a.	n. a.	S
Tetracyclines	tigecycline	n. a.	n. a.	n. a.	n. a.	n. a.	S
Oxazolidinones	linezolid	n. a.	n. a.	n. a.	n. a.	n. a.	S

Abbreviations: resistant: R; intermediate: I (susceptible, increased exposure); susceptible: S; n. a.—not applicable; *—devoid of fluoroquinolone resistance mechanisms.

The *P. aeruginosa* isolate was observed to exhibit susceptibility to cefiderecol, meropenem, tobramycin, and amikacin. Intermediate levels (susceptible, but with increased expo-

sure) were also found for piperacillin, ceftadizime, imipenem, levofloxacin, ciprofloxacin, ticarcillin-clavulanic acid, and cefepime.

E. faecalis strains demonstrated susceptibility to all the antibiotics tested. Susceptibility with increased exposure was found for imipenem, with an inhibition zone diameter of 30.5 mm. For moxifloxacin, there are no clinical breakpoints, but acquired resistance should be excluded (when acquired resistance is excluded, the isolate should be reported as "devoid of fluoroquinolone resistance mechanisms" but not as susceptible to moxifloxacin).

Testing the antimicrobial susceptibility of *K. oxytoca* rods to 15 different antibiotics also revealed susceptibility to most of the applied agents. The tested bacteria exhibit resistance to cefepime (inhibition zone: 8 mm) and susceptibility with increased exposure to imipenem. The testing results for the *K. pneumoniae* strain indicated susceptibility with increased exposure to imipenem (33.5 mm). Examination of other agents belonging to six antibiotic classes resulted in the absence of phenotypic bacterial resistance. For cefepime, no resistance was found; the zone of inhibition reached 38 mm. The *S. marcescens* strain demonstrated susceptibility with increased exposure to agents from three antibiotic classes: imipenem (32.5 mm), tobramycin (19.5 mm), and amoxicillin-clavulanic acid (19.5 mm). The isolate tested was susceptible to all the remaining antibiotics. The results revealed that the *P. vulgaris* strain was generally also susceptible to all antibiotics tested. Similarly to the previous results, the isolate was classified as imipenem-susceptible with increased exposure.

4. Discussion

Research on microbial contamination and cross-contamination of minimally processed fruit and vegetables has focused the attention of researchers for years. There are limited studies that have explored bacterial isolation from wash waters, with additional assessment of phenotypic antibiotic resistance, conducted in several diverse fruit and vegetable processing plants. The current study addresses this gap by conducting a thorough analysis across different facilities, offering a broader perspective on the presence and resistance patterns of bacteria in these environments. The water recycling and implementation of the closed-loop economy principles are recognized as an essential aspect of food processing and a part of a sustainable development strategy. However, there is a consensus highlighting that recycled water must meet high quality standards to ensure the safety of the final product [34].

Regarding the levels of microbial contamination with total bacterial counts, our results revealed relatively low and medium levels of contamination, compared to previous studies, which reported contamination levels of up to 5 to 9 log units [8,34,35]. The results correlate with an earlier assessment of microbial contamination at an apple processing unit, where a total contamination level of 4 log units was obtained [7].

Differences in microbial contamination levels are explained by variable factors related to geographical location, type of raw material processed, technological process, disinfection approach, season, etc. As noted by Zhou et al., secondary bacterial multiplication is linked to the presence of post-harvest residues and damaged vegetables in the water, which may be the main driver of bacterial contamination [36,37].

The identification of bacterial species via mass spectrometry, mostly applied in clinical microbiological investigations, indicates the presence of bacteria of diverse origins. In the current study, the isolated strains were soil bacteria, fecal contamination indicators, as well as plant and human pathogenic bacteria. The findings correlate with an investigation by Liu et al. on species analysis of the production environment in a fresh-cut processing plant. The study found the presence of high bacterial biodiversity originating from various sources [38].

Staphylococcus spp., represented in our study by *Staphylococcus equorum* and *Staphylococcus sciuri*, confirm previously reported detections of representatives of this bacterial family. An investigation by Sun et al. on the microbial diversity of fresh-cut lettuce during processing and storage demonstrated the occurrence of pathogenic *S. aureus* [39]. This supports the idea that inadequate washing of fresh-cut vegetables and fruits can lead to contamination, which continues during transportation and distribution. It is important to note

that *S. equorum* (coagulase-negative staphylococci) are frequently found in both processing units and various foods, including ready-to-eat products [40,41]. *S. sciuri* bacteria, on the other hand, may present a specific threat due to its potential opportunistic pathogenicity [42]. Given that it is principally an animal-associated bacterial species (inhabiting, e.g., the skin of free-living rodents), it is possible to indicate that the raw material (in the case of our study, cucumbers) may have been subjected to potential animal exposure [43].

The current study also indicates frequent isolation of members of the *Enterobacteriaceae* family, including typical opportunistic pathogens. Research conducted by Pintor-Cora et al. suggests that contamination with *Enterobacteriaceae* representatives occurs as early as during cultivation. *Enterobacteriaceae* rods, exceeding the detection limit, were found in 82.9% of vegetable samples and 36.8% of environmental samples (study based on 117 vegetable samples and 57 farm locations) [44]. The detected isolates of *K. oxytoca* and *K. pneumoniae* may originate from soil, natural fertilizer, and water, owing to the widespread presence of *Klebsiella* spp. in the natural environment [45].

The detection of *K. pneumoniae* in food products is alarming since it is classified among the ESKAPE bacteria group. Opportunistic infections caused by the above pathogens can include urinary tract infections, pneumonia, liver abscesses, bacteremia, soft tissue infections, endophthalmitis, and meningitis [46]. Liu et al. emphasize the biofilm-forming potential of *K. pneumoniae* rods in the processing environment of fresh fruits and vegetables. This could pose a threat due to the bacterium's ability to persist and propagate for extended periods despite disinfection measures [38]. Other strains of the *Enterobacteriaceae* family isolated in our study, such as *P. vulgaris* and *S. marcescens*, could also pose threats to public health and safety. Hence, their presence in the environment of fresh-cut vegetable and fruit processing facilities is undesirable and therefore should be constantly monitored and kept to a minimum [47,48].

The presence of *Enterococcus* spp. in the wash water indicates most often fecal contamination, as these bacteria are commonly found in such environments. It is consistent with our earlier studies, which also demonstrated the presence of *Enterococcus* spp. in wash water, indicating the widespread fecal contamination of fresh vegetables and fruits [7]. The distribution and ecology of the *E. casseliflavus* isolate are less understood compared to *E. faecalis*; however, the pathogen also colonizes the human intestinal tract due to the regularity of its isolation in nosocomial infections with vancomycin-resistant strains [49]. The problematic nature of *Enterococcus* spp. is due to both natural and easily acquired and maintained resistance to a broad spectrum of antibiotics [50]. Research by Xie et al. also indicates the ease of transfer of these pathogens due to workers' loss of hygiene, particularly during the step of packaging of fresh fruit and vegetables [51].

Our study also revealed the presence of representatives of *Pseudomonas* spp. in the tested water samples. While *P. putida*, a rhizosphere-borne classical root colonizer, does not pose a major threat to vegetable and fruit sanitization processes, the detection of *P. aeruginosa* may provide additional production challenges. Due to their ubiquity, *P. aeruginosa* bacteria are also already isolated in final fresh-cut products, tolerating well the washing and preprocessing procedures of the raw material [52,53]. It is noteworthy that *P. aeruginosa* intercalates into plant tissues and can colonize them for long periods without visible disease symptoms (or manifesting soft-rot symptoms) [54]. Due to its genome plasticity, broad adaptability, high biofilm production capacity, as well as advanced secretion systems, *P. aeruginosa* is regarded as a significant pathogen. The bacterium is particularly concerning since it causes several infections that include nosocomial pneumonia, surgical wound infections, urinary tract infections, and bacteremia [55,56].

The second part of this study, related to the assessment of the susceptibility of selected isolates to various antibiotics, revealed the resistance of *K. oxytoca* to the application of cefepime. An increase in antibiotic resistance level in clinical strains may have a substantial impact on the spread of resistance in environmental strains. Clinical studies on resistance trends in *K. pneumoniae* causing urinary tract infections, conducted on 1543 *K. pneumoniae* isolates from 2011 to 2019, have shown a notable rise in cefepime resistance

levels. Specifically, the level of resistance to cefepime increased from 18.2% to 30.5% by 2017 [57]. As noted by Okaiyeto et al., the contamination of food chains by antibiotic-resistant bacteria is a growing global problem that requires enhanced action and efforts to implement an integrated approach. This strategy should include not only preventive measures but also novel monitoring systems, along with the provision of antibacterial agents [58]. Noteworthy is that cefepime can be degraded by some extended-spectrum β-lactamases (ESBLs) and carbapenemases (although it shows moderate resistance to hydrolysis by OXA-48) [59,60]. In this context, it is also crucial to control the transfer of antibiotics to the environment. As noted by Wang et al., exposure to low concentrations of cefepime can lead to significant antimicrobial resistance levels in environmental bacteria [61]. Overall, the low detection rate of phenotypic resistance is encouraging, particularly considering the numerous reports of AMR strains found on fresh fruits and vegetables, where washing waters can serve as a source of contamination. It is important to highlight that geographical location should not be overlooked in this context. Research by Salmanov et al. (Ukraine) demonstrated the isolation of various concerning pathogens, such as methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus* spp., and third-generation cephalosporin-resistant *K. pneumoniae* and *E. coli* [62]. Saksena et al. (India) also highlight the frequent isolation of AMR coliforms from fresh vegetables and fruits, exhibiting ESBLs and carbapenem resistance [63]. As noted by Tiedje et al., the food production system may be an underestimated reservoir for antibiotic-resistant bacteria and antibiotic-resistance genes [64]. An example is the research conducted on the analysis of contamination of vegetables by *Pseudomonas* spp. in Jamaica. In antimicrobial susceptibility tests, it was found that isolates were resistant or had reduced susceptibility to ampicillin, chloramphenicol, sulfamethoxazole/trimethoprim, and aztreonam, and up to 35% were resistant to four different antibiotics [65].

A limitation of this study is that the MALDI Biotyper is primarily designed for the identification of clinical strains, which led to the inability to identify four species. Nonetheless, this study is relevant to public health safety aspects, such as monitoring and controlling pathogens and identifying potential threats.

In summary, our research contributes to understanding the microbiological contamination of minimally processed vegetables and fruits and underscores the importance of adhering to high sanitary standards. The isolated bacteria, including potentially pathogenic ones, pose a challenge to health safety. The findings from our study can contribute to further actions aimed at improving hygiene and safety in food production. Future research could explore the impact of various disinfection methods on microbiological contamination in wash waters. An interesting area of investigation is the use of non-invasive biological methods, which, by reducing the reliance on traditional chemical methods, could help mitigate microbiological risks and support sustainable food production [66].

5. Conclusions

In conclusion, bacterial species identification studies carried out by mass spectrometry have shown that the wash waters of fresh-cut processing plants can be a constant habitat for a diverse range of bacteria.

Opportunistic pathogens such as *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Proteus vulgaris* were found among the isolated strains. The isolation of cefepime-resistant *K. oxytoca* indicates that the waters of the agri-food industry may be considered a site for the potential development and maintenance of antibiotic resistance. Broader research is needed to investigate the mechanisms of bacterial contamination in washing water further and develop more effective sanitation methods. If contaminated washing water is not properly managed, potential risks to consumers include contamination of food chains and the consequent threat to consumer safety. These findings highlight the importance of monitoring the microbial microflora in minimally processed foods and the need for appropriate sanitary control procedures to minimize the risk of pathogen contamination.

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Antimicrobial Activity and Phytochemical Profiling of Natural Plant Extracts for Biological Control of Wash Water in the Agri-Food Industry

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Featured Application: This study explores the potential of natural plant extracts as antimicrobial agents for the biological control of wash water pathogens in the agri-food industry. The findings support the development of eco-friendly alternatives to chlorine-based disinfection, contributing to safer and more sustainable food processing practices.

Abstract: Water used in cleaning processes within the agri-food industry can be a vector for post-harvest contaminants, thus contributing to cross-contamination. The contamination risk is increased when water is not replaced between batches or when disinfection protocols are insufficient. Given the increasing focus in recent years on the potential of natural, non-invasive plant extracts to combat a variety of pathogens, including multidrug-resistant bacteria, environmental strains, and clinical isolates, this study aimed to evaluate the antibacterial activity of selected water-ethanol plant extracts against six opportunistic pathogens isolated from wash water in the agri-food industry, along with chromatographic analyses of the selected extracts. Plant extracts were obtained from the fruits, leaves, shoots, roots, and bark of 13 species. Antibacterial activity was assessed using the well diffusion method. The results indicated that antimicrobial activity was exhibited by six extracts: *Tilia cordata* Mill., *Camellia sinensis*, *Quercus robur* L., *Betula pendula* Roth, *Rubus idaeus* L., and *Salix alba* L. The extracts showed strain-dependent antimicrobial activity, with *C. sinensis* and *R. idaeus* up to 4.0 mm and 8.0 mm inhibition zones, respectively. *P. aeruginosa* and *E. faecalis* were the most susceptible strains, demonstrating the largest inhibition zones. In contrast, *P. vulgaris* and *K. oxytoca* were more resistant. The efficacy of the most active extracts can be linked to the presence of phytochemicals identified via GC-MS, including epicatechin, shikimic acid, quinic acid, gallic acid, and caffeine. These metabolites are known to interfere with bacterial cell structures and metabolic pathways. These studies may serve as a preliminary step toward the development of non-invasive water treatment methods for wash water.

Keywords: water-born pathogens; plant extracts; gas chromatography analysis; antimicrobial agents

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1. Introduction

One of the major challenges facing the modern world is the balanced use of water resources [1]. The increasing impacts of climate change, manifested through water scarcity,

droughts, and extreme flooding, affect the security of crop and livestock production, human health, and socioeconomic well-being [2,3]. The reuse of water within the framework of a circular economy is a critical component of strategies to reduce water consumption, thereby promoting more efficient management of the resources [4]. In this context, agriculture, as the most water-intensive sector, is responsible for approximately 70% of global freshwater consumption [5,6]. The fresh-cut fruits and vegetables processing sector is a major consumer of water, particularly during the initial stages of production. Water consumption and wastewater generation in the production of fresh-cut products typically range from 2 to 11 m^3 of water per ton of product and from 11 to 23 m^3 of wastewater per ton, respectively. This means that the production of one ton of fresh-cut fruits or vegetables requires several to over ten cubic meters of clean water, while the volume of wastewater generated can be up to twice as high. Such significant water use and wastewater generation are mainly associated with intensive washing and rinsing processes, which are essential for maintaining hygiene and ensuring the quality of products intended for direct consumption [7–9]. These processes primarily aim to remove organic matter residues and soil particles and reduce microbiological contamination of raw material [10].

From a microbiological risk perspective, the raw material can become contaminated at both the pre-harvest and post-harvest stages [11]. Wash water may act as a carrier of post-harvest contamination, contributing to cross-contamination, especially when water is not replaced between batches. Reusing the same water multiple times allows microbiological contaminants from one batch of fruits or vegetables to transfer to subsequent batches, thereby increasing the risk of pathogen propagation [12,13]. From a processing perspective, microorganisms that affect the quality of the final product are particularly undesirable. These include filamentous fungi, yeasts, and saprotrophic bacteria, which contribute to raw material spoilage and shorten its shelf life, leading to premature product deterioration [14]. The second group comprises pathogens that pose a risk to public health, including the ESKAPE pathogens—*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.—as well as *Salmonella* spp., *Listeria monocytogenes*, *Clostridium* spp., *Escherichia coli*, and coliforms [15–18] (Figure 1).

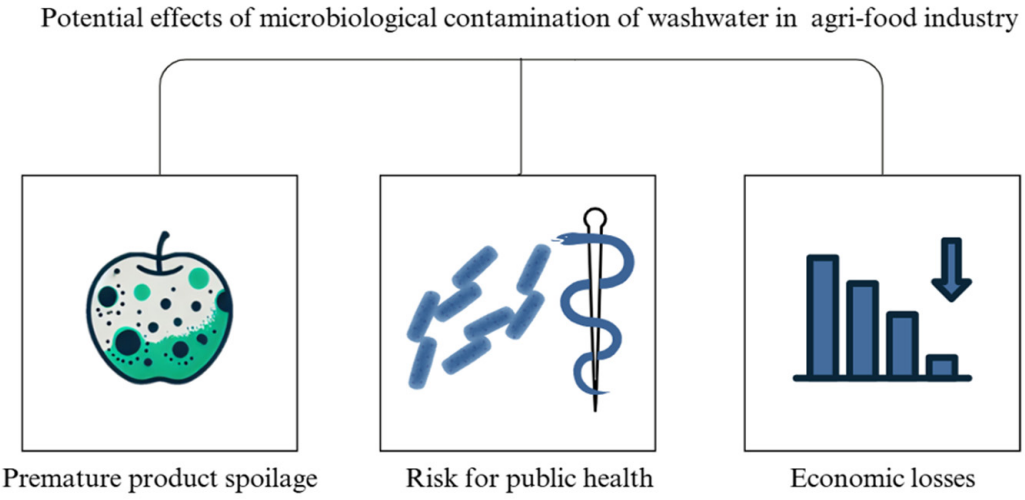

Figure 1. Diagram presenting the potential main risks associated with contamination of the wash water.

Considering the rational use of water within the principles of the circular economy, the optimal solution would be the reuse of rinse water following proper treatment. This approach minimizes wastewater generation in facilities within the agri-food sector [8]. A variety of methods are employed to purify rinse water, including physicochemical

processes such as sedimentation, flocculation, and filtration using sand, silica, and carbon, as well as advanced techniques like nano-ultrafiltration [19,20]. Pathogen inactivation is typically achieved through traditional methods involving chlorine compounds, which, although highly effective, can have adverse effects due to the formation of harmful by-products [21]. Consequently, there has been a growing body of research focused on the use of natural, non-invasive plant extracts against various strains, including multidrug-resistant bacteria, virulent strains, and clinical isolates [22–24]. However, to the best of the authors' knowledge, a significant research gap exists regarding the use of plant extracts against environmental strains isolated from wash water in the agri-food sector.

The research hypothesis suggests that extracts from various plant materials are effective against pathogens isolated from wash water in the agri-food industry. It further posits that combining bark extracts, which contain heavy, non-volatile organic compounds, with extracts rich in volatile organic compounds (from leaves, shoots, flowers, and fruits) may result in an increased effect, thereby enhancing antibacterial activity against the tested bacteria.

The aim of the study was to evaluate the antibacterial activity of selected plant extracts against opportunistic pathogens derived from rinse water in the agri-food industry. The second phase of the research involved determining the qualitative composition of the antibacterial extracts, which are characterized by a high content of volatile compounds (from extracts of leaves, fruits, flowers, and shoots), using gas chromatography-mass spectrometry (GC-MS). This technique was selected due to its high sensitivity and specificity for the identification of volatile and semi-volatile organic compounds, which can affect bacterial cells by disrupting membrane integrity, inhibiting DNA and protein synthesis, suppressing biofilm formation, and interfering with efflux pump activity [25–27].

2. Materials and Methods

2.1. Research Material

The selection of the 13 plant species was based on a multi-criteria approach combining ethnobotanical relevance, particularly their traditional use in herbal infusions or food preservation; reported antimicrobial properties in the scientific literature; availability in the commercial or local market; and diversity in phytochemical profiles to ensure a broad representation of chemical groups potentially involved in microbial inhibition. The research material consisted of 13 plant-based raw materials selected based on previous reports (Table 1, Figure 2). These materials included various plant parts, such as leaves (*Urtica dioica* L.), flowers (*Tilia cordata* Mill.), bark (*Quercus robur* L., *Betula pendula* Roth, *Salix alba* L., *Quercus suber* L.), fruits (*Rosa canina* L.), roots (*Taraxacum officinale* F.H. Wiggers coll.), shoots (*Rubus idaeus* L., *Pinus sylvestris* L.), herbs (*Verbena officinalis* L.), and waste products—coffee grounds (*Coffea* spp.) and tea grounds (*Camellia sinensis*). These plants have not yet been used in the control of opportunistic pathogens isolated from water.

Table 1. Summary of reports on the antibacterial properties of selected plant materials.

N°	Name	Common Name	Material	Traditional Usage	Examples of In-Vitro Tested Pathogens	References
1	*Coffea* L.	Coffee	grounds	common cold, diarrhea, wound treatment	*Salmonella enterica*, *Streptococcus mutans*, *K. oxytoca*, *E. coli*	[28–31]
2	*Camellia sinensis*	Tea	grounds	diarrhea, streptococcal pharyngitis,	*E. faecalis*, *S. aureus*, *E. coli*, *S. typhimurium*, *Bacillus cereus*, *Shigella dysenteriae*	[32–35]
3	*Urtica dioica* L.	Common nettle	leaves	skin diseases, urinary disorders, respiratory diseases	*Bacillus cereus*, *S. aureus*, *Staphylococcus epidermidis*, *E. coli*	[36–39]
4	*Tilia cordata* Mill.	Small-leaved lime	flowers	pneumonia, sore throat, diarrhea	*Candida glabrata*, *S. aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*	[40,41]

Table 1. *Cont.*

N°	Name	Common Name	Material	Traditional Usage	Examples of In-Vitro Tested Pathogens	References
5	*Quercus robur* L.	English oak	bark	tonsillitis, wound treatment, diarrhea	*E. coli*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Serratia liquefaciens*, *S. aureus*	[42–45]
6	*Rosa canina* L.	Dog rose	fruits	common colds, influenza, diarrhea, cough, urinary tract infections	*S. aureus*, *K. pneumoniae*, *E. coli*	[46–49]
7	*Betula pendula* Roth	Silver birch	bark	urinary tract infections, wound treatment	*Enterococcus faecalis*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*	[44,50,51]
8	*Taraxacum officinale* F.H. Wiggers coll.	Dandelion	root	urinary infections	*S. aureus*, *K. pneumoniae*, *E. coli*, *Proteus mirabilis*	[52–54]
9	*Quercus suber* L.	Cork oak	expanded bark	diarrhea, gastritis, ulcer, and skin infection	*S. aureus*, *B. cereus*, and *Listeria monocytogenes*	[55–57]
10	*Rubus idaeus* L.	Red Raspberry	shoots	common cold, fever, and flu-like infections	*Proteus mirabilis*, *A. baylyi*, *P. aeruginosa*	[58–61]
11	*Salix alba* L.	White Willow	bark	flu symptoms including fever, and generalized pain	*S. aureus*, *E. coli*	[62–64]
12	*Verbena officinalis*	Common Vervain	shoots	anti-inflammatory, anti-fungal, common cold	*P. aeruginosa*, *Citrobacter freundii*, *S. aureus.*	[65,66]
13	*Pinus sylvestris* L.	Scots Pine	shoots	colds, cough, wound treatment	*E. coli*, *S. aureus*, *K. pneumoniae*, *B. subtilis*	[67–70]

Figure 2. Plant materials selected for the preparation of extracts used in this study (Order: 1—*Coffea* L., 2—*Camellia sinensis*, 3—*Urtica dioica* L., 4—*Tilia cordata* Mill., 5—*Quercus robur* L., 6—*Rosa canina* L., 7—*Betula pendula* Roth, 8—*Taraxacum officinale* F.H. Wiggers coll., 9—*Quercus suber* L., 10—*Rubus idaeus* L., 11—*Salix alba* L., 12—*Verbena officinalis*, 13—*Pinus sylvestris* L.).

The study on antibacterial activity utilized six isolates of opportunistic bacteria obtained from the collection of the Department of Microbiology and Plant Ecology (Bydgoszcz University of Science and Technology, Bydgoszcz, Poland): *P. aeruginosa*, *E. faecalis*, *K. oxy-*

toca, *K. pneumoniae*, *S. marcescens*, and *P. vulgaris*. Detailed descriptions of these isolates were provided in previous research conducted by the department [71].

2.2. Preparation of Extracts

Seventy percent water-ethanol extracts were prepared by measuring 25 g of cleaned and dried plant material (pre-dried for 12 h at 30 °C). The material was then ground into a fine powder and soaked in 250 mL of solvent. The extracts were agitated at 150 rpm for 24 h and subsequently filtered using laboratory filter paper. The filtered extracts (150 mL) were concentrated three-fold (50 mL) using a Laborota 4000 efficient rotary evaporator (Heidolph, Schwabach, Germany) at 90 rpm and 65 °C. The extracts were sterilized using syringe filters with a pore size of 0.22 µm and a diameter of 25 mm to ensure sterility.

Extracts exhibiting antibacterial activity, as assessed by the well-diffusion method, were further processed via spray drying at an air intake temperature of 180 °C and an air outflow temperature of 91.9 °C, using a Labspray SD-18A spray dryer (Changsha, China). The percentage recovery of the dried extract was estimated by using the formula:

$$Extract\ recovery = \frac{C}{D} \times 100$$

where C is the mass (g) of the dried extract, and D is the mass (g) of the powdered plant material.

2.3. The Well Difusion Method

The 24-h bacterial cultures were grown on TSA (Tryptic Soy Agar, Merck, Darmstadt, Germany) slants at 36 ± 0.5 °C. After incubation, 1 mL of sterile saline solution (0.9% NaCl) was added to each test tube. The bacterial culture was then collected from the surface of the medium using a sterile loop. The final bacterial inoculum density was determined spectrophotometrically (DEN-1 McFarland Densitometer, Biosan, Riga, Latvia) and adjusted to 1.0–2.0 × 10^8 CFU/mL, which corresponds to the standard cell density used in antimicrobial susceptibility testing. The resulting suspension was transferred into cooled, still-liquid Mueller-Hinton agar (Merck) and gently mixed. The inoculated medium was then poured into sterile Petri dishes and allowed to solidify. After the agar solidified, six wells (8 mm in diameter) were made on each plate using sterile cork borers. In the final step, 100 µL of the test extracts were added to each well, and the plates were incubated for 24 h at 36 ± 0.5 °C. After incubation, the inhibition zones were measured, excluding the well diameter.

Based on the analysis, extracts with antibacterial properties were identified and subsequently subjected to tests assessing their combined effect. These tests involved combining selected extracts in pairs according to a defined matrix and applying 100 µL of the resulting mixture to the designated wells in the well-diffusion method [72]. The result was presented as an average of two repetitions.

2.4. Gas Chromatography-Mass Spectrometry Analysis

The GC/MS analysis was performed as follows: 10 mg of the tested plant extract was dissolved in 1 mL of 99.8% anhydrous pyridine, and 100 µL of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was then added. The mixture was heated at 60 °C for 30 min to allow derivatization. The resulting silylated samples were then analyzed using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass selective detector (Agilent, Santa Clara, CA, USA). A 1 µL sample injection was performed using an Agilent 7693A autosampler. Analyte separation was achieved on an HP-5MS fused silica column (30 m × 0.25 mm × 0.25 µm film thickness) with a helium flow rate of 1 mL/min. The injector was operated in split mode (1:10), with the temperature set to 300 °C. The initial

column temperature was 50 °C, which was increased to 325 °C at a rate of 3 °C/min and held for 10 min. The ion source temperature was set to 230 °C, and the quadrupole temperature was maintained at 150 °C. Electron ionization mass spectrometry (EIMS) was performed at an ionization energy of 70 eV. Detection was carried out in full-scan mode, with a mass range of 41 to 800 m/z. After data integration, the relative contributions of each compound to the total ion current (% TIC) were calculated. Retention indices for the analytes were determined using the retention times of alkanes as standards. The mass spectra and calculated retention indices were used for compound identification. Mass spectral identification was facilitated by an automated GC-MS data processing system provided by the National Institute of Standards and Technology (NIST) [73], using data from the "Identification of Biologically and Environmentally Significant Organic Compounds Mass Spectra and Retention Indices Library of Trimethylsilyl Derivatives". The retention indices of the identified compounds were compared with reference values.

3. Results

3.1. Evaluation of Extract Activity

The extracts exhibited antimicrobial activity with varying effectiveness depending on the tested strain (Table 2). For the purpose of this study, inhibition zones were classified as follows: strong activity (≥7 mm), moderate (2–6 mm), minimal (1 mm), and no activity (0 mm). Based on this classification, *C. sinensis* and *R. idaeus* demonstrated the largest activity, with maximum inhibition zones reaching 8.0 mm for *P. aeruginosa* and *E. faecalis* and 8.0 mm for *P. aeruginosa*, respectively. Extracts from *Q. robur*, *B. pendula*, *T. cordata*, and *S. alba* displayed moderate antimicrobial activity, with inhibition zones ranging from 2.0 mm to 6.0 mm. No antimicrobial activity was observed for the extracts from *Coffea* L., *R. canina*, *T. officinale*, *Q. suber*, *V. officinalis*, and *P. sylvestris*, with inhibition zones of 0.0 mm for all tested strains. The *U. dioica* extract exhibited only minimal activity against *P. aeruginosa*, with an inhibition zone of 1.0 mm.

Table 2. Inhibition zone diameter (mm) of tested plant extracts in the well diffusion method.

		Tested Bacteria					
		P. aeruginosa	***P. vulgaris***	***K. pneumoniae***	***K. oxytoca***	***E. faecalis***	***S. marcescens***
Plant Extract	**Material**	**Mean of Inhibition Zone [mm] (After Exclusion of the Well Diameter)**					
Coffea L.	grounds	0.0	0.0	0.0	0.0	0.0	0.0
Camellia sinensis	grounds	**8.0**	**7.0**	**4.0**	**2.0**	**8.0**	**4.0**
Urtica dioica L.	leaves	**1.0**	0.0	0.0	0.0	0.0	0.0
Tilia cordata Mill.	flowers	**6.0**	**4.0**	**3.0**	**2.0**	0.0	**3.0**
Quercus robur L.	flowers	**6.0**	**2.0**	**4.0**	**5.0**	**6.0**	**4.0**
Rosa canina L.	fruit	0.0	0.0	0.0	0.0	0.0	0.0
Betula pendula Roth	bark	**4.0**	**4.0**	**5.0**	**6.0**	**4.0**	**4.0**
Taraxacum officinale F.H. Wiggers coll.	root	0.0	0.0	0.0	0.0	0.0	0.0
Quercus suber L.	expanded bark	0.0	0.0	0.0	0.0	0.0	0.0
Rubus idaeus L.	shoots	**8.0**	**4.0**	**6.0**	**6.0**	**5.0**	**4.0**
Salix alba L.	bark	**4.0**	**4.0**	**4.0**	**4.0**	**4.0**	**2.0**
Verbena officinalis	shoots	0.0	0.0	0.0	0.0	0.0	0.0
Pinus sylvestris L.	shoots	0.0	0.0	0.0	0.0	0.0	0.0
* Postive control:		37.5	36.5	38.0	30.0	22.5	41.5

* Ciprofloxacin (5 µg); inhibition zones are marked in bold font.

The susceptibility analysis indicated that *P. aeruginosa* and *E. faecalis* were more susceptible to the tested extracts, whereas *P. vulgaris* and *K. oxytoca* exhibited higher resistance.

For further studies on the assessment of combined effects, extracts from the following raw materials were selected: *C. sinensis*, *T. cordata*, *Q. robur*, *B. pendula*, *R. idaeus*, and *S. alba*. Combinations of plant extracts were prepared in a 1:1 ratio and tested at the same total volume (100 µL) as single extracts. The percentage increase in antimicrobial activity of the combined extracts was calculated relative to the most active single extract in each pair. This approach assumes that any observed enhancement beyond the highest individual activity may indicate a potential synergistic or additive effect between the extracts. This method provides a conservative estimate of enhanced antimicrobial activity, as it compares the mixture directly with the stronger of the two components. To achieve this, an experimental setup was prepared in which the extracts were combined according to the proposed matrix (Table 3).

Table 3. Matrix of extract combinations for interaction testing.

Cs × Tc				
Cs × Qr	Tc × Qr			
Cs × Bp	Tc × Bp	Qr × Bp		
Cs × Ri	Tc × Ri	Qr × Ri	Bp × Ri	
Cs × Sa	Tc ×Sa	Qr × Sa	Bp × Sa	Sa × Ri

Cs—*Camellia sinensis*; Tc—*Tilia cordata* Mill.; Qr—*Quercus robur* L.; Bp—*Betula pendula*; Ri—*Rubus idaeus*; Sa—*Salix alba* L.

The recovery yield of dried extracts obtained after spray drying varied among the plant species, with *Camellia sinensis* exhibiting the highest recovery (10.59%), followed by *Rubus idaeus* (7.86%) and *Tilia cordata* (5.34%) (Table 4).

Table 4. Recovery percentage of dried plant extracts after spray drying.

Plant Species	Initial Plant Mass (g)	Dried Extract Mass (g)	Recovery (%)
Tilia cordata	25	1.335	5.34
Rubus idaeus	25	1.965	7.86
Camellia sinensis	25	2.648	10.59
Quercus robur	25	0.385	1.54
Betula pendula	25	0.265	1.06
Salix alba	25	0.390	1.56

Accordingly, 100 µL of each extract applied in the well-diffusion assay contained 8.9 mg of dry extract for *Tilia cordata*, 13.1 mg for *Rubus idaeus*, 17.7 mg for *Camellia sinensis*, 2.6 mg for *Quercus robur*, 2.6 mg for *Salix alba*, and 1.8 mg for *Betula pendula*.

The effect of the combination of *Q. robur* × *B. pendula* against the *P. vulgaris* strain, resulting in a 125% increase in the inhibition zone, was observed. Also, a 50% increase for the combination of *B. pendula* × *S. alba* against *S. marcescens* and *T. cordata* × *R. idaeus* against *P. aeruginosa* was noted. An intermediate effect (33% increase) for the combination of *T. cordata* × *B. pendula* against *K. oxytoca* and *Q. robur* × *R. idaeus* against *K. pneumoniae* was recorded. The lowest effect (8%) was observed for the combination of *Q. robur* × *R. idaeus* against *E. faecalis*. The remaining combinations—did not result in an increased diameter of inhibition zones compared to the individual extracts. This lack of enhanced activity may stem from limited compatibility between active compounds or the absence of additive or synergistic effects in these specific pairings (Figure 3, Table 5).

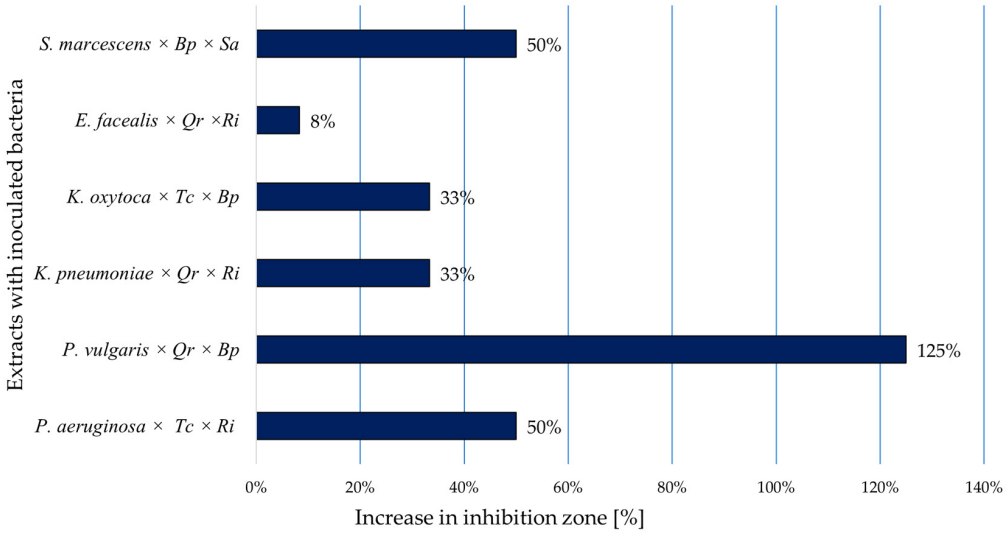

Figure 3. Increase in diameter of inhibition zones, following application of a mixture of two extracts [%] (Abbreviations: Tc—*Tilia cordata* Mill.; Qr—*Quercus robur* L.; Bp—*Betula pendula*; Ri—*Rubus idaeus*; Sa—*Salix alba* L.).

Table 5. Summary of results for combinations of extracts exhibiting growth inhibition of the bacteria tested.

Combination	*P. aeruginosa*	*P. vulgaris*	*K. pneumoniae*	*K. oxytoca*	*E. faecalis*	*S. marcescens*
Cs × *Tc*	8.0	7.0	4.0	2.0	8.0	4.0
Cs × *Qr*	8.0	7.0	4.0	5.0	8.0	4.0
Tc × *Qr*	6.0	4.0	4.0	5.0	6.0	4.0
Cs × *Bp*	8.0	7.0	5.0	6.0	8.0	4.0
Tc × *Bp*	6.0	4.0	5.0	**4.0**	4.0	4.0
Qr × *Bp*	6.0	**4.5**	5.0	6.0	6.0	4.0
Cs × *Ri*	12.0	7.0	6.0	6.0	8.0	4.0
Tc × *Ri*	**8.0**	4.0	6.0	6.0	5.0	4.0
Qr × *Ri*	8.0	4.0	**4.0**	6.0	**6.5**	4.0
Bp × *Ri*	8.0	4.0	6.0	6.0	5.0	4.0
Cs × *Sa*	8.0	7.0	4.0	4.0	8.0	4.0
Tc × *Sa*	6.0	4.0	4.0	4.0	4.0	3.0
Qr × *Sa*	6.0	4.0	4.0	5.0	6.0	4.0
Bp × *Sa*	4.0	4.0	5.0	6.0	4.0	**6.0**
Sa × *Ri*	8.0	4.0	6.0	6.0	5.0	4.0

Abbreviations: Cs—*Camellia sinensis*; Tc—*Tilia cordata* Mill.; Qr—*Quercus robur* L.; Bp—*Betula pendula*; Ri—*Rubus idaeus*; Sa—*Salix alba* L.; an increase relative to the highest inhibition zone in each pair of tested extracts is shown in bold.

3.2. GC-MS Analysis of the Extracts

Chromatographic analysis using gas chromatography-mass spectrometry revealed multiple peaks in the tested samples, with retention times of 203, 223, and 239 for tea, linden, and raspberry extracts, respectively (Figure 4). Detailed data for the individual extracts, including the compounds, retention times, experimental retention indices, literature retention indices, and total ion chromatogram (TIC) (%) for the linden, raspberry, and tea extracts, are provided in the Supplementary Materials (S1).

Among the analyzed chemical groups, amino acids and their derivatives had the highest proportion in tea (23.3%) and the lowest in raspberries (19.6%). Organic acids occurred in similar proportions in all plants, with a slight predominance in lime (27.5%). Sugars and their derivatives dominated raspberries (28.6%), while in tea they accounted for 25.0%. Similarly, in the case of alcohols and polyols, the extract from raspberries showed a

14.3% share, while in tea it was 11.7%. Aromatic and phenolic compounds were present in the highest proportion in tea (7.5%) and the lowest in raspberries (5.4%) (Figure 5).

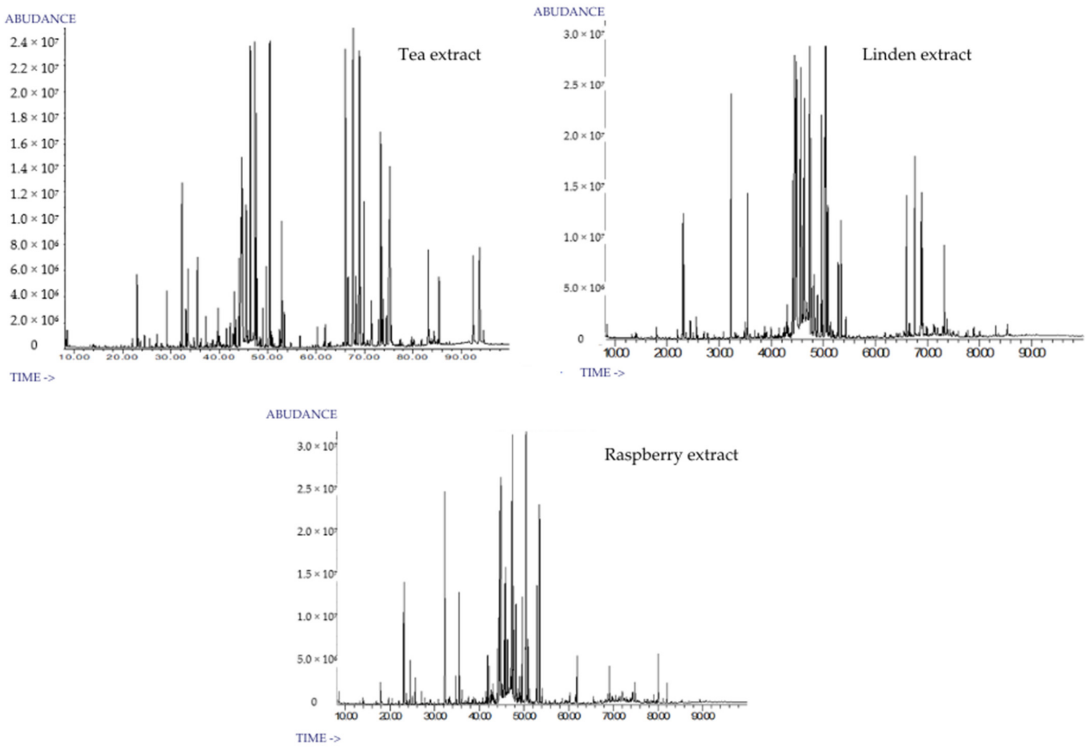

Figure 4. Comparison of GC-MS chromatographic profiles obtained for the tested extracts.

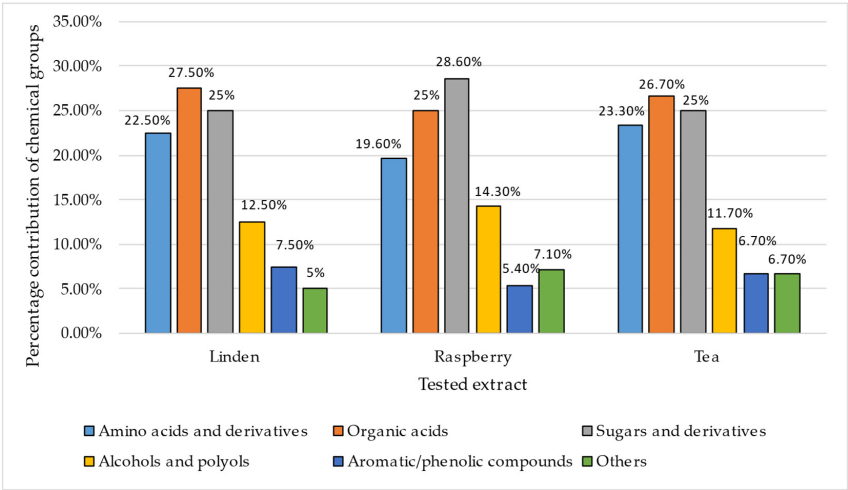

Figure 5. Distribution of main chemical groups in linden, raspberry, and tea extracts [%].

In the linden extract, the most abundant compound was shikimic acid, constituting 8.81% of TIC, followed by fructose (8.16%) and glucose (7.92% for α-D-glucopyranose and 6.20% for β-D-glucopyranose). Other major compounds included quinic acid (6.74%) and inositol (3.37%). Malic acid accounted for 4.46% TIC, while methyl glucofuranoside contributed 4.42% TIC. The remaining identified compounds each constituted less than 4% TIC. Additionally, p-Coumaric acid (0.76%) and epicatechin (1.34%) were also detected, both of which are known for their bioactivity.

The raspberry shoots extract was characterized by a high content of sugars, including glucose (a combination of β-D-glucopyranose and α-D-glucopyranose, totaling 21.74%), β-fructofuranose (10.08%), and fructose (6.06%). Malic acid constituted 6.00% of TIC, while

myo-inositol accounted for 4.45%. Additionally, quinic acid (1.73%) and gallic acid (0.56%) were present, both contributing to the antimicrobial potential of the extract.

The tea extract contained glucose (α-D-glucopyranose—5.13%, β-D-glucopyranose—4.50%) as one of the dominant sugars, along with sucrose (7.44% TIC). Among the organic acids, quinic acid (6.33%) and shikimic acid (1.52%) were present. The extract also contained notable amounts of epicatechin (3.46%) and epicatechin gallate (1.50%), both known for their antibacterial properties. Additionally, caffeine (1.60%) was detected, which has been reported to exhibit antimicrobial activity against certain bacterial strains. The compounds identified in the analyzed spectra, each contributing more than 1% of the total ion current (TIC), are summarized in Table 6.

Table 6. Summary of identified compounds (>1% TIC) in analyzed spectra.

	Compound	TIC (%)		Compound	TIC (%)		Compound	TIC (%)
Linden (SIL)	Shikimic acid, tetra-TMS	8.81	Tea (SIL)	Sucrose, octa-TMS	7.44	Raspberry (SIL)	β-D-Glucopyranose, penta-TMS	11.26
	β-Fructofuranose, penta-TMS	8.16		Carbohydrate, TMS	7.31		α-D-Glucopyranose, penta-TMS	10.48
	α-D-Glucopyranose, penta-TMS	7.92		Quinic acid, penta-TMS	6.33		β-Fructofuranose, penta-TMS	10.08
	Quinic acid, penta-TMS	6.74		α-D-Glucopyranose, penta-TMS	5.13		α-Fructofuranose, penta-TMS	6.06
	β-D-Glucopyranose, penta-TMS	6.20		Carbohydrate, TMS	4.89		Malic acid, tri-TMS	6.00
	Malic acid, tri-TMS	4.46		NN	4.87		myo-Inositol, hexa-TMS	4.45
	Methyl glucofuranoside, tetra-TMS, isomer 2	4.42		β-D-Glucopyranose, penta-TMS	4.50		β-Glucofuranose, penta-TMS	2.96
	deoxy-Inositol, penta-TMS	3.37		Methyl glucofuranoside, tetra-TMS, isomer 1	4.14		Carbohydrate, TMS	2.61
	Glucopyranose, penta-TMS	3.22		NN	3.61		β-Mannopyranose, penta-TMS	2.35
	Methyl glucofuranoside, tetra-TMS, isomer 1	3.10		Epicatechin, penta-TMS	3.46		Methyl glucofuranoside, tetra-TMS, isomer 2	2.27
	Carbohydrate, TMS	3.02		β-Fructofuranose, penta-TMS	3.04		deoxy-Inositol, penta-TMS	2.16
	Carbohydrate, TMS	2.76		β-Mannopyranose, penta TMS	2.82		Glycerol, tri-TMS	2.08
	β-Mannopyranose, penta-TMS	2.66		NN	2.00		NN	2.02
	Sucrose, octa-TMS	2.27		Carbohydrate, TMS	1.77		2,3,4-Trihydroxybutyric acid, isomer 2, tetra-TMS	1.96
	Carbohydrate, TMS	2.01		Malic acid, tri-TMS	1.75		Gluconic acid, δ-lactone, tetra-TMS	1.73
	β-Glucofuranose, penta-TMS	2.00		Glucopyranose, penta-TMS	1.73		Quinic acid, penta-TMS	1.73
	2,3,4-Trihydroxybutyric acid, isomer 2, tetra-TMS	1.81		Caffeine	1.60		Carbohydrate, TMS	1.70
	myo-Inositol, hexa-TMS	1.59		Methyl glucofuranoside, tetra-TMS, isomer 2	1.55		Phosphotic acid, tri-TMS	1.62
	Carbohydrate, TMS	1.57		Shikimic acid, tetra-TMS	1.52		Glucopyranose, penta-TMS	1.38
	Scyllo-Inositol, penta-TMS	1.53		Epicatechin gallate, hepta-TMS	1.50		Carbohydrate, TMS	1.37
	Glycerol, tri-TMS	1.44		Carbohydrate, TMS	1.40		Methyl glucofuranoside, tetra-TMS, isomer 1	1.25
	Phosphotic acid, tri-TMS	1.42		Raffinose, TMS	1.39			
	Epicatechin, penta-TMS	1.34		Carbohydrate, TMS	1.27			
	Carbohydrate, TMS	1.33		Aspartic acid, tri-TMS	1.21			
	Gluconic acid, hexa-TMS	1.30		Carbohydrate, TMS	1.16		<1% TIC	
	Gluconic acid, δ-lactone, tetra-TMS	1.02		Carbohydrate, TMS	1.06			
	Carbohydrate, TMS	1.01		-	-			

Abbreviations: SIL—Silylated; TMS—Trimethylsilyl; NN—Not Named.

4. Discussion

In our study, we focused on evaluating the antibacterial activity of plant extracts obtained from various parts of plants, including bark, flowers, leaves, and shoots, against bacteria isolated from water used in the food processing industry. Our findings demon-

strated antibacterial activity against opportunistic pathogens, which could contribute to the development of alternative methods for combating bacteria in this context. Research on the effects of various plant extracts on bacteria has been of significant interest to researchers worldwide for many years [74–79]. However, to the best of the authors' knowledge, there is a gap in the research concerning the testing of bacteria isolated from water used in the food processing industry. The second part of the study aligns with numerous scientific reports describing the combined antibacterial effects of various plant parts. However, much of the existing research focuses on extracts from different parts of a single plant, similar extracts from different plant materials, or their interactions with antibiotics [80–84]. In contrast, our study explores combinations of extracts obtained both from different plant parts and different species. This mixed approach may offer a broader spectrum of bioactive compounds and diverse mechanisms of action, potentially enhancing antibacterial effects. While the selection of plant materials was informed by ethnobotanical and antimicrobial literature, the novelty of our study lies in the application of interspecies and inter-part combinations. Furthermore, it is noteworthy that natural substances could serve as an alternative to traditional chemical agents, whose production is energy-intensive and contributes to an increased carbon footprint, negatively impacting the environment [85]. In our study, the 13 plant species were selected based on their traditional use in herbal infusions or food preservation, documented antimicrobial potential, commercial availability, and phytochemical diversity to ensure a broad representation of compounds possibly involved in microbial inhibition.

The medicinal properties of *Tilia* L. flowers have been utilized for centuries in traditional folk medicine. The most commonly used raw material is dried linden flowers, which are employed in the form of infusions and herbal teas to alleviate symptoms such as colds, sore throats, and coughs [86]. Studies conducted by Pavlović et al. (2020) demonstrated that *T. cordata* extract exhibited the highest antimicrobial activity against most of the tested pathogens. Notably, some of the strains used in that study were isolated from clinical samples, including urine cultures, vaginal swabs, and oral swabs [40]. Although the antimicrobial properties of linden flower extracts have been confirmed, the current literature lacks studies evaluating their effects on a broader spectrum of pathogens. In contrast, similar research on honey has demonstrated antibacterial activity against reference strains (ATCC®) of *P. aeruginosa*, *E. coli*, and *S. aureus* [87]. Our study did not reveal any effect of the linden flower extract on *E. faecalis*, which may be attributed to the high environmental adaptability and unique cell wall structure of enterococci [88]. On the other hand, the study by Atanasova et al. did not confirm the antimicrobial activity of the tested linden flower extract against *B. cereus*, *Citrobacter diversus*, *S. aureus*, *Pseudomonas* spp., and *Candida* spp. One notable difference compared to our study is the use of a CO_2 extract from flowers with bracts. Additionally, a different species, *T. tomentosa* Moench, was used in their tests [89]. Nevertheless, our study confirmed the antibacterial activity against the other five environmental strains tested. As noted by Oniszczuk et al., the main components of linden flowers include flavonoids (such as quercetin glycosides, kaempferol, and tiliroside), phenolic acids, essential oils, phytosterols, organic acids, tannins, mucilage, and vitamins [90]. GC-MS analysis revealed a high content of shikimic acid, which may have contributed to the inhibition of bacterial growth due to its known biological activity and antibacterial properties [91]. Similarly, malic acid (4.46% TIC) may have contributed to the interaction with pathogens. Research by Adamczak et al. demonstrated the impact of malic acid on *E. faecalis*, *E. coli*, and *P. aeruginosa*, with a minimum inhibitory concentration ranging from 500 to 1000 µg/mL [92]. The tested extract also revealed the presence of p-coumaric acid (0.76%) and epicatechin (1.34%), both of which have demonstrated established antimicrobial activity [93,94].

The shoots, leaves, and buds of raspberries are less studied compared to the commonly consumed fruits, which are rich in vitamins, minerals, organic acids, pectins, sugars, and fiber [95]. Nevertheless, traditional medicine utilizes infusions of young raspberry shoots to treat flu-like infections [96]. Studies on the composition of methanol extracts from raspberry shoots, conducted by Krauze-Baranowska et al., indicate the presence of various phenolic compounds, including ellagic acid and other polyphenols. Inhibitory effects were also observed against *Bacillus subtilis*, *Clostridium sporogenes*, *Staphylococcus epidermidis*, *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Helicobacter pylori* [61]. Our studies also confirm the presence of antibacterial activity in raspberry extracts, with the strongest effect observed against *P. aeruginosa* and the weakest against *S. marcescens*. The previously mentioned ellagic and gallic acids were also identified in the tested raspberry shoot extract, at 0.38% and 0.56% TIC, respectively. These polyphenols exhibit antibacterial properties, as demonstrated in studies conducted on animal models [97]. The presence of quinic acid (1.73% TIC in our study) may also promote the antibacterial effect, particularly against *Klebsiella* spp. and *P. aeruginosa* [98].

The use of tea waste, specifically tea leaves, to produce extracts with confirmed antimicrobial properties aligns with sustainable resource management and zero-waste policies. The health benefits of green tea are well-documented and have been utilized for centuries in traditional medicinal practices [99]. Our studies demonstrated the antimicrobial activity of green tea extract on all tested pathogens, with the strongest activity against *P. aeruginosa* and *E. faecalis.* Research by Sharma et al. also highlighted the efficacy of tea extracts on a broad spectrum of bacteria, including skin pathogens (or pathobionts) such as *S. epidermidis*, *Micrococcus luteus*, *Brevibacterium linens*, *P. fluorescens*, and *B. subtilis* [100]. Studies have also confirmed that tea extracts exhibit antibacterial activity against resistant and multidrug-resistant strains, highlighting their potential for future use [101]. One of the key bioactive compounds in green tea extracts is epigallocatechin gallate (EGCG), a predominant catechin known for its strong antibacterial properties, accounting for 1.15% of the total ion current (TIC) in our study. Previous research has also demonstrated that the bioactive compounds in green tea extracts exhibit antimicrobial activity against *E. coli* and bacteria from the *Salmonella* genus [102].

Tree bark is a rich source of substances used in the treatment of diseases and the direct combat of pathogens [103]. Studies on tree bark extracts traditionally used by First Nations in North America have demonstrated that 86% of the extracts exhibited activity against methicillin-sensitive *S. aureus*, 71% against *Bacillus subtilis*, and 79% against *Mycobacterium phlei* [104]. Our study demonstrated the effectiveness of extracts from birch, oak, and willow bark against six distinct opportunistic pathogens. This finding is consistent with existing literature, which reports that bark extracts are effective against a variety of bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* [105,106]. The antimicrobial properties of oak bark extracts are primarily attributed to the presence of phenolic compounds, such as gallic and ellagic acids, which exhibit both bacteriostatic and bactericidal activity [43,107]. Our studies did not confirm antibacterial activity for *Quercus suber* bark, contrary to the findings of Gonçalves et al., who demonstrated antibacterial activity against *S. aureus* and *E. coli* [108]. In our studies, we utilized expanded cork, a material produced by exposing cork to high temperatures (typically around 180–200 °C) during its manufacturing process. This thermal treatment could have significantly altered the chemical composition of the cork bark, potentially leading to the degradation or volatilization of thermolabile bioactive compounds responsible for the antimicrobial properties. As for the antibacterial effect of *B. pendula* extract, our findings are in line with the existing literature. Chemical compounds in birch bark, such as betulin and other triterpenes, have demonstrated antibacterial properties and may inhibit the growth of various bacterial strains [109]. Polyphenols

found across the *Betula* L. genus include catechin, salidroside, rhododendrin, and platyphylloside, which are known for their antioxidant, anti-inflammatory, and antimicrobial properties [110].

The interaction extends beyond bacteria; extracts from birch bark have also shown antiviral and antifungal activity against various pathogenic strains [51]. The presence of salicin is a significant factor contributing to the widespread investigation and utilization of *Salix* species extracts in therapeutic applications. Alicin, along with other phenolic compounds such as procyanidins, can inhibit bacterial growth through various mechanisms, including antioxidant activity [111]. However, other bioactive compounds present in willow bark may also play a significant role in its antibacterial activity. Research by Piątczak et al. has identified quercetin glycosides, monomeric, dimeric, and trimeric flavan-3-ol derivatives, including B-type procyanidins, as well as cavoylquinone pseudodepsides, all of which may contribute to the observed antimicrobial effects [63].

Our study also revealed that the combination of plant extracts exhibiting antimicrobial activity in the initial screening phase can lead to enhanced inhibition of pathogen growth. All extract mixtures were prepared in a 1:1 (w/w) ratio, and the same total volume (100 µL) was applied in each assay. This means that the individual extracts were present at half the concentration used in the single-extract tests. Therefore, any observed increase in antimicrobial activity in the combinations, despite the lower concentration of each extract, may indicate an increased effect. Notably, five of the six extract mixtures that demonstrated increased antibacterial effects contained at least one bark extract. A similar observation was made in the study by Jeong et al., where the combination of extracts from *Solanum torvum* and *Uncaria gambir* significantly augmented their antibacterial activity against methicillin-resistant *S. aureus* strains [112]. Similarly, Ncube et al., in their research, highlight that utilizing the synergistic effects of plant extracts may have potential therapeutic applications [113]. Individual extracts within the mixture may target distinct cellular mechanisms of the pathogen. For instance, one extract could disrupt the integrity of the bacterial cell membrane, thus facilitating the penetration of a second extract, which could act on other key metabolic processes, such as protein synthesis or DNA replication. Alternatively, one of the extracts may inhibit the pathogen's resistance mechanisms, such as efflux pumps, thus enhancing the effectiveness of the second component within the mixture [114,115].

Several factors may explain the lack of confirmed antimicrobial activity for the seven extracts despite existing literature reports. The primary factors include the plant species, the type of material used, and the storage conditions of the raw material [116]. The method of extract preparation is also crucial. As noted by Sánchez et al., methanolic extracts consistently exhibited higher antimicrobial activity compared to ethanolic or aqueous extracts. They attributed this difference to the solvent's ability to dissolve a greater number of bioactive compounds [117]. The selection of strains is equally important, as their origin can significantly influence the variation in response to the applied extracts. The limitations of our study include the use of a single extraction method (ethanol:water), which may not have captured the full spectrum of bioactive compounds. Additionally, the thermal processing of extracts at 65 °C could have led to the degradation of heat-sensitive compounds, potentially reducing their antibacterial activity. Furthermore, the study focused solely on antibacterial activity without testing other bioactivities, and solvent control was not implemented in the study. However, these limitations should be considered in the context of this being a pilot study, which aimed to provide preliminary insights into the antimicrobial potential of plant extracts. The agar-well diffusion method was selected for this study as it provides a practical and reproducible approach for the preliminary screening of antimicrobial activity of plant extracts. This technique allows for the direct

application of a defined volume of extract into wells, facilitating better diffusion into the medium. The study was limited to the well-diffusion method; however, complementary techniques such as MIC, MBC, or other quantitative assays could enhance the evaluation of antimicrobial efficacy in future research. Additionally, the use of reference strains would provide a clearer understanding of the differences in the effects of the extracts on environmental strains.

Our study confirmed the research hypothesis, which postulated that the application of extracts from various plant materials is effective against pathogens isolated from rinse water in the agri-food industry. Furthermore, it was established that combining bark extracts with those rich in volatile organic compounds (derived from leaves, shoots, flowers, and fruits) results in an increased effect, enhancing the antibacterial activity against the examined bacterial strains.

The presence of various secondary metabolites in the plant species analyzed in this study has been reported in the literature. Although these compounds have been previously identified, their antimicrobial potential against opportunistic pathogens isolated from water has not been extensively investigated.

5. Conclusions

The study demonstrated that plant extracts exhibit differential antimicrobial activity. *C. sinensis* and *R. idaeus* showed the highest efficacy, particularly against *P. aeruginosa* and *E. faecalis*, while *Q. robur*, *B. pendula*, *T. cordata*, and *S. alba* exhibited lower effects. No antimicrobial activity was observed for *Coffea* L., *R. canina*, *T. officinale*, *Q. suber*, *V. officinalis*, and *P. sylvestris*. GC-MS analysis identified various bioactive compounds in the most effective extracts, including epicatechin, shikimic acid, quinic acid, gallic acid, and caffeine, which are known for their antimicrobial properties. The presence of these compounds may explain the observed inhibitory effects and supports further investigation into their mechanisms of action. From an economic perspective, the tested plant materials are locally available and low-cost, which makes them promising candidates for sustainable and accessible antimicrobial applications in water treatment systems. In conclusion, this study highlights the potential of plant-derived extracts as natural antimicrobial agents against strains isolated from wash water in the agri-food industry. In a broader context, this study investigated the potential of natural plant extracts for application in non-invasive water treatment approaches relevant to the agri-food sector. The research was exploratory in nature and represents a preliminary phase toward the development of a practical and scalable solution. The broader project constitutes a research continuum that will ultimately lead to the design and evaluation of a pilot-scale filtration bed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app15095199/s1.

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Article

Pilot-Scale Evaluation of a Filter Prototype for Bacterial Inactivation in Agro-Food Processing Wastewater

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Abstract

The processing of freshly cut fruits and vegetables represents an important niche for implementing circular economy principles, particularly through the reuse of washing water. This is especially relevant as post-wash water is often treated as wastewater and discarded without reuse. One promising research avenue is the use of plant-derived extracts in water sanitation processes. Their antimicrobial properties offer a natural alternative to conventional disinfectants while reducing the formation of harmful disinfection by-products. The aim of this study was to evaluate the effectiveness of different filter bed configurations in removing pathogens from water. These configurations included a hydrogel saturated with natural plant extracts, an ion exchange resin layer, and an activated carbon layer. The most effective composite was also tested using real process water from a fruit washing line. The test materials included concentrated extracts from oak bark (*Quercus robur*), willow (*Salix alba*), birch (*Betula pendula*), raspberry shoots (*Rubus idaeus*), tea leaves (*Camellia sinensis*), and linden flowers (*Tilia cordata*), all immobilized in hydrogel, along with activated carbon and ion-exchange resin. Water samples were artificially inoculated with six opportunistic pathogens and collected process water was also used. Samples were analyzed microbiologically at six time intervals. The composite filter (hydrogel–resin–carbon) achieved a reduction of over 2 $\log_{10}$ in heavily inoculated water (~10^8 CFU mL^{-1}) and maintained at least a 1 $\log_{10}$ reduction in real process effluents. The proposed solution supports blue water footprint reduction strategies (as the system aims to decrease the demand for freshwater resources through the reuse of treated wastewater) and aligns with the principles of green processing.

Keywords: plant extracts; agri-food waste water; closed-loop economy; water recycling

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1. Introduction

The integration of circular economy principles into zero-waste policies contributes directly to mitigating the adverse effects of anthropogenic climate change [1]. Particularly important in this context is the need to intensify efforts aimed at water conservation and recovery, as both water scarcity, and the disruption of hydrological systems are becoming increasingly apparent worldwide—not only in the Global South, but also across the Northern Hemisphere, affecting both rural areas and expanding urban centers [2,3]. The expanding awareness of environmental issues among the public not only shapes consumer

behavior but can also facilitate the adoption of legal frameworks aimed at lowering climate stress over time [4,5].

Agriculture is one of the most water-intensive sectors of the economy and plays a crucial role in the design, planning, and implementation of measures aimed at water conservation [6,7]. According to data from the European Environment Agency, the average annual water use for agricultural purposes across the EEA-39 countries between 2008 and 2017 was 92 billion m^3. In 2017 alone, the EU-28 member states used an average of 50 billion m^3 of water per year, while Turkey reported usage of approximately 40 billion m^3 [8]. In the European Union, the adoption of diverse water-saving solutions has been facilitated through the Common Agricultural Policy, including measures such as irrigation automation, precision farming, the reuse of treated water, the promotion of less invasive cultivation methods, and the use of crops with increased resilience to water stress [9].

The processing of freshly harvested fruits and vegetables is recognized as a relevant niche for implementing circular economy solutions, particularly through the reuse of water employed in raw material washing [10,11]. This issue is particularly important given that wash water is often discharged as wastewater and not reused. However, this approach is primarily driven by concerns over preventing microbiological cross-contamination, which may lead to outbreaks that pose risks to public health and compromise the quality of the final product. An alternative strategy involves the use of conventional chemical disinfection, typically based on chlorine compounds such as sodium and calcium hypochlorite [12]. However, the use of this conventional approach may lead to the formation of harmful disinfectant byproducts (DBPs), some of which exhibit carcinogenic, cytotoxic, teratogenic, or neurotoxic properties (Table 1). In the early cleaning stages at agri-food-processing facilities, fruits and vegetables often carry soil particles, dust, and remnants of dead organic matter. These materials can react with disinfectants, which raises concerns about the potential formation of toxic or otherwise undesirable compounds [13].

Table 1. Characteristics of the main disinfection by-products.

DBP Type	Examples of Compounds	Sources of Formation	Toxicity	References
Carbon-based DBPs	Chloroform, Dichloroacetic acid, Trichloroacetic acid	Reaction of disinfectants with natural organic matter and anthropogenic contaminants	Cytotoxicity, genotoxicity (compound-specific), some carcinogenic effects	[14–16]
Nitrogen-based DBPs	Halonitromethanes, Haloacetamides, Haloacetonitriles	Reactions with nitrogen-containing compounds (e.g., proteins, amino acids)	Strong genotoxicity, mutagenicity, neurotoxicity	[15,17]
Inorganic DBPs	Bromate, Chlorite, Chlorate	Byproducts of ozone and chlorine dioxide disinfection	Variable; bromate with confirmed genotoxicity and carcinogenicity	[15,18]
Iodinated DBPs	Iodoacetic acid, Iodoform, Iodo-trihalomethanes	Presence of iodide in water during disinfection	Very high toxicity; strong cytotoxicity and genotoxicity	[15,19]
Brominated DBPs	Bromodichloromethane, Bromoform, Dibromoacetic acid	Presence of Bromide in water	Higher toxicity than chlorinated analogs; including genotoxic and carcinogenic	[15,20]
Chlorinated DBPs	Chloroform, Trichloroacetic acid, Chloroacetic acid	Typical products of chlorine-based disinfection	Lower toxicity than Br- and I-DBPs; variable toxicological profiles	[13,15]

Due to the wide availability and convenience of chemical disinfection, alternative methods—such as mechanical filtration using carbon, sand, or silica filters, UV irradiation, or cold plasma—tend to attract less interest among producers [11,21]. The technical problem addressed in this study is the lack of alternative, non-invasive methods for the effective disinfection of wastewater generated in the fresh-cut industry.

In recent years, there has been growing interest in the use of less invasive methods involving natural plant extracts, which may offer a more appealing alternative [22]. Research on the use of plant-based raw materials as natural preservatives, and antimicrobials has gained importance in response to the growing demand for safe and environmentally friendly alternatives to synthetic additives [23,24]. Plant extracts, due to their antibacterial and antifungal properties, are used not only in the food industry, but also in pharmaceuticals and cosmetology [25–29]. The active compounds found in plant extracts—such as flavonoids, alkaloids, and tannins—exhibit diverse antimicrobial mechanisms. Flavonoids, for example, can inhibit DNA gyrase activity, destabilize bacterial cell membranes, and interfere with cellular energy metabolism [30]. Alkaloids, on the other hand, have been shown to inhibit bacterial cell wall formation, alter membrane permeability, suppress the synthesis of proteins and nucleic acids, and disrupt various metabolic pathways [31]. Tannins exert antibacterial activity through several mechanisms, including iron chelation, disruption of the bacterial cell membrane, inhibition of cell wall synthesis and fatty acid biosynthesis. The compounds also demonstrate antivirulence effects, such as the suppression of quorum sensing and biofilm formation [32]. In light of the above, the use of plant extracts in water sanitization processes represents a promising area of research, as their antimicrobial properties may serve as a natural alternative to conventional disinfectants while simultaneously reducing the formation of harmful disinfection byproducts [33].

The research hypothesis assumes that the use of a multilayer filtration bed composed of hydrogel infused with natural plant extracts, ion-exchange resin, and activated carbon significantly enhances the efficiency of pathogen removal from wastewater compared to single-layer filtration systems.

The aim of this study was to experimentally evaluate the efficiency of a pilot-scale filtration bed designed for the treatment of wastewater under semi-technical conditions. The research focused on assessing the effectiveness of pathogen removal using different bed configurations, which included hydrogel infused with natural plant extracts, an ion-exchange resin layer, and an activated carbon layer. The objective was to determine which configuration offers the highest microbiological purification efficiency while maintaining operational stability and demonstrating potential for application in environmental engineering practice. The second phase of the study involved preliminary validation on an industrial scale. The most effective configuration identified during the pilot tests was applied to the treatment of real process water collected from a fruit processing facility.

2. Materials and Methods

2.1. Preparation of Filter Material

Based on previous research, the selected plant material included bark of *Quercus robur*, *Salix alba*, and *Betula pendula*, stems of *Rubus idaeus*, leaves of *Camellia sinensis*, and flowers of *Tilia cordata* [34].

Hydroethanolic extracts (70%) were prepared from the selected plant material. For this purpose, 25 g of cleaned and dried plant material (pre-dried for 12 h at 30 °C) were ground into a fine powder and then soaked in 250 mL of solvent. The mixtures were shaken for 24 h at 150 rpm, then filtered using standard laboratory-grade filter paper. The filtered extracts (150 mL) were concentrated threefold to 50 mL using a high-efficiency rotary evaporator

(Laborota 4000, Heidolph, Schwabach, Germany) at 65 °C and 90 rpm. To ensure sterility, the extracts were further filtered through 25 mm syringe filters with a pore size of 0.22 µm.

Subsequently, the extracts were applied for hydrogel hydration to prepare the material for further analysis (Figure 1).

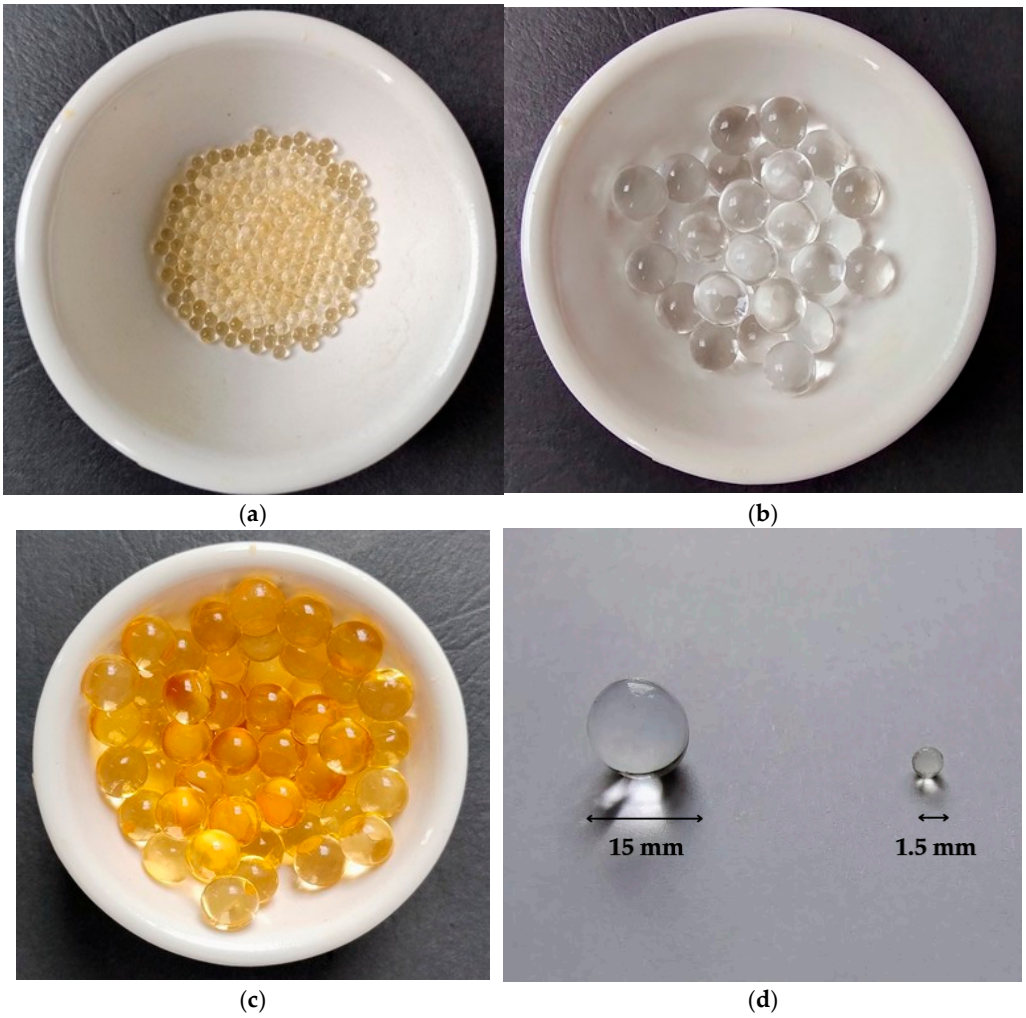

Figure 1. Graphical representation of the hydrogel in various forms used during the study: (**a**) dry, non-hydrated hydrogel; (**b**) hydrated hydrogel; (**c**) hydrogel hydrated with plant extracts; (**d**) comparison of hydrogel volume before and after hydration.

The remaining filtration materials included ion-exchange resin and activated carbon. According to the manufacturer Formaster S.A., Kielce, Poland), the macroporous resin removes calcium and magnesium ions from water through ion exchange, while the activated carbon, derived from coconut shells, effectively adsorbs chlorine and other contaminants due to its high porosity, thereby improving the taste and odor of the water. According to the manufacturer, the service life of activated carbon cartridges is estimated at 4–6 months, while ion-exchange resin cartridges lose efficiency once their exchange capacity is exhausted. For medium-hard water this corresponds to approximately 700–1000 L, with higher capacities for lower hardness. In this study, these values were considered as reference information, while the experimental evaluation focused on microbiological performance.

2.2. Filtration System

The filtration system (Figure 2) consisted of an initial tank containing 25 L of inoculated water with bacteria at a specified concentration, a water pump ensuring constant flow, control valves regulating both the direction and intensity of flow, manometers monitoring pressure

at various stages, and a main flow-through filter filled with a suitable filtration medium—activated carbon with ion-exchange resin, hydrogel, or a mixture of these, depending on the setup. Filtered water was collected in a receiving tank for further analysis.

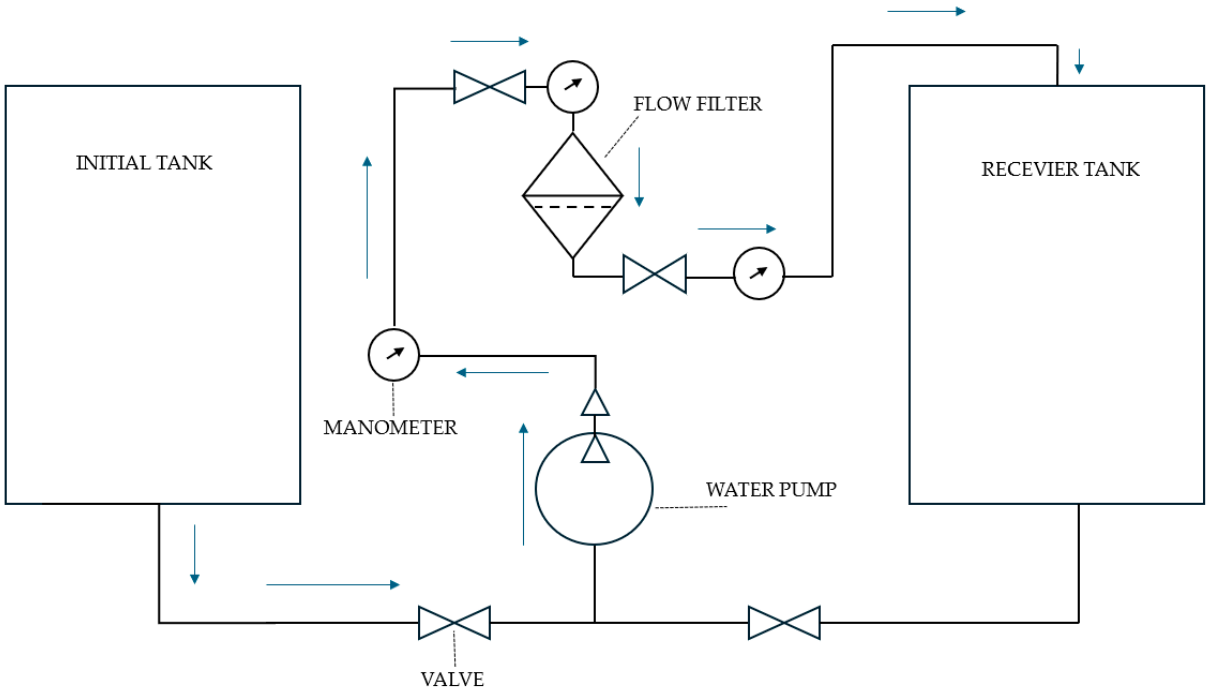

Figure 2. Scheme of the filtration system used in the study.

2.3. Bacterial Inoculum

As identified in prior studies, the test strains comprised opportunistic pathogens isolated from wash waters in agri-food processing facilities. These included *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens*, and *Enterococcus faecalis* [34,35]. To prepare the bacterial inoculum, strains stored in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) with glycerol at −20 °C were revived on general-purpose medium (Tryptic Soy Agar, TSA; Merck, Germany) and subcultured twice. Bacteria were subsequently collected with a sterile inoculation loop and suspended in 9 mL of sterile 0.9% sodium chloride solution. The suspension was adjusted to a concentration of 1.0–2.0 × 10^8 CFU/mL, consistent with the cell density typically applied in antimicrobial susceptibility assays. A 1 mL aliquot from each bacterial suspension was inoculated into 250 mL of sterile Nutrient Broth (Merck, Germany) and mixed thoroughly to achieve uniform dispersion of the cells. The same procedure was carried out for each strain tested.

To prepare the inoculum for further testing, a total of 1.5 L of bacterial culture (6 × 250 mL), each at a concentration of 2 × 10^8 CFU/mL, was added to 25 L of sterile water. This resulted in a final bacterial concentration of approximately 1.2 × 10^7 CFU/mL. The actual concentration was confirmed by determining colony-forming units (CFU/mL) in samples collected immediately after inoculation.

2.4. Testing Procedure

Twenty-five liters of sterile water were inoculated with 1.5 L of bacterial suspensions containing the test strains (6 × 250 mL) and transferred into the preliminary tank. After opening the control valves and activating the pump, the water was passed through filtration columns in one of the following three configurations: (1) a filter filled with activated carbon and ion-exchange resin; (2) a filter containing hydrogel; and (3) a filter composed of a mixture of activated carbon, ion-exchange resin, and hydrogel (Figure 3).

Water samples were collected and analyzed at six intervals: 1 h, 2 h, 3 h, 6 h, and 12 h. Approximately 4 L of water were circulated through the system during each test. Following filtration, microbiological analyses were performed in duplicate: 1 mL of filtered water was plated on appropriate agar medium and spread using a sterile spreader. The results were compared across the different filter configurations.

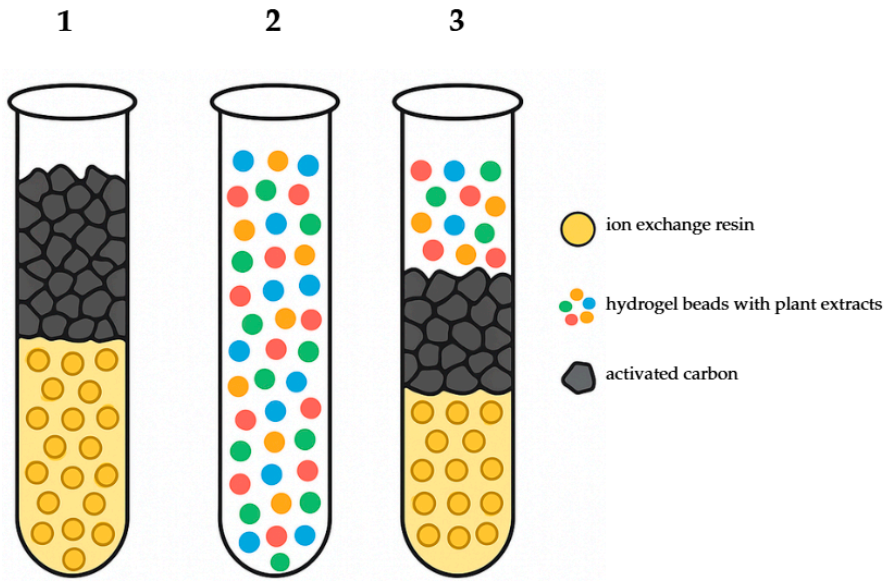

Figure 3. Schematic overview of the filter media used in the tested configurations: (**1**) filter filled with activated carbon and ion-exchange resin; (**2**) filter containing hydrogel; and (**3**) filter composed of a mixture of activated carbon, ion-exchange resin, and hydrogel.

2.5. Log Reduction and Statistical Analysis

Filter performance was evaluated by monitoring the decrease in bacterial counts in the outflow over time. Results were described as a $\log_{10}$ reduction in colony-forming units (CFU), calculated using the following equation:

$$log_{reduction} = log_{10}\left(C_{start}\right) - log_{10}\left(C_{after\ filtartion}\right)$$

C_{start}—initial bacterial concentration in water before filtration (CFU/mL).

$C_{after\ filtartion}$—bacterial concentration in the sample collected from the filter outflow at a given time point (CFU/mL)

The concentration of CFU/mL was determined using colony counts from serial dilution plates, in accordance with standard microbiological procedures. When the number of colonies exceeded 300 (designated as "TNTC"—too numerous to count), the result was estimated using data from the next highest dilution that produced countable growth.

The obtained microbiological data were subjected to statistical analysis using two-way analysis of variance (ANOVA). The first factor (A) corresponded to the type of filter medium: H—hydrogel; C-IER—activated carbon with ion-exchange resin; H-C-IER—a combination of hydrogel, activated carbon, and ion-exchange resin. The second factor represented the sampling time interval (1, 2, 3, 6, 12, and 24 h). To assess the significance of the individual effects and their interaction, Tukey's post hoc test with a 95% confidence interval was performed, allowing for comparison of the mean values of the analyzed parameters.

2.6. Validation of the System on Water from the Agri-Food Industry

The facility studied operates under an integrated food safety management system, with certification covering all stages of the production chain—from field cultivation to

delivery of fruit to the end consumer. Annually, approximately 10,000 metric tons of fruit are produced, mainly apples and pears. Process water samples were collected from the main drainage channel of a fruit processing plant located in the Kuyavian-Pomeranian Voivodeship (northern Poland).

3. Results

3.1. Results for Inoculated Water

Results demonstrated that the composite filter (H-C-IER) achieved a reduction by approximately one order of magnitude in microbial load within the first hour, outperforming both the single- and double-layer filters, and continued to do so over the entire observation period. The resin–carbon configuration (C-IER) demonstrated moderate yet steady performance, while the hydrogel-only filter (H) had the highest CFU/mL levels at all time points (Figure 4).

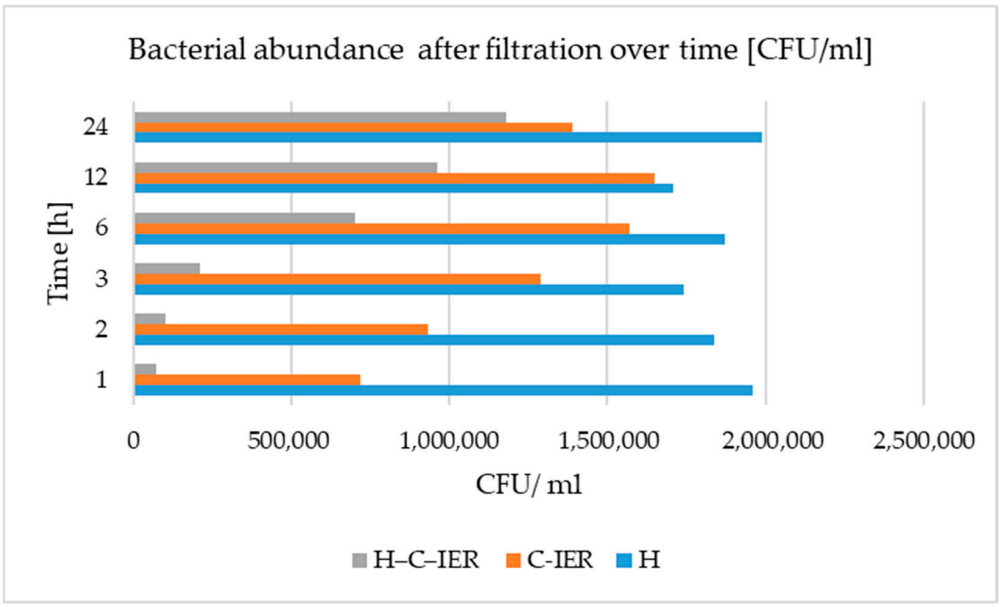

Figure 4. Summary of bacterial counts (CFU/mL) after filtration at specific intervals. Abbreviations: H—hydrogel; C-IER—activated carbon with ion-exchange resin; H-C-IER—a combination of hydrogel, activated carbon, and ion-exchange resin.

For the H configuration, bacterial reduction after 1 h did not exceed 0.8 $\log_{10}$. During the 24 h operation, an increase in bacterial counts in the outflow was observed—from approximately 2×10^6 to nearly 2×10^7 CFU/mL. At the two last time points (12–24 h), colony counts were higher than in samples collected during the first hour of filtration (Table 2).

The filter setup based on C-IER layer exhibited moderate effectiveness in reducing bacterial concentrations in water initially containing around 1.2×10^7 CFU/mL. The highest reduction was observed during the first filtration time point, with an average colony count of 37 at a 10^{-4} dilution—equivalent to 3.7×10^5 CFU/mL—and a log reduction of 1.51.

Over time, bacterial levels in the outflow gradually increased. After 3 h, the CFU/mL value rose to 9.3×10^5, yielding a log reduction of 1.11. By the sixth hour, bacterial reduction dropped to 0.97 log, and after 24 h, the system's effectiveness stabilized below 1 log, with a final concentration of 1.39×10^6 CFU/mL (Table 3).

The composite filter H-C-IER demonstrated the highest efficiency among all tested configurations. At the first time point, a log reduction of 2.23 was recorded, corresponding to a decrease in bacterial count to 7.0×10^4 CFU/mL (an average of 7 colonies at a 10^{-4} dilution).

Table 2. Summary of bacterial count reduction over time for the hydrogel-based filter with natural extracts.

Time Point [h]	Mean Colony Count (10^{-4})	CFU/mL	$\log_{10}$ CFU/mL	Log-Reduction
1	196	1.96×10^6	6.29	0.79
2	184	1.84×10^6	6.27	0.81
3	174	1.74×10^6	6.24	0.84
6	187	1.87×10^6	6.27	0.81
12	171	1.71×10^6	6.23	0.85
24	199	1.99×10^6	6.30	0.78

Table 3. Summary of bacterial count reduction over time for the ion-exchange resin and activated carbon filter.

Time Point [h]	Mean Colony Count (10^{-4})	CFU/mL	$\log_{10}$ CFU/mL	Log-Reduction
1	37	3.70×10^5	5.57	1.51
2	72	7.20×10^5	5.86	1.22
3	93	9.30×10^5	5.97	1.11
6	129	1.29×10^6	6.11	0.97
12	157	1.57×10^6	6.20	0.88
24	139	1.39×10^6	6.14	0.94

In subsequent sampling points, retention remained high—during the first three time points, the log reduction exceeded 1.7 $\log_{10}$. Despite a slight increase in CFU/mL in later samples (reaching a maximum of 1.18×10^6 CFU/mL after 24 h), the filter's effectiveness remained stable. At the final time point, the log reduction was 1.01, indicating removal of over 90% of microorganisms from the flowing water (Table 4).

Table 4. Summary of bacterial count reduction over time for the hydrogel, ion-exchange resin and activated carbon filter.

Time Point [h]	Mean Colony Count (10^{-4})	CFU/mL	$\log_{10}$ CFU/mL	Log-Reduction
1	7	7.00×10^4	4.85	2.23
2	10	1.00×10^5	5.00	2.08
3	21	2.10×10^5	5.32	1.76
6	70	7.00×10^5	5.85	1.23
12	96	9.60×10^5	5.98	1.10
24	118	1.18×10^6	6.07	1.01

Changes in filter performance over time were evaluated using linear regression based on the log reduction in bacterial counts ($\log_{10}$(CFU/mL)). The hydrogel filter provided a low but consistent level of bacterial removal. Its regression slope (−0.0009) indicated that operating time had little effect on performance. However, the inactivation efficiency remained limited (around 0.8 log). The C-IER filter demonstrated higher initial performance. Over time, its effectiveness decreased slightly, with a slope of −0.0177. The composite filter delivered the strongest reduction at the start of the test (1.51 log). Its efficiency declined more noticeably, as reflected by a steeper slope (−0.0487). This drop was likely caused by gradual saturation of the filter media (Figure 5).

In the comparative analysis of filtration efficiency between different bed configurations (C-IER and H-C-IER), it was demonstrated that the use of a filter containing a hydrogel layer enriched with plant extracts (H-C-IER) significantly reduced bacterial abundance compared to the bed lacking this layer (C-IER). The average CFU value for the H-C-IER configuration was 0.52×10^6 CFU/mL, while for C-IER it was 1.05×10^6 CFU/mL. These

differences were statistically significant ($LSD_{0.05}$ = 0.336 × 10^6 CFU/mL). The mean values across individual time points (levels of Factor B) also varied significantly. The lowest bacterial counts were recorded at the initial time points (B1–B3), while the highest values were observed at later stages (B5–B6), indicating a decline in filtration efficiency with prolonged exposure to biologically loaded water.

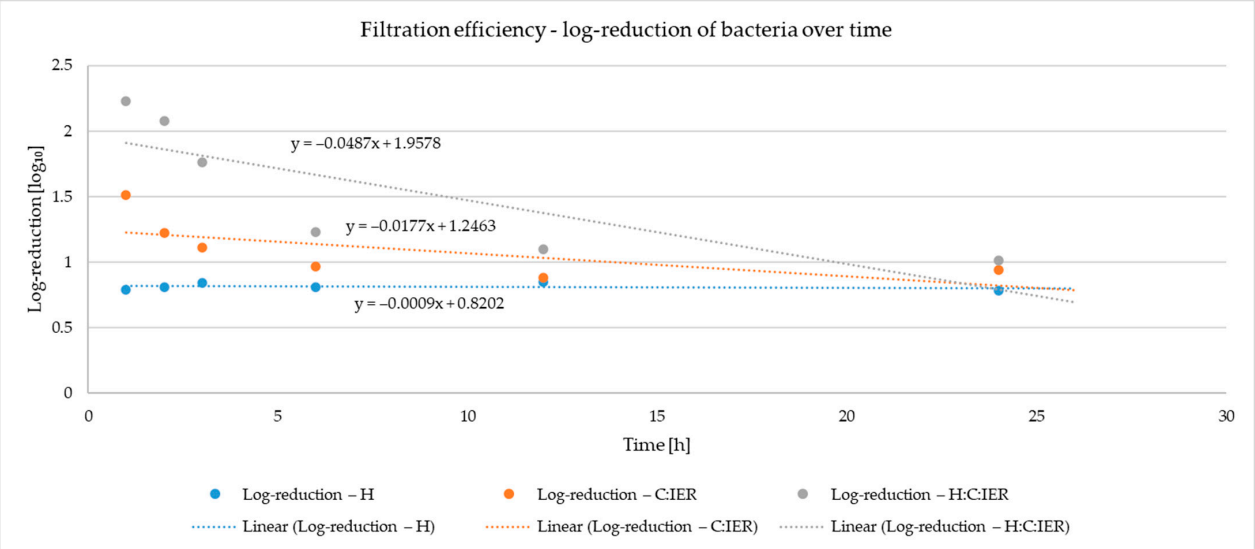

Figure 5. Analysis of the linear relationship between time and bacterial log reduction (log_{10}(CFU/mL)).

A comparison of H vs. H-C-IER configurations showed that the application of the composite filter significantly enhanced antibacterial performance at every time point. The average bacterial count for the hydrogel-only filter was 1.85 × 10^6 CFU/mL, compared to 0.52 × 10^6 CFU/mL for the variant with plant extracts ($LSD_{0.05}$ = 0.340 × 10^6 CFU/mL) (Table 5).

Table 5. Comparative bacterial abundance over time between experimental filter configurations.

		Level of Factor A		
		A1 (C-IER)	**A2 (H-C-IER)**	**Mean**
		Bacterial abundance (×10^6 CFU mL^{-1})		
Level of Factor B	**B1**	0.37	0.07	0.22 c
	B2	0.72	0.09	0.41 c
	B3	0.93	0.12	0.53 c
	B4	1.29	0.70	1.00 b
	B5	1.57	0.96	1.27 b
	B6	1.39	1.18	1.29 a
	Mean	1.05 a	0.52 b	
C-IER vs. H-C-IER: $LSD_{0.05}$ = 0.336 × 10^6 CFU mL^{-1}, $LSD_{0.05}$ (Tukey); A = 0.158 × 10^6, B = 0.421; B/A = n.s.; A/B = n.s.				
		A1 (H)	**A2 (H-C-IER)**	**Mean**
		Bacterial abundance (×10^6 CFU mL^{-1})		
Level of Factor B	**B1**	1.96 $^{c, A}$	0.07 $^{a, B}$	1.02 b
	B2	1.84 $^{c, A}$	0.09 $^{a, B}$	0.97 b
	B3	1.74 $^{c, A}$	0.12 $^{a, B}$	0.93 b
	B4	1.87 $^{c, A}$	0.70 $^{a, A}$	1.28 b
	B5	1.71 $^{b, A}$	0.96 $^{a, B}$	9.03 a
	B6	1.99 $^{b, A}$	1.18 $^{a, B}$	1.59 a
	Mean	1.85 a	0.52 b	
H vs. H-C-IER: $LSD_{0.05}$ = 0.340 × 10^6 CFU mL^{-1}; A = 0.127; B = 0.340; B/A = 0.480; A/B = 0.311.				

Notes: Different lowercase letters (a, b, c) within columns indicate statistically significant differences between means according to Tukey's test (α = 0.05). Means followed by the same lowercase letter do not differ significantly. Capital letters within rows denote homogenous groups; values sharing the same letter are not significantly different.

3.2. Results for Agro-Industrial Process Water

Microbiological analysis of the process water samples exhibited a lower overall bacterial concentration compared to the laboratory-inoculated model. Simple staining with crystal violet applied to selected colonies indicated the presence of bacilli, cocci, and clustered cells characteristic of staphylococci, suggesting a mixed microbial community. This composition is typical for wastewater derived from vegetable processing lines (Figure 6).

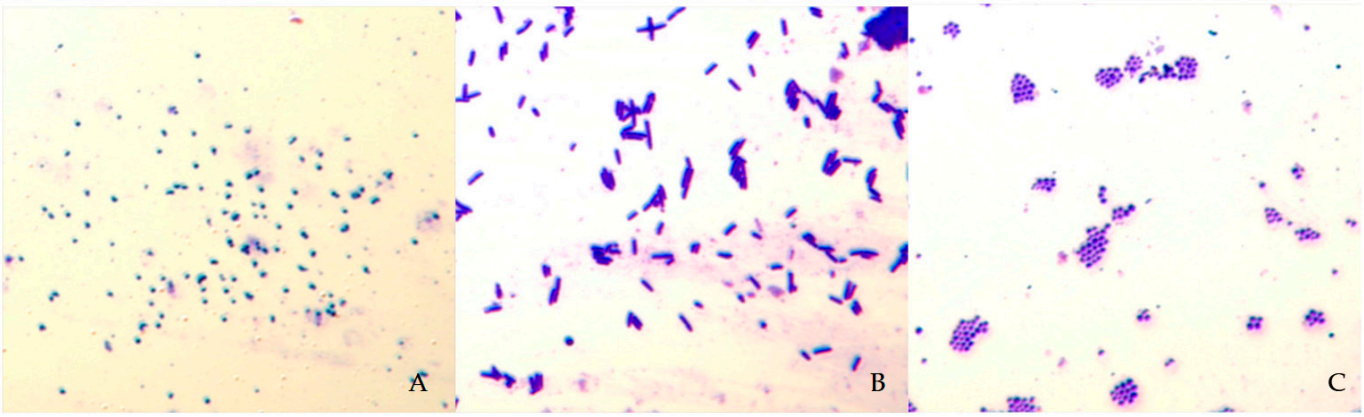

Figure 6. Microscopic images showing diverse morphological forms of bacteria isolated from selected colonies cultured from process water ((**A**)—cocci, (**B**)—bacilli, and (**C**)—staphylococci; simple crystal violet staining, microscope: Olympus BX53, Tokyo, Japan).

The data indicate that the composite H-C-IER filter reduced bacterial concentrations in process water from 1.07×10^4 CFU mL^{-1} (control) to 1.1×10^2–1.0×10^3 CFU mL^{-1} within the first 6 timepoints of operation, corresponding to a reduction of 1.2–2.0 $\log_{10}$ (Table 6). The highest efficiency (≈2 $\log_{10}$) was observed after the first hour, followed by a gradual decline in performance, stabilizing at approximately 0.8–1.2 $\log_{10}$ from the third test interval onward. This trend suggests that while the filter retains over 90% of its retention capacity its efficiency declines over time.

Table 6. Log-reduction in bacterial counts in process water after filtration through the composite bed at selected time intervals.

Time Point	CFU mL^{-1}	$\log_{10}$ CFU mL^{-1}	Log-Reduction *
1 h	110	2.04	1.99
2 h	200	2.30	1.73
3 h	630	2.80	1.23
6 h	640	2.81	1.22

Notes: * The control sample showed 107 colonies at the 10^{-2} dilution, corresponding to 1.07×10^4 CFU mL^{-1}, or 4.03 $\log_{10}$ CFU mL^{-1}.

During the first two sampling intervals, the composite removed about 11% (1 h) and 17% (2 h) more bacteria from the artificially contaminated matrix than from the actual rinse water. After three hours the difference rose to roughly 30%, suggesting that suspended solids and organic matter in the plant effluent had begun to block active bed surfaces, thereby diminishing performance. By the sixth interval, the log-reductions were virtually the same (≈1% difference), indicating that with longer contact times the composite provides a comparable bacterial barrier regardless of water-matrix complexity, although its advantage over the process water declines as the contaminant load increases.

4. Discussion

Increasing efforts to implement a circular economy and to minimize losses—particularly in preserving and conserving valuable water resources—have become a prominent research focus [36,37]. The main driver of this trend is increasing anthropogenic pressure, along with progressing climate change, which adversely affects water resources through prolonged droughts and short periods of intense rainfall. These phenomena disrupt the hydrological balance in natural reservoirs and watercourses, as well as in groundwater systems [38,39]. In this context, the fresh-cut fruit and vegetable industry is receiving growing attention due to its high water consumption, low water recycling rates, and the associated potential microbiological risks [40].

According to the FDA Produce Safety Rule, water used during and after harvest must contain no detectable *E. coli* in 100 mL of water, reflecting the use of this bacterium as the primary indicator of microbiological safety [41]. In the European Union, by contrast, water intended for contact with food must meet the criteria of potable water, as defined by Directive (EU) 2020/2184, which requires the absence of *E. coli* and *Enterococcus* spp. in 100 mL [42]. However, our earlier field observations indicated that in some processing facilities, the wash water was not always replaced between product batches, which significantly increases the risk of microbial accumulation and highlights the practical relevance of developing effective treatment strategies [35].

In our study, we designed and evaluated the performance of multilayer filters composed of activated carbon, ion exchange resin, and hydrogel hydrated with natural plant extracts. The system was subsequently validated using industrial process water. To the best of the authors' knowledge, this is the first report to apply a filtration approach incorporating natural plant extracts immobilized within the filter bed for use in the fresh-cut industry. Conventional disinfection techniques are typically employed, including chlorine-based compounds (which are predominant), ozonation, peracetic acid, ultraviolet (UV) irradiation, hydrogen peroxide, as well as combined or hybrid methods [11]. In addition, the study was carried out using environmental bacterial isolates obtained from food-processing facilities and real industrial process water, rather than the reference strains or model systems employed in most previous studies [43–45]. This choice reflects real operating conditions and provides a preliminary indication of the applicability of the proposed approach in an industrial context.

The study was carried out under several assumptions that informed both its design and the interpretation of the findings. It was assumed that the bacterial isolates obtained from food-processing facilities are representative of the microbial contaminants typically present in wastewater from the fresh-cut industry. The multilayer filtration bed was considered to act through the complementary effects of hydrogel with natural extracts, ion-exchange resin, and activated carbon. The pilot-scale system was regarded as sufficiently representative of industrial conditions, although the experiments were conducted over limited operating intervals. Finally, the industrial process water used for validation was assumed to reflect the composition of effluents commonly generated in agro-food processing environments.

The study by Ignat et al. [46] on the disinfection of wash water using acidic electrolyzed water (30 mg Cl_2 L^{-1}) demonstrated a reduction in mesophilic bacteria of approximately 1.0–1.2 $\log_{10}$ CFU mL^{-1} after 10 min of contact. In contrast, the composite filter proposed in the present study reduced the number of inoculated bacteria by 2.2 $\log_{10}$ CFU mL^{-1} in the initial interval, without the addition of chlorine. This suggests that the multilayer system saturated with natural plant extracts may offer higher microbiological efficacy while remaining free of chlorine-based compounds. A similar level of effectiveness was reported by López-Gálvez et al. [47], who applied 2–3 mg L^{-1} ClO_2 in the wash water recirculation system at a tomato-sorting facility, achieving a reduction in mesophilic bacteria, coliforms,

and *E. coli* in process water by slightly more than 1 $\log_{10}$ CFU mL^{-1}. Importantly, the authors also did not detect any disinfection by-products in the final product. This highlights the critical importance of selecting an appropriate dosage when using conventional disinfection methods. Higher levels of microbial reduction were achieved by Banach et al. [48] in a pilot-scale setup at an iceberg lettuce sorting facility. The study confirmed the high effectiveness of adding a low-chlorine oxidant (5 mg ClO_2 L^{-1} or 3 mg ClO_2 L^{-1}) to the recirculating process water, resulting in a $\geq$5-$\log_{10}$ reduction in inoculated *E. coli* in less than 1 min.

Another example of disinfectants used in agri-food processing facilities is ozone. Compared to chlorine, its stability in water is significantly lower due to its spontaneous decomposition, which is initiated by reactions with hydroxyl ions or reduced substances [49]. Nevertheless, studies conducted by Selma et al. [50] demonstrated that 60 min of exposure to O_3, or its combination with UV-C, resulted in a 5.9–6.6 $\log_{10}$ reduction in microbial load in wastewater from vegetable processing. It is important to note, however, that ozonation generates a wide range of secondary inorganic and organic compounds. Ozonation by-products can be classified into two main groups: oxidation by-products, formed from naturally occurring constituents of the water itself (e.g., bromide ions), and transformation products, resulting from reactions between ozone and trace contaminants present in the solution [51].

In contrast, our study was based on the use of non-invasive methods involving natural filter components—namely, activated carbon and natural plant extracts—supported by an ion exchange resin. In tests using inoculated water, this system demonstrated greater stability and a higher level of bacterial reduction. Activated carbon filters are widely used as a medium for water purification. While their primary function is the removal of contaminants through adsorption onto a highly porous surface, they may also exert effects on microbial populations [52]. Microorganisms can become immobilized within the pores of activated carbon, which consequently leads to a reduction in their abundance in the flowing water [53]. As noted by Sbardella et al. [54], over time, colonization of the filter bed occurs, along with the stabilization of interactions between microorganisms and the carbon phase. This leads to the phenomenon of biological activated carbon (BAC) filtration, in which sessile microorganisms degrade immobilized contaminants. While this process can be considered beneficial in the case of natural colonization or the use of microbial inoculants, it may be highly undesirable in the context of our study, due to the use of opportunistic pathogens in the inoculated water. Therefore, a subsequent step in the development of the system should include a detailed analysis of biofilm formation within the filtration unit, together with the implementation of risk management strategies aimed at minimizing potential adverse effects. Many authors highlight one of the major limitations associated with the use of activated carbon filters—their potential to become reservoirs of antibiotic resistance genes and multidrug-resistant bacteria, which may pose a significant risk to public health safety [55–57]. Nevertheless, activated carbon remains one of the longest-used and most thoroughly documented sorbents in environmental engineering, with its industrial application dating back to the early 20th century [58].

Ion exchange resins, traditionally used in water softening and demineralization processes, have in recent years gained increasing attention as components of active antibacterial barriers [59]. However, in our study, they were employed as a conventional component complementing the composite filter bed, with the aim of enhancing its functionality by improving sorption capacity and potentially contributing to overall water quality improvement.

The final component of the filter bed was a hydrogel hydrated with natural plant extracts—an approach that has been scarcely described in the scientific literature to date. Previous in vitro studies on the extracts [34] demonstrated their effectiveness under lab-

oratory conditions; however, the present work represents the first report in which this solution has been implemented in the context of process water treatment. Importantly, most available publications concerning hydrogels enriched with plant extracts primarily relate to biomedical applications, particularly wound healing and localized therapy [60–62]. Compounds identified in the earlier GC-MS analysis—such as epigallocatechin gallate, shikimic, ellagic, and gallic acids, as well as betulin, salicin, and procyanidins—may be responsible for the observed antimicrobial activity of the extracts.

Filtration is commonly employed in environmental engineering as a final polishing step to lower particle-associated microbial loads and enhance the effectiveness of subsequent disinfection [63,64]. An example of effective bacterial removal is the study by Zhang et al., which showed that adding biochar to sand columns enhances the elimination of *E. coli* and *B. subtilis* under both slow and rapid filtration, mainly due to the material's higher adsorption capacity [65]. Other studies have shown that the depth of the sand bed influences both filter performance and flow rate. Deeper beds improve the removal of suspended solids, turbidity and coliform bacteria, while dissolved contaminants are less effectively reduced [66]. These findings demonstrate the effectiveness of conventional granular media mainly through physical straining and adsorption. By contrast, our multilayer bed integrates these mechanisms with the antimicrobial activity of plant-extract-infused hydrogel, offering an additional mode of bacterial reduction beyond polishing alone.

Sodium polyacrylate-based hydrogels are superabsorbents that undergo extensive swelling in aqueous environments. As a result of this process, a porous structure is formed within the polymer network. This feature enables the gradual release of immobilized compounds into the surrounding medium, both in medical applications and in our own studies with plant extracts [67,68]. The release of active substances (e.g., in medicines) from hydrogels can occur through different mechanisms: diffusion of the active substance with the absorbed liquid, erosion of the polymer matrix leading to pore enlargement, or swelling of the matrix due to water uptake [67]. Although we did not perform a kinetic analysis of the release process in our study, the literature clearly indicates that sodium polyacrylate-based systems typically exhibit non-Fickian diffusion behavior and therefore require models that account for both diffusive transport and polymer network relaxation [69,70].

With an initial load of approximately 10^7 CFU mL^{-1}, the laboratory-inoculated water represented an extremely challenging test scenario, significantly exceeding the level of contamination observed in the wastewater collected from the fruit processing facility. Despite this high microbial burden, the H-C-IER composite filter achieved a reduction in more than 2 $\log_{10}$ within the first three hours, and after 24 h, it still maintained an effectiveness greater than 1 $\log_{10}$. The bacterial concentrations used in our study are consistent with common research practice. For example, Faith et al. employed an inoculum concentration of 10^7 CFU of *Salmonella enterica* in their experimental design. An alternative approach was presented by Yesil et al. [71], who applied a broader range of inoculum concentrations—10^8, 10^7, and 10^5 CFU/g—for the inoculation of spinach leaves. In real process water—characterized by a lower bacterial concentration but a higher content of organic matter following apple-washing—the observed log reductions were lower ($\approx$1–1.3 $\log_{10}$), indicating that such contaminants may partially affect the performance of the composite. Nevertheless, the filter met the minimum efficiency criterion of 90%. As noted in the study by López-Gálvez et al., the physicochemical quality of process water in a fresh produce facility varied between processing lines, which was attributed to differences in the types of raw materials being washed as well as the uneven efficiency of the washing systems themselves [72]. This, in turn, can lead to differences in the microbial load of the process water itself.

A limitation of the present study lies in the barrier properties of the hydrogel. The observed antibacterial effect is primarily attributable to the diffusion of plant extracts from the hydrogel matrix into the surrounding medium. While this release mechanism is intrinsic to the functioning of the system, it makes it difficult to assess the potential barrier effect of the hydrogel itself. A comprehensive characterization of both the release kinetics and the barrier properties will be required in future studies to optimize the hydrogel formulation and to gain a clearer understanding of its role in microbial inactivation. In this preliminary study, the operational time of the prototype was limited to 24 h, during which a marked decrease in the efficiency of the C and IER components was observed. Although this time frame allowed us to obtain a first evaluation of the system, further investigations are needed to assess its performance over longer operating periods. Future optimization of the filter will focus not only on testing extended operational times but also on increasing the effective filtration surface, with the aim of improving both efficiency and durability. In this context, the H component appears particularly promising, and further studies will be directed toward refining its performance and stability.

An interesting approach aimed at enhancing the efficiency of bacterial inactivation in process water could involve the integration of additional modules into the proposed system—for example, a sedimentation tank, which reduces suspended solids that may serve as a habitat for microorganisms [73]. Another strategy involves increasing the filtration surface area by adding a greater number of filter tubes, in order to adjust the performance and lifespan of the filtration unit to the specific conditions of a given production facility—such as the type of processed crop, continuous vs. seasonal operation, or geographical location. In addition, future work should include long-term performance tests under continuous operation to better reflect industrial practice. Further studies will also be necessary to characterize the release kinetics of natural extracts from the hydrogel matrix and to optimize the balance between diffusion and barrier properties.

The proposed filter system in our study may serve as an alternative or complementary solution to conventional disinfection methods, which are known to generate undesirable by-products.

5. Conclusions

The results showed that the composite filter bed—comprising hydrogel, ion exchange resin, and activated carbon, saturated with natural plant extracts from tea waste, willow bark, oak bark, birch bark, raspberry shoots, and linden flowers—reduced the bacterial load by more than 2 $\log_{10}$ in highly inoculated water (1.2×10^7 CFU mL^{-1}) and maintained at least a 1 $\log_{10}$ reduction in real wastewater effluents from fruit processing. This effect was achieved without the use of chlorine, thereby eliminating the risk of forming undesirable disinfection by products. While the filter maintained its efficacy, long-term operation requires further evaluation of durability and potential regeneration. In the future, the system could be enhanced with preliminary clarification modules (e.g., sedimentation tanks), which may further increase filter lifespan and performance. The proposed solution aligns with the development of modern methods for reducing contamination in wastewater for potential reuse in the agri-food industry—supporting blue water footprint reduction strategies and compliance with "green processing" principles.

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

Abbreviations

The following abbreviations are used in this manuscript:

DBPs	Disinfectant byproducts
CFU	Colony-forming unit
H	Hydrogel
C-IER	Carbon-ion exchange resin filter

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Compound	$t_{ret.}$ (min.)	$RI_{exp.}$	$RI_{lit.}$	linden SIL		rapsberry SIL		tea SIL	
				Area	TIC (%)	Area	TIC (%)	Area	TIC (%)
L-Alanine, TMS	8.195	939	N/A	10775105	0,03	12326561	0,05	17280530	0,06
Ethylamine, di-TMS	8.484	964	966[a]	45309538	0,14	55158698	0,20	47888109	0,16
Carbodiimide, di-TMS	9.126	964	966[a]	-	-	4735125	0,02	6642265	0,02
1,2-Dihydroxyethylene, di-TMS	9.663	978	N/A	-	-	4710395	0,02	-	-
Lactic acid, di-TMS	13.251	1071	1073[a]	15899762	0,05	13855944	0,05	7011491	0,02
Glycolic acid, di-TMS	13.873	1080	1083[a]	20709973	0,06	28732266	0,11	10189061	0,03
L-Valine, TMS	14.128	1086	1083[b]	18550441	0,06	19778687	0,07	6925883	0,02
Pyruvic acid, enol, di-TMS	14.387	1092	1095[a]	-	-	-	-	6634275	0,02
L-Alanine, di-TMS	15.082	1108	1112[a]	-	-	-	-	5305633	0,02
Oxalic acid, di-TMS	16.528	1141	1143[a]	-	-	-	-	7016845	0,02
β-Lactic acid, di-TMS	16.945	1151	1151[b]	6671205	0,02	15518959	0,06	-	-
L-Leucine, TMS	17.235	1158	1156[a]	5658388	0,02	5912213	0,02	-	-
L-Proline, TMS	17.926	1173	1174[a]	54532213	0,17	112429730	0,41	-	-
L-Isoleucine, TMS	18.118	1178	1178[b]	17728133	0,05	14693330	0,05	-	-
Malonic acid, di-TMS	19.700	1214	1216[a]	6671707	0,02	32529528	0,12	8462788	0,03
L-Valine, di-TMS	20.213	122	1226	-	-	7592328	0,03	-	-

		6	[a]						
1,3-Dihydroxyacetone, di-TMS	20.469	1231	N/A	6261343	0,02	29617745	0,11	7048647	0,02
Urea, di-TMS	21.318	1251	1255 [a]	-	-	10951967	0,04	8115525	0,03
L-Serine, di-TMS	21.979	1266	N/A	41352796	0,13	19046359	0,07	29524381	0,10
Phosphotic acid, tri-TMS	22.966	1288	1289 [a]	458749485	1,42	439250590	1,62	217585561	0,74
Glycerol, tri-TMS	23.162	1292	1293 [a]	465083833	1,44	565061454	2,08	32262955	0,11
L-Threonine	23.661	1304	1305 [a]	24328342	0,08	60253101	0,22	24233593	0,08
Succinic acid, di-TMS	24.464	1323	1324 [a]	71181892	0,22	198521973	0,73	41856718	0,14
Glyceric acid, tri-TMS	25.593	1349	1348 [a]	85445160	0,26	118834060	0,44	27190564	0,09
Fumaric acid, di-TMS	25.912	1356	1355 [a]	12861276	0,04	13101634	0,05	5019008	0,02
cis-Dihydro-3,4-dihydroxy-2(3H)-furanone, di-TMS	27.084	1384	1384 [a]	29163946	0,09	66716393	0,25	42636348	0,15
L-Threonine, tri-TMS	28.014	1406	1406 [a]	-	-	6062836	0,02	15270726	0,05
Tartronic acid, tri-TMS	28.211	1411	1408 [a]	-	-	7949314	0,03	-	-
L-Aspartic acid, di-TMS	29.125	1433	1427 [b]	-	-	18010185	0,07	179137623	0,61
1,2-Ethanediol phenyl, di-TMS	29.379	1439	N/A	6864235	0,02	-	-	-	-
2,3,4-Trihydroxybutyric acid, γ-lactone, di-TMS	29.460	1441	1441 [a]	-	-	5501204	0,02	-	-

3,4-Dihydroxybutanoic acid, tri-TMS	29.942	1453	1456[a]	10070711	0,03	11683924	0,04	5199064	0,02
4-Methoxyphenethoxy, TMS	30.680	1471	N/A	7889854	0,02	-	-	-	-
Malic acid, tri-TMS	32.297	1512	1512[a]	1440925254	4,46	1628278669	6,00	512599740	1,75
Salicylic acid, di-TMS	32.579	1519	1522[a]	7006645	0,02	20074314	0,07	6658834	0,02
Pyroglutamic acid, di-TMS	33.065	1531	1532[a]	22238040	0,07	-	-	133799509	0,46
Threitol, tetra-TMS	33.350	1539	1540[a]	19122084	0,06	34871623	0,13	-	-
4-Aminobutyric acid, tri-TMS	33.486	1542	1541[a]	-	-	28929446	0,11	-	-
Aspartic acid, tri-TMS	33.504	1543	1540[a]	-	-	-	-	353006336	1,21
Phenylalanine, TMS	33.709	1548	1549[a]	15093138	0,05	-	-	14534236	0,05
2,3,4-Trihydroxybutyric acid, isomer 1, tetra-TMS	34.729	1574	1575[a]	32093776	0,10	124213840	0,46	32923961	0,11
NN	34.997	1581	-	70170775	0,22	-	-	-	-
2,3,4-Trihydroxybutyric acid, isomer 2, tetra-TMS	35.456	1593	1595[a]	584776158	1,81	531572865	1,96	275296529	0,94
α-Hydroxyglutaric acid, tri-TMS	35.566	1596	1597[a]	10454051	0,03	21291024	0,08	-	-
L-Asparagine, di-TMS	35.977	1607	N/A	27376795	0,08	31773863	0,12	20304456	0,07
Carbohydrate, TMS	36.162	1612	-	-	-	67541905	0,25	-	-
Phenylalanine, di-TMS	37.099	163	1640	-	-	6963925	0,03	-	-

		7	[a]						
Glutamine, tri-TMS	37.209	1640	1642 [a]	7159051	0,02	17012653	0,06	104606827	0,36
Arabinofuranose, tetra-TMS	37.394	1645	1647 [a]	6301291	0,02	8177055	0,03	9720978	0,03
Arabinose, tetra-TMS	37.536	1649	1648 [a]	26111264	0,08	41999022	0,15	18629911	0,06
Arabinoic acid, γ-lactone, tri-TMS	37.707	1654	1650 [a]	5249880	0,02	6952497	0,03	-	-
Rhamnose, tetra-TMS	38.002	1662	1657 [a]	-	-	-	-	25189208	0,09
Phloroglucinol, tri-TMS	38.040	1663	1664 [a]	18992019	0,06	-	-	-	-
Xylofuranose, tetra-TMS	38.139	1666	1668 [a]	10048082	0,03	7724196	0,03	-	-
Tartaric acid, tetra-TMS	38.383	1672	1667 [a]	-	-	22838662	0,08	14938090	0,05
α-Ribofuranose, tetra-TMS	38.701	1680	1680 [a]	59552164	0,18	17263447	0,06	25674128	0,09
Carbohydrate, TMS	38.705	1681	-	-	-	48851133	0,18	-	-
L-Asparagine, tri-TMS	39.075	1691	1690 [a]	-	-	35444750	0,13	-	-
L-Ribose, tetra-TMS	39.152	1693	1694 [a]	28118834	0,09	19473397	0,07	-	-
L-Theanine	39.697	1708	N/A	-	-	-	-	199575739	0,68
Suberic acid, di-TMS	39.738	1709	1710 [a]	4126176	0,01	-	-	-	-
Carbohydrate, TMS	39.965	1716	-	-	-	-	-	41651569	0,14

Levoglucosan pyranose, tri-TMS	40.446	1729	1730 [a]	-	-	-	-	7850410	0,03
α-Xylopyranose, tetra-TMS	40.795	1739	1740 [a]	20682526	0,06	42466510	0,16	13954855	0,05
Xylitol, penta-TMS	41.064	1747	1750 [a]	-	-	6125129	0,02	-	-
Ribitol, penta-TMS	41.504	1760	1761 [a]	43445158	0,13	63216543	0,23	58609928	0,20
Lyxofuranose, tetra-TMS	41.836	1769	1770 [a]	16068041	0,05	239312696	0,88	-	-
Carbohydrate, TMS	42.182	1779	-	-	-	211958578	0,78	95707661	0,33
Carbohydrate acid, TMS	42.580	1790	-	-	-	75454110	0,28	-	-
β-Xylopyranose, TMS	42.742	1795	1796 [a]	20056662	0,06	53208792	0,20	55611236	0,19
Carbohydrate, TMS	42.938	1800	-	101381609	0,31	88214575	0,33	56105131	0,19
Ribonic acid, penta-TMS	43.102	1805	1805 [a]	161973389	0,50	133675354	0,49	192520297	0,66
Azelaic acid, di-TMS	43.219	1809	1808 [a]	30118642	0,09	27828475	0,10	-	-
Carbohydrate acid, TMS	43.372	1813	-	47611810	0,15	35536818	0,13	98575315	0,34
Gulose, penta-TMS	43.988	1832	1834 [a]	-	-	-	-	104918215	0,36
Methyl glucofuranoside, tetra-TMS , isomer 1	44.096	1835	1836 [a]	1002492625	3,10	338587705	1,25	1209751993	4,14
Caffeine	44.463	1846	1842 [a]	-	-	-	-	468850176	1,60
α-Fructofuranose, penta-TMS	44.507	184	1845	-	-	16441152	6,06	-	-

		7	[a]			93			
Shikimic acid, tetra-TMS	44.521	1847	1843 [a]	2847293813	8,81	-	-	444023097	1,52
β-Fructofuranose, penta-TMS	44.826	1857	1854 [a]	2636745262	8,16	2736528282	10,08	888536398	3,04
α-Galactofuranose, penta-TMS	45.158	1866	1865 [a]	73347034	0,23	189063773	0,70	71568102	0,24
Glucopyranose, penta-TMS	45.562	1879	N/A	1040375411	3,22	375014499	1,38	504887385	1,73
Methyl glucofuranoside, tetra-TMS , isomer 2	45.694	1882	1882 [b]	1427496505	4,42	616215890	2,27	452880195	1,55
β-Glucofuranose, penta-TMS	45.921	1889	1889 [a]	645484998	2,00	803778856	2,96	80056406	0,27
L-Tyrosine, di-TMS	46.147	1896	N/A	139972066	0,43	-	-	-	-
Quinic acid, penta-TMS	46.396	1904	1901 [a]	2179338477	6,74	468934361	1,73	1852243296	6,33
2-Amino-2-deoxyglucose, tetra-TMS	46.596	1910	1907 [a]	118413556	0,37	-	-	-	-
Carbohydrate, TMS	46.720	1914	-	169065377	0,52	177236917	0,65	-	-
Carbohydrate, TMS	46.870	1918	-	152515676	0,47	-	-	-	-
Gluconic acid, δ-lactone, tetra-TMS	46.960	1921	1920 [a]	330305866	1,02	470152652	1,73	137502547	0,47
α-D-Glucopyranose, penta-TMS	47.353	1933	1935 [a]	2558717864	7,92	2844880574	10,48	1501447692	5,13
β-Mannopyranose, penta-TMS	47.664	1943	1943 [a]	858464643	2,66	637012043	2,35	825075574	2,82
Carbohydrate, TMS	47.746	1946	-	-	-	461849545	1,70	222288195	0,76

p-Coumaric acid, di-TMS	47.748	1946	1947[a]	246793840	0,76	-	-	-	-
Coniferyl alcohol, di-TMS	47.962	1952	1954[a]	-	-	-	-	14192868	0,05
D-Gulonic acid, γ-lactone, tetra-TMS	48.018	1954	1958[a]	-	-	42710517	0,16	31947380	0,11
NN	48.170	1959	-	243337656	0,75	547660259	2,02	-	-
Carbohydrate, TMS	48.263	1962	-	298934507	0,93	-	-	51440918	0,18
Mannitol, hexa-TMS	48.481	1969	1973[a]	132359224	0,41	98729149	0,36	-	-
Carbohydrate, TMS	48.558	1971	-	-	-	-	-	31690689	0,11
Glucitol, hexa-TMS	48.891	1981	1880[a]	181960217	0,56	44112211	0,16	16254241	0,06
Gallic acid, tetra-TMS	48.995	1985	1985[a]	28617282	0,09	151888237	0,56	124939390	0,43
D-chiro-Inositol, hexa-TMS	49.369	1996	N/A	-	-	147495981	0,54	-	-
deoxy-Inositol, penta-TMS	49.678	2006	2004[a]	1090257604	3,37	585435089	2,16	256349534	0,88
Carbohydrate, TMS	49.854	2012	-	144164348	0,45	-	-	-	-
Carbohydrate, TMS	50.331	2028	-	976785285	3,02	-	-	-	-
β-D-Glucopyranose, penta-TMS	50.458	2032	2032[a]	2002536827	6,20	3056014642	11,26	1317948431	4,50
scyllo-Inositol, penta-TMS	50.697	2040	2040[a]	495286942	1,53	43977854	0,16	93724828	0,32
Carbohydrate, TMS	50.887	204	-	508704950	1,57	37216902	1,37	-	-

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Gluconic acid, hexa-TMS	50.945	2048	2046 [a]	421586516	1,30	254358235	0,94	64199877	0,22
Carbohydrate acid, TMS	51.248	2058	-	52321310	0,16	78233601	0,29	-	-
Carbohydrate acid, TMS	51.371	2062	-	76940225	0,24	-	-	-	-
scyllo-Inositol, hexa-TMS	51.696	2072	2071 [a]	32887220	0,10	15988370	0,06	-	-
Carbohydrate, TMS	51.818	2076	-	35338523	0,11	-	-	-	-
Carbohydrate, TMS	52.003	2082	-	33032392	0,10	-	-	-	-
Traumatic acid, di-TMS	52.070	2084	2086 [a]	-	-	12806295	0,05	-	-
Galactaric acid, hexa-TMS	52.218	2089	2090 [a]	11739138	0,04	10919424	0,04	5411040	0,02
NN	52.413	2096	-	-	-	-	-	63677740	0,22
Carbohydrate acid, TMS	52.750	2107	-	-	-	84920989	0,31	29622393	0,10
Carbohydrate, TMS	52.898	2112	-	326539041	1,01	707330592	2,61	409869619	1,40
myo-Inositol, hexa-TMS	53.450	2131	2129 [a]	512302132	1,59	1206869591	4,45	122480575	0,42
(E)-Caffeic acid, tri-TMS	54.111	2153	2154 [a]	16152173	0,05	69715249	0,26	-	-
Carbohydrate, TMS	54.349	2161	-	89450964	0,28	-	-	-	-
Carbohydrate, TMS	56.637	2241	-	-	-	-	-	58578273	0,20

Carbohydrate, TMS	58.585	2311	-	-	-	37194233	0,14	-	-
2-O-Glycerol-α-D-galactopyranoside, hexa-TMS	60.284	2373	2375[a]	15992205	0,05	75443868	0,28	75257998	0,26
Glycerol glycoside, TMS	61.571	2422	-	-	-	39357635	0,15	-	-
Glycerol glycoside, TMS	61.696	2427	-	-	-	54494568	0,20	29831000	0,10
β-D-Glucopyranuronic acid, penta-TMS	61.878	2434	2437[a]	21572585	0,07	232449161	0,86	96852363	0,33
Uridine, tri-TMS	62.825	2470	2469[a]	12930106	0,04	4685321	0,02	-	-
Carbohydrate, TMS	65.552	2578	-	-	-	46045508	0,17	-	-
Carbohydrate, TMS	66.003	2596	-	651102172	2,01	-	-	1431052610	4,89
Carbohydrate, TMS	66.559	2619	-	71551518	0,22	-	-	287553126	0,98
Carbohydrate, TMS	67.446	2655	-	-	-	-	-	116640245	0,40
Carbohydrate, TMS	67.579	2661	-	891584791	2,76	14986771	0,06	2138668333	7,31
Xylobiose, penta-TMS	68.209	2687	2691[a]	-	-	-	-	250027332	0,85
Carbohydrate, TMS	68.317	2691	-	-	-	28886622	0,11	147928424	0,51
β-Lactulose, octa-TMS	68.754	2710	2705[a]	154390771	0,48	-	-	-	-
Sucrose, octa-TMS	68.870	2715	2716[a]	734132691	2,27	72239771	0,27	2178090248	7,44
Carbohydrate, TMS	69.069	272	-	428842425	1,33	20389007	0,75	244025473	0,83

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Carbohydrate, TMS	69.457	2740	-	-	-	29969441	0,11	-	-
Carbohydrate, TMS	69.761	2753	-	-	-	48996101	0,18	517520034	1,77
α-Cellobiose, octa-TMS	69.971	2762	2761[a]	44644582	0,14	49553329	0,18	-	-
Carbohydrate, TMS	70.549	2787	-	-	-	83612544	0,31	-	-
Carbohydrate, TMS	71.245	2817	-	50261642	0,16	57520886	0,21	-	-
Carbohydrate, TMS	71.410	2824	-	45059588	0,14	-	-	309137632	1,06
Carbohydrate, TMS	71.542	2830	-	-	-	66020256	0,24	-	-
Carbohydrate, TMS	71.958	2849	-	-	-	69068572	0,25	-	-
Carbohydrate, TMS	72.066	2853	-	-	-	129149427	0,48	-	-
Carbohydrate, TMS	72.856	2888	-	-	-	-	-	117421497	0,40
epi-Catechin, penta-TMS	73.246	2906	2907[a]	432335889	1,34	31038680	0,11	1011754787	3,46
Carbohydrate, TMS	73.498	2917	-	-	-	-	-	338488127	1,16
Catechine, penta-TMS	73.829	2932	2936[a]	92258972	0,29	29775569	0,11	186010505	0,64
α-Isomaltose, octa-TMS	74.383	2958	2953[a]	42354557	0,13	72795292	0,27	-	-
Carbohydrate, TMS	74.502	2963	-	-	-	-	-	149416726	0,51

Gentibiose, octa-TMS	74.901	2981	2990 a	-	-	175864312	0,65	-	-
NN	74.947	2983	-	-	-	-	-	249992132	0,85
NN	75.058	2989	-	-	-	-	-	586378926	2,00
NN	75.285	2999	-	-	-	-	-	1424799074	4,87
β-Isomaltose, octa-TMS	75.300	3000	3005 a	-	-	18109531	0,07	168739979	0,58
Galactinol, nona-TMS	75.607	3014	3012 a	-	-	-	-	93981078	0,32
Kaempherol, tetra-TMS	77.678	3112	3114 a	-	-	22458157	0,08	8232058	0,03
Carbohydrate, TMS	78.913	3172	-	48969273	0,15	-	-	-	-
Chlorogenic acid, hexa-TMS	79.073	3180	3183 a	15076790	0,05	48057840	0,18	5771582	0,02
Quercetin, tetra-TMS	79.734	3213	3213 a	8284743	0,03	29946846	0,11	37106949	0,13
Carbohydrate, TMS	80.046	3228	-	-	-	261350548	0,96	-	-
Quercetin, penta-TMS	80.174	3235	3239 a	9513015	0,03	52351917	0,19	27632044	0,09
Neochlorogenic acid, hexa-TMS	80.776	3265	3268 a	13236590	0,04	5069309	0,02	-	-
Carbohydrate, TMS	81.210	3287	-	-	-	28859775	0,11	-	-
Ellagic acid, tetra-TMS	82.060	3330	3329 a	-	-	103263073	0,38	-	-
Carbohydrate, TMS	83.138	338	-	55765514	0,17	-	-	372396902	1,27

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Carbohydrate, TMS	83.295	3394	-	-	-	-	-	78088510	0,27
Carbohydrate, TMS	84.299	3447	-	-	-	-	-	54994775	0,19
Carbohydrate, TMS	84.434	3454	-	-	-	-	-	35636030	0,12
Raffinose, TMS	85.418	3506	3504 [a]	78316465	0,24	21522041	0,08	408009805	1,39
Epicatechin gallate, hepta-TMS	92.439	3888	3892 [a]	-	-	-	-	438954886	1,50
NN	93.756	3900	-	-	-	-	-	1056527932	3,61
				32314247025	**100,00**	**27135515048**	**100,00**	**29255758666**	**100,00**

[a] - Isidorov (2020)

[b] - https://webbook.nist.gov/