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

Zastosowanie cytometrii przepływowej do oceny jakości preparatów probiotycznych wytwarzanych za pomocą suszenia fluidalnego

Flow cytometric assessment of fluid bed-dried probiotic preparations

mgr inż. Jakub Kiepś

Rozprawa doktorska w dziedzinie nauk rolniczych
w dyscyplinie technologia żywności i żywienia

Promotor:
dr hab. Radosław Dembczyński

Poznań, 2023 rok

Składam serdeczne podziękowania

Promotorowi,

dr hab. Radosławowi Dembczyńskiemu

za ukształtowanie mojej postawy naukowej

oraz ogromne zaangażowanie i życzliwość

Kierownikowi, Pracownikom i Doktorantom

Katedry Biotechnologii i Mikrobiologii Żywności

za koleżeńską pomoc i wsparcie

Rodzicom i Siostrze

za *wsparcie w rozwoju osobistym,*

cierpliwość i wyrozumiałość

Malwinie

za nieustająca inspirację i motywację

do podejmowania nowych wyzwań

i ogromne wsparcie w ich realizacji

Publikacje wchodzące w skład rozprawy doktorskiej

Publikacja I:

Kiepś J, Dembczyński R. Current Trends in the Production of Probiotic Formulations. *Foods*. 2022; 11(15):2330. https://doi.org/10.3390/foods11152330

IF: 5.2

MEiN$_{2023}$: 140

Publikacja II:

Kiepś J, Juzwa W, Dembczyński R. Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups. *International Journal of Molecular Sciences*. 2023; 24(7):6841. https://doi.org/10.3390/ijms24076841

IF: 5.6

MEiN$_{2023}$: 140

Publikacja III:

Kiepś J, Juzwa W, Olejnik A, Sip A, Tomaszewska-Gras J, Dembczyński R. The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line. *Nutrients*. 2023; 15(15):3484. https://doi.org/10.3390/nu15153484

IF: 5.9

MEiN$_{2023}$: 140

Publikacja IV: Kiepś J, Olejnik A, Juzwa W, Dembczyński R. Economic analysis of the production process of probiotics based on the biological and physiological parameters of the cells. – publikacja załączona do cyklu jako manuskrypt w trakcie recenzji.

Sumaryczny Impact Factor dla publikacji wchodzących w skład rozprawy doktorskiej wyniósł 16.7

Suma punktów wg wykazu czasopism MEiN$_{2023}$ dla publikacji wchodzących w skład rozprawy doktorskiej wyniosła 420.

Spis Treści

Wykaz skrótów

CCD – charge coupled device – urządzenie o sprzężeniu ładunkowym

DMEM – Dulbecco's modified Eagle medium – pożywka Eagle'a w modyfikacji Dulbecco

DSC - differential scanning calorimetry - różnicowa kalorymetria skaningowa

FSC – forward scatter – rozproszenie przednie

GRAS - generally recognized as safe - uznane za bezpieczne

IFC – imaging flow cytometry – cytometria przepływowa z obrazowaniem

MRS – De Man, Rogosa, Sharpe

PBS – phosphate-buffered saline **–** sól fizjologiczna buforowana fosforanami

PI – propidium iodide – jodek propidyny

RMS – root mean square – średnia kwadratowa

SEM – scanning electron microscopy – skaningowa mikroskopia elektronowa

SGF – simulated gastric fluid – symulowany płyn żołądkowy

SIF – simulated intestinal fluid – symulowany płyn jelitowy

SSC – side scatter – rozproszenie boczne

TEER – trans-epithelial electrical resistance – przeznabłonkowy opór elektryczny

TG/DTA – thermogravimetry differential thermal analysis - termograwimetria z różnicową analizą termiczną

VBNC – viable but nonculturable – żywe, niezdolne do wzrostu

Streszczenie

Preparaty zawierające probiotyczne szczepy bakterii korzystnie wpływają na zdrowie człowieka. Wyniki badań klinicznych potwierdzają pozytywny wpływ przyjmowania probiotyków na choroby przewodu pokarmowego, w tym na zespół jelita drażliwego, biegunkę, zapalenie jelit i stany alergiczne, takie jak atopowe zapalenie skóry. Wykazano również, że probiotyki zwiększają odporność organizmu poprzez immunomodulację. Przekłada się to na wzrost zainteresowania metodami zarówno utrwalania, jak i oceny mikroorganizmów.

Celem badań była analiza cech fizjologicznych i morfologicznych mikroorganizmów probiotycznych po suszeniu w złożu fluidalnym, z wykorzystaniem cytometrii przepływowej z obrazowaniem, sortowania komórek i uczenia maszynowego. Metody te pozwalają na uwzględnienie, oprócz subpopulacji komórek żywych i martwych, także subpopulacji komórek o pośredniej aktywności metabolicznej i zróżnicowanym stopniu uszkodzeń błony komórkowej. W pracy ponadto oceniono wpływ ekspozycji na stresy subletalne podczas hodowli bakterii probiotycznych na ich przeżywalność w trakcie suszenia i długotrwałego przechowywania i po symulowanym trawieniu *in vitro*, a także na ich adhezję do nabłonka jelitowego. Wykazano, że stopień uszkodzeń błony komórkowej istotnie wpływa na zdolność adhezji bakterii probiotycznych do nabłonka jelitowego. Stwierdzono również, że zastosowanie warunków stresowych w trakcie hodowli bakterii pozwala na zwiększenie przeżywalności komórek w preparacie probiotycznym w trakcie jego utrwalania i przechowywania. Na trwałość preparatu w trakcie długotrwałego przechowywania wpływały też takie czynniki jak zastosowanie w procesie technologicznym powlekania wysuszonego preparatu dodatkową warstwą ochronną i warunki przechowywania (temperatura, obecność tlenu). Opracowano także projekt procesu produkcji preparatów probiotycznych metodą suszenia fluidalnego wraz z analizą ekonomiczną oraz oceną ryzyka przedsięwzięcia w skali przemysłowej.

Słowa kluczowe: bakterie kwasu mlekowego, żywotność, adhezja, trawienie, proces technologiczny

Abstract

Preparations containing probiotic strains of bacteria have a beneficial effect on human health. The results of clinical trials show the positive effects of taking probiotics on diseases of the gastrointestinal tract, including irritable bowel syndrome, diarrhea, enteritis, and allergic conditions, such as atopic dermatitis. Probiotics have also been shown to increase the body's immune resistance through immunomodulation. This translates into an increased interest in methods for both preservation and assessment of microorganisms.

The aim of this research was to analyze the physiological and morphological analysis of probiotic microorganisms after fluid bed drying using imaging flow cytometry, cell sorting, and machine learning. These methods allow to take into account also the subpopulations of cells with intermediate metabolic activity and cellular membrane damage in addition to subpopulations of living and dead cells. Additionally, the study also assessed the impact of exposure to sublethal stresses during culturing of probiotic bacteria on their viability during drying, long-term storage and *in vitro* simulated digestion, as well as their adhesion to the intestinal epithelium. It was proven that the degree of cell membrane damage significantly affects the adhesion ability of probiotic bacteria. It was also found that the use of stress conditions during bacterial cultivation allows for increased cell survival in the probiotic preparation during its drying and storage. The durability of the preparation during long-term storage was also influenced by factors such as the use of a protective substance during coating and storage conditions (temperature, presence of oxygen). A simulation of the production process of probiotic preparations using fluid bed drying was also prepared, along with an economic analysis and risk assessment of the project on an industrial scale.

Keywords: lactic acid bacteria, viability, adhesion, digestion, technological process

1. Wprowadzenie

1.1. Opis podjętego problemu badawczego

Probiotyki, zgodnie z definicją WHO, określane są jako żywe mikroorganizmy, które podane we właściwej ilości przynoszą korzyść zdrowotną (FAO/WHO, 2006). Skoncentrowane preparaty bakterii probiotycznych stosowane w żywieniu zwierząt jak i spożywane przez ludzi mają najczęściej postać wysuszonej biomasy. Bakterie kwasu mlekowego o statusie GRAS to grupa, w obrębie której odnotowano najwięcej bakterii o właściwościach probiotycznych. Najczęściej preparaty probiotyczne występują w formie kapsułek, proszków lub zawiesin. Zawierają żywe bakterie - przede wszystkim z rodzajów *Lactobacillus* i *Bifidobacterium*. W zdecydowanej większości probiotyki stosowane jako suplementy zarówno w żywieniu zwierząt, jak i ludzi przygotowywane są w postaci suszonych granulatów biomasy. Wyniki badań klinicznych potwierdzają pozytywne skutki przyjmowania probiotyków w schorzeniach przewodu pokarmowego, takich jak zespół jelita drażliwego, biegunka i zapalenie jelit, infekcje układu moczowego oraz stany alergiczne, np. atopowe zapalenie skóry (Kerry et al., 2018; Lopez-Santamarina et al., 2021). Wykazano również, że probiotyki zwiększają odporność organizmu poprzez immunomodulację (Gill & Prasad, 2008). Aby wywrzeć korzystny efekt zdrowotny, preparat probiotyczny powinien zawierać minimalną liczbę żywych bakterii (jednostek tworzących kolonie) – co najmniej 10^6 jtk/g.

Parametry jakościowe utrwalonych komórek (liczba żywych komórek, aktywność biologiczna) zwykle zależą od rodzaju zastosowanej metody suszenia (Kiepś & Dembczyński, 2022). W przypadku materiałów termolabilnych, w tym także komórek bakterii, najmniejsze obniżenie jakości po suszeniu obserwuje się w przypadku zastosowania suszenia sublimacyjnego (Mehanna & Abla, 2022). Jest to jednak metoda kosztowna i mało wydajna (Broeckx et al., 2016). W związku z tym liofilizację coraz częściej próbuje się zastąpić suszeniem rozpyłowym. Podczas suszenia rozpyłowego bardzo często jednak trudno jest uzyskać jakość preparatów bakterii zbliżoną do otrzymywanej w trakcie suszenia sublimacyjnego. Jedną z przyczyn jest stosunkowo wysoka temperatura suszenia, która zazwyczaj nie będzie niższa niż 60-70ºC. Tymczasem w suszarniach fluidalnych możliwe jest prowadzenie procesu w znacznie niższych temperaturach, zbliżonych do 40ºC. Można zatem założyć, że znacznie zmniejszy się degradacja termiczna struktur komórkowych, a tym samym zachowana będzie większa przeżywalność bakterii (Fu & Chen, 2011). Suszarki pracujące w warunkach złoża fluidalnego posiadają bardzo dobre parametry przenoszenia masy i energii w związku z tym czas suszenia jest krótki (Assari et al., 2013). W złożu fluidalnym matryca z

komórkami może zostać łatwo powleczona dodatkową warstwą ochronną. Powlekanie stanowi jedną ze strategii stosowanych w celu poprawy przeżywalności probiotyków w procesie trawienia, a także wydłużenia ich trwałości i stabilności w trakcie przechowywania (Safeer Abbas et al., 2023; Yang et al., 2023). W celu ochrony przed niskim pH powszechnie stosuje się polisacharydy, takie jak alginian, oraz powłoki na bazie celulozy i lipidów. Dodatkowo, koszty inwestycyjne i operacyjne suszenia fluidalnego są niższe niż suszenia sublimacyjnego czy rozpyłowego (Mohideen Batcha et al., 2023). W związku z tym wzrasta zainteresowanie tą metodą suszenia, czego dowodem jest rosnąca liczba publikacji w przedmiocie utrwalania szczepów probiotycznych za pomocą suszenia fluidalnego.

W trakcie procesu suszenia na mikroorganizmy oddziałuje szereg czynników stresowych, takich jak stres termiczny, mechaniczny, osmotyczny oraz oksydacyjny (Santivarangkna et al., 2008). Ekspozycja na warunki stresowe może prowadzić do denaturacji białek, uszkodzenia kwasów nukleinowych i zniszczenia struktur błony komórkowej. Celem redukcji wpływu wymienionych czynników na przeżywalność bakterii w procesie suszenia stosuje się środki zapobiegawcze, w postaci dodatku substancji ochronnych, optymalizacji parametrów procesu, pozyskiwanie komórek znajdujących się w stacjonarnej fazie wzrostu (Fu & Chen, 2011) oraz adaptacji komórek na etapie hodowli (Huang et al., 2017). Wśród powszechnie stosowanych substancji ochronnych wymienia się inulinę, trehalozę, oligosacharydy, białka mleka, a także gumę arabską (Ananta et al., 2005; Arepally & Goswami, 2019; Avila-Reyes et al., 2014; Morgan et al., 2006; Perdana et al., 2014). Ekspozycja na subletalny stress na etapie hodowli zwiększa odporność bakterii na czynniki stresowe na dalszych etapach procesu technologicznego poprzez indukcję mechanizmów obronnych (Broeckx et al., 2016). Wydzielone w odpowiedzi na stres białka pomagają w nabyciu odporności krzyżowej, zwiększając przeżywalność nie tylko podczas ekspozycji na dany czynnik, ale także na późniejszych etapach procesu, np. zwiększając odporność bakterii na niskie pH w środowisku przewodu pokarmowego (Bucka-Kolendo & Sokołowska, 2017).

Probiotyki definiuje się jako żywe mikroorganizmy, jednak zdolność do wywoływania efektów prozdrowotnych posiadają również bakterie probiotyczne o pośrednich stanach fizjologicznych, takie jak grupa bakterii pozbawionych zdolności do wzrostu na klasycznych podłożach mikrobiologicznych (Fiore et al., 2020). Oznacza to, że klasyczne metody oznaczania liczby żywych mikroorganizmów w produktach probiotycznych nie biorą pod uwagę wszystkich komórek o potencjale prozdrowotnym. W celu precyzyjnego oznaczenia bakterii w preparatach należy uzupełnić klasyczne metody mikrobiologiczne, takie jak posiewy

na płytki z agarem metodą Kocha o bardziej zaawansowane metody. Należą do nich takie techniki analityczne jak cytometria przepływowa z obrazowaniem, umożliwiająca nie tylko dokładne zliczanie komórek, niezależnie od ich zdolności do wzrostu, ale również ocenę ich morfologii i fizjologii. Ważnym czynnikiem w ocenie preparatów probiotycznych jest również zdolność bakterii do adhezji do komórek nabłonka jelitowego (Kiepś, et al., 2023). Efektywna adhezja pozwala komórkom probiotycznym na zasiedlenie układu pokarmowego i regulację mikrobioty jelitowej (Gorreja & Walker, 2022; Wang et al., 2018). Po adhezji bakterie wydzielają również szereg metabolitów o pozytywnym oddziaływaniu na organizm gospodarza, do których należą krótkołańcuchowe kwasy tłuszczowe (Chang et al., 2021) i bakteriocyny (Mohanty et al., 2019). Ponadto bakterie probiotyczne po adhezji do nabłonka jelitowego konkurują z patogenami, ograniczając ich rozwój i negatywne oddziaływanie (Jessie Lau & Chye, 2018; Walsham et al., 2016). Posiadają one również zdolność wzmacniania funkcji barierowej błony sluzowej jelita (Bron et al., 2017). Obecność probiotyków pozytywnie wpływa także na regulację odpowiedzi immunologicznej organizmu i może łagodzić stany zapalne (Monteagudo-Mera et al., 2019).

1.2. Nowatorstwo i oryginalność podjętej tematyki

Mimo znacznego wzrostu zainteresowania metodami utrwalania mikroorganizmów większość badań skupia się na suszeniu rozpyłowym i liofilizacji. Suszenie fluidalne jest znacznie rzadziej stosowaną metodą. Wobec tego brak jest kompleksowego opisu procesu wytwarzania preparatów probiotycznych za pomocą tej metody. Dostępne dane literaturowe na temat suszenia fluidalnego z reguły koncentrują się jedynie na technologicznych aspektach procesu suszenia komórek i wpływie parametrów operacyjnych na żywotność mikroorganizmów. Zaproponowany w pracy pełny opis procesu przygotowania stabilnego preparatu - od etapu hodowli, poprzez suszenie aż po ocenę warunków rehydratacji i zbadanie przeżywalności w trakcie przechowywania, a także adhezji do komórek nabłonkowych jelit - wyróżnia się oryginalnością na tle innych badań. Takie szczegółowe podejście pozwala na lepsze zrozumienie wpływu poszczególnych czynników biologicznych i technologicznych na parametry jakościowe końcowego produktu. Oprócz tradycyjnych, powszechnie stosowanych technik analitycznych (np. oznaczanie liczebności komórek za pomocą posiewów na płytkach) w badaniach wykorzystywane są nowoczesne metody i narzędzia analityczne takie jak: IFC, SEM, DSC, symulacja trawienia *in vitro* oraz pomiar adhezji komórek bakterii w kulturach komórkowych.

Należy także zauważyć, że obecnie zdecydowana większość badań koncentruje się na występujących w preparatach komercyjnych bakteriach należących do rodzajów *Lactobacillus, Bifidobacterium* i grzybach z rodzaju *Saccharomyces* (Sanders et al., 2018). Dlatego, do eksperymentów wytypowano szczepy rzadko spotykane w preparatach probiotycznych. Wybrane szczepy bakteryjne nie zostały jeszcze szczegółowo zbadane i opisane pod względem ich żywotności i aktywności probiotycznej po suszeniu fluidalnym.

Kolejny aspekt stanowi protokół oceny morfologii i fizjologii komórek metodą cytometrii przepływowej z obrazowaniem i sortowania komórek. Przeprowadzono złożoną charakterystykę morfologiczną: w pierwszym etapie wykorzystano parametr Gradient RMS z sygnałów jasnego pola (Ch01), co umożliwiło rozróżnienie obrazów komórek o wysokiej rozdzielczości, w drugim etapie parametry przetwarzania obrazu cyfrowego: współczynnik proporcji i obszar w celu scharakteryzowania kształtu i wielkości analizowanych komórek bakteryjnych w połączeniu z rozróżnianiem komórek bakteryjnych od resztek pozakomórkowych (pozostałości pożywki po hodowli oraz matrycy po suszeniu) i pojedynczych komórek od agregatów. Celem poprawy dokładności rozróżniania komórek i elementów pozakomórkowych zastosowano również moduł uczenia maszynowego. Po manualnym wybraniu dwóch przykładowych populacji wyjściowych algorytm wygenerował najlepiej je rozróżniające klasyfikatory. Zostały one następnie przedstawione w formie histogramu i zastosowane do rozróżnienia między komórkami i elementami pozakomórkowymi we wszystkich pomiarach. Komórki w próbkach zliczano i oceniano pod kątem morfologii (obraz mikroskopowy), aktywności (sygnał dla RedoxSensorTM Green) i integralności błony komórkowej (sygnał dla PI). Po wykonaniu wspomnianych etapów komórki posortowano w celu wyizolowania czterech subpopulacji i dalszej analizy populacji będących przedmiotem zainteresowania (np. komórek o obniżonej aktywności metabolicznej – VBNC), a także w celu analizy wzrostu tych subpopulacji w pożywce stałej i płynnej. Sorter umożliwia wyizolowanie określonej liczby komórek do płytek 96-dołkowych z bulionem MRS lub płytek Petriego z podłożem MRS-Agar. Sortowanie komórek przeprowadzono na podstawie odczytów uzyskanych za pomocą barwienia fluorescencyjnego. W próbkach zaobserwowano cztery różne grupy komórek, mianowicie komórki aktywne, mid-active I, mid-active II i komórki martwe. Określano je na podstawie dwóch odrębnych parametrów – aktywności metabolicznej (RedoxSensorTM Green) i żywotności, rozumianej jako integralność błony komórkowej (PI). Zastosowany protokół szczegółowo opisano w Publikacji II (Kiepś, et al., 2023).

Skuteczną ocenę jakości preparatów probiotycznych utrudnia brak standaryzowanych wymagań dotyczących ich skuteczności i liczby żywych komórek (jednostek tworzących kolonie) w produkcie. Co ważniejsze, nie bierze się pod uwagę spadku żywotności komórek, który występuje podczas pasażu w przewodzie pokarmowym (Hathi et al., 2021). Dlatego opracowano komputerową symulację procesu technologicznego wytwarzania proszków probiotycznych za pomocą suszenia fluidalnego i dokonano oceny ekonomicznej procesu w celu oszacowania kosztów produkcji preparatu. W kolejnej analizie, odniesiono koszt uzyskania końcowego produktu nie tylko do końcowej masy uzyskanego produktu. Ostateczny koszt został skorygowany na podstawie danych pozyskanych w trakcie oznaczeń cytometrycznych, tak aby uwzględniał on liczbę wszystkich żywych i aktywnych metabolicznie komórek w preparacie przed i po procesie trawienia. W ten sposób obliczono rzeczywisty koszt produkcji preparatu uwzględniający tylko te komórki, które nawet po procesie trawienia zachowają właściwości probiotyczne i prozdrowotne. Takie podejście umożliwia także łatwiejszą ocenę zasadności stosowania takich zabiegów technologicznych jak powlekanie dodatkową substancją ochronną czy implementacja subletalnych stresów na etapie hodowli.

2. Hipoteza badawcza i cel badań

W trakcie przygotowań do badań oraz na podstawie przeglądu literaturowego zawartego w Publikacji I sformułowano następujące hipotezy badawcze:

H.1: Cytometria przepływowa z obrazowaniem pozwala na podział komórek na subpopulacje o zróżnicowanym stanie fizjologicznym (aktywność metaboliczna) i morfologii (stopień uszkodzeń błony komórkowej).

H.2.: Suszenie fluidalne pozwala na uzyskanie preparatu zawierającego rekomendowaną dawkę komórek probiotycznych – na minimalnym poziomie 10^6 komórek na gram preapratu.

H.3.: Stopień uszkodzeń błony komórkowej istotnie wpływa na zdolność adhezji bakterii probiotycznych do komórek nabłonka jelitowego.

H.4: Zastosowanie warunków stresowych w trakcie hodowli bakterii pozwala na zwiększenie przeżywalności komórek w preparacie probiotycznym w trakcie jego utrwalania i przechowywania.

H.5: Proces technologiczny i warunki przechowywania wpływają na trwałość preparatu w trakcie długotrwałego przechowywania.

H.6: Realne oszacowanie kosztu wytworzenia preparatu probiotycznego wymaga uwzględnienia trawienia.

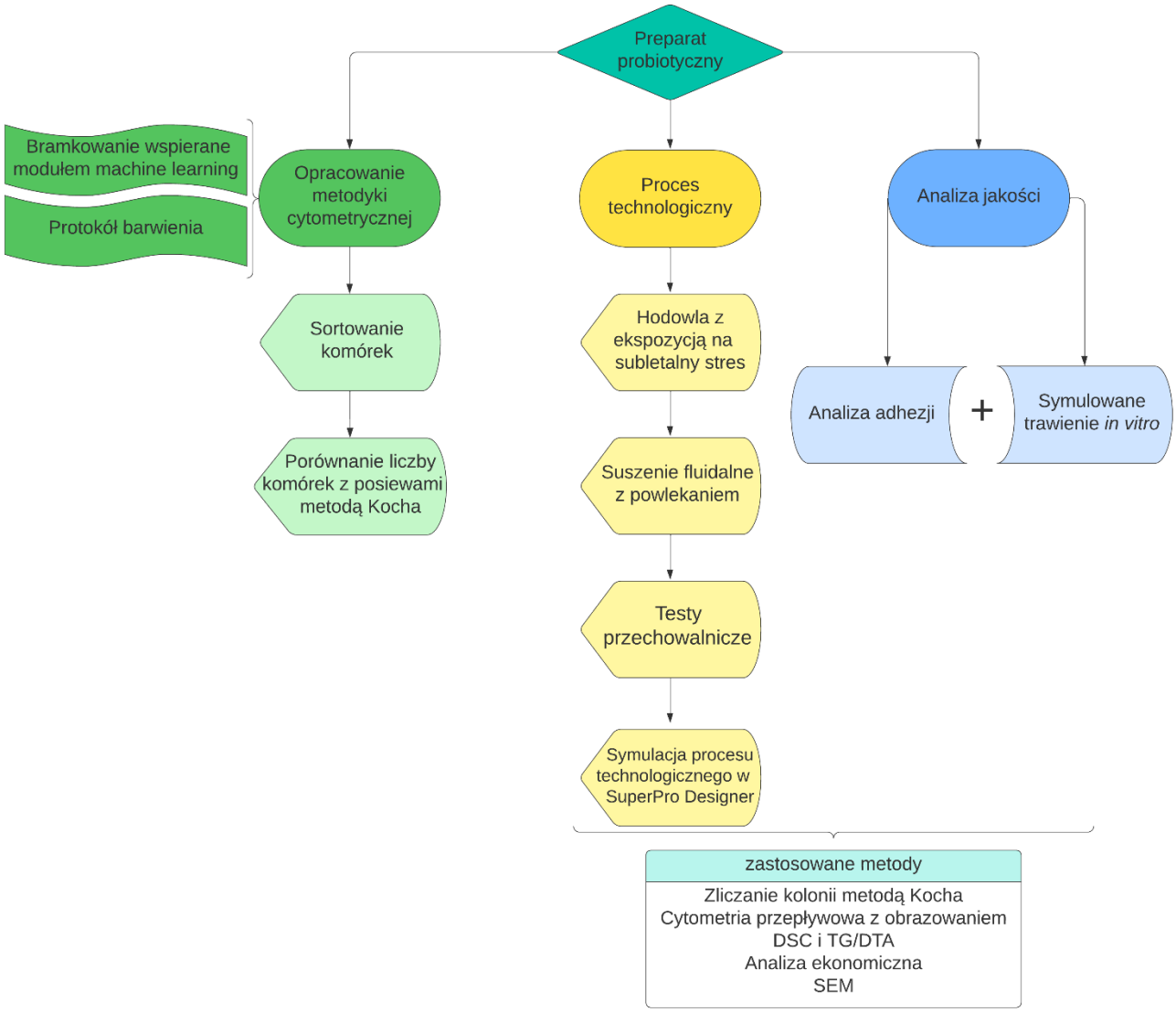
Ponadto zrealizowano dodatkowe cele badawcze, do których należały:

- Ocena wpływu warunków stresowych podczas hodowli na stabilność i żywotność bakterii po suszeniu i podczas przechowywania w różnych warunkach, a także na ich zdolność do adhezji i przeżywalność w symulowanych warunkach przewodu pokarmowego.
- Porównanie posiewów metodą Kocha i cytometrii przepływowej z obrazowaniem jako metod oceny jakości preparatów probiotycznych.
- Pomiar zmian żywotności i trwałości podczas przechowywania w różnych temperaturach i atmosferach ochronnych.
- Określenie temperatury przemiany szklistej wybranych substancji powlekających oraz temperatury denaturacji komórek bakterii za pomocą różnicowej

kalorymetrii skaningowej, oraz analizy termograwimetrycznej z różnicową analizą termiczną.

- Ocena stanu fizjologicznego i aktywności metabolicznej komórek w symulowanych warunkach trawienia.
- Analiza adhezji bakterii do nabłonka jelitowego *in vitro*.
- Przygotowanie projektu procesu produkcji preparatów probiotycznych metodą suszenia fluidalnego wraz z analizą ekonomiczną.

3. Metodologia badań



Rysunek 1. Model badań

3.1. Selekcja szczepów

Jako przedmiot badań wybrano trzy szczepy bakterii fermentacji mlekowej o potencjale probiotycznym i silnych właściwościach przeciwdrobnoustrojowych, tj. *Enterococcus faecium* 73KBiMŻ, *Leuconostoc mesenteroides* 5tKBiMŻ *Carnobacterium divergens* 3cdKBiMŻ. Pierwsze dwa wymienione szczepy zostały wyizolowane z żywności, odpowiednio z gołki owczej otrzymanej metodami gospodarskimi i surowego mleka. Szczep *C. divergens* 3cdKBiMŻ pozyskano natomiast z treści jelitowej prosiąt. Wszystkie wytypowane szczepy są zdeponowane w kolekcji Czystych Kultur Katedry Biotechnologii i Mikrobiologii Żywności Uniwersytetu Przyrodniczego w Poznaniu (KBiMŻ), zidentyfikowane metodami

molekularnymi i dokładnie scharakteryzowane pod kątem bezpieczeństwa zdrowotnego (antybiotykoodporności, syntezy amin biogennych, hemolizyn, występowania wybranych czynników wirulencji, zdolności rozszczepiania soli kwasów żółciowych). Wyniki przeprowadzonych badań w połączeniu z analizą historii stosowania szczepów należących do gatunków, których przedstawicielami są wybrane szczepy, potwierdziły, że nie stanowią one zagrożenia dla zdrowia ludzi.

3.2. Przechowywanie szczepów

Bakterie uzyskane z kolekcji Czystych Kultur Katedry Biotechnologii i Mikrobiologii Żywności Uniwersytetu Przyrodniczego w Poznaniu (KBiMŻ) przechowywane są w kriobankach. Szczepy po inkubacji przenoszone są do 1,5 ml probówek Eppendorf zawierających koraliki do mrożenia kultur. Probówki są następnie oznaczane numerem szczepu, umieszczone w kriopudełku i przechowywane do późniejszego użycia w niskotemperaturowej laboratoryjnej zamrażarce w temperaturze -76°C.

3.3. Przygotowanie inokulum

Aby uzyskać najlepszą wydajność biomasy, inokulum przygotowano w objętości 10% pożywki wprowadzanej do bioreaktora. Dla hodowli o objętości 1 l przygotowanie przebiegało w trzech etapach, ponieważ stopniowe zwiększenie objętości pozwala na lepszą adaptację mikroorganizmów i skraca fazę spoczynkową w trakcie hodowli. Inokulum inkubowano w 30°C kolejno w 1 ml, 10 ml i 100 ml bulionu MRS (po 24h dla każdej objętości). Bulion MRS wybrano do namnażania jako pożywkę zapewniającą optymalne warunki wzrostu dla bakterii kwasu mlekowego.

3.4. Hodowla bioreaktorowa

W bioreaktorze zaszczepionym przygotowanym inokulum hodowlę prowadzono w temperaturze 30 ° C, przy prędkości mieszadła 200 obr./min i pH dostosowanym do optymalnej wartości dla hodowanego szczepu. Objętość hodowli wynosiła 1l, a jako pożywkę zastosowano bulion MRS. Wartość pH monitorowano i regulowano 30% roztworem NaOH. W trakcie hodowli komórki poddane zostały również warunkom stresowym (stres termiczny, kwasowy). Wprowadzenie szoku polega na krótkotrwałej (30 min) zmianie konkretnego parametru hodowli (zwiększenie temperatury lub zmiana pH) w stacjonarnej (szok temperaturowy) lub logarytmicznej (szok pH) fazie wzrostu. Kulturę bakterii prowadzono do osiągnięcia stacjonarnej fazy wzrostu. Jako wyznacznik wskazujący na zatrzymanie wzrostu drobnoustrojów wykorzystano sygnał z elektrody pH. Stabilizacja pH na ustalonym poziomie

przy jednoczesnym braku zużycia zasady wskazuje na koniec wzrostu drobnoustrojów i zahamowanie produkcji zakwaszających metabolitów.

3.5. Wirowanie

Biomasa uzyskana podczas hodowli była odwirowywana w wirówce Hettich Rotanta 460R przez 20 min, z przyspieszeniem 3,478 g w 4°C. Supernatant usuwano, a pozostała biomasa była zawieszana w 0,9% roztworze NaCl i ponownie dwukrotnie zwirowywana, aby usunąć pozostałości pożywki z biomasy komórkowej.

3.6. Suszenie i powlekanie w złożu fluidalnym

Do procesu suszenia w złożu fluidalnym zastosowano laboratoryjną suszarkę GEA Strea-1. W pierwszym kroku matryca była dodawana do zbiornika suszarki. Matrycę dobrano spośród komercyjnie dostępnego granulatu mikrocelulozy krystalicznej Cellets, o średnicy 200-350 μm lub 350-500 μm i jej zamiennika w postaci kaszy manny. Po wprowadzeniu matrycy przeprowadzano etap suszenia - strumień przefiltrowanego powietrza, ogrzewany przez nagrzewnicę do zadanej temperatury (od 35°C do 50°C) przepływa przez perforowaną płytę na dnie komory i pozwala utrzymać matrycę w fazie fluidalnej, co zapewnia równomierne suszenie, aż do uzyskania wymaganej wilgotności. Równolegle wprowadzana jest zawiesina komórek i substancji ochronnej, rozpylana przez dyszę pod ciśnieniem atomizacji regulowanym w zakresie od 0 do 5 bar. Procesy suszenia i powlekania przeprowadzano w dwóch osobnych etapach. Mikroorganizmy po wirowaniu były zawieszane w roztworze substancji ochronnej i podawane do dyszy rozpylającej suszarki za pomocą zewnętrznej pompy perystaltycznej. Następnie w celu naniesienia powłoki na już wysuszony granulat przeprowadzano ponowne suszenie, tym razem z rozpylaniem roztworu powlekającego zamiast zawiesiny komórek.

3.7. Przechowywanie

Gotowy preparat probiotyczny był przechowywany w podwójnie zapakowanych w workach strunowych. Próbki po wysuszeniu przechowywano przez rok w różnych temperaturach (-4°C, 4°C i 20°C) i atmosferach ochronnych (powietrze, próżnia, azot). Takie przechowywanie umożliwiało dalsze testy w celu określenia żywotności i aktywności mikroorganizmów oraz stabilności preparatu w czasie.

3.8. Rehydratacja

Próbki po suszeniu i powlekaniu, które wykorzystywano w kolejnych analizach poddawano rehydratacji. W tym celu umieszczano 1 g preparatu w 9 ml 0,9% NaCl, worteksowano i inkubowano w stałej temperaturze 30°C. Próbki po rehydratacji wirowano celem usunięcia z zawiesiny pozostałości matrycy.

3.9. Oznaczanie liczebności komórek

Liczebność komórek oznaczano przy użyciu klasycznej metody płytkowej w celu ustalenia ilości żywych mikroorganizmów obecnych w próbkach z hodowli i po suszeniu, oraz w celu wykluczenia zakażenia produktu bakteriami innymi niż zastosowany szczep. Były one przeprowadzane w sterylnych warunkach pod komorą laminarną, w dwóch powtórzeniach w celu uzyskania wiarygodnych wyników. Próbki do posiewu przygotowywano metodą rozcieńczeń dziesiętnych, a jako rozcieńczalnik zastosowano 0,9% roztwór soli fizjologicznej. Płytki po posiewie były inkubowane w temperaturze 30°C przez 48 godzin, po czym widoczne kolonie były zliczane. Wyniki oznaczenia uznawano za statystycznie znaczące tylko dla płytek z rozcieńczeń, dla których liczba kolonii wynosiła od 30 do 300. W przeciwnym razie wynik odrzucano. Jako uzupełnienie klasycznej metody płytkowej do zliczania i oceny aktywności metabolicznej komórek zastosowano także cytometrię przepływową z obrazowaniem.

3.10. Cytometria przepływowa i sortowanie komórek

Cytometria przepływowa z obrazowaniem (IFC) to metoda analizy komórek, która była stosowana jako metoda alternatywna dla klasycznych hodowli płytkowych. Komórki bakteryjne badano pod kątem aktywności metabolicznej i żywotności komórek przy użyciu obrazowego cytometru przepływowego Amnis FlowSight™ (Luminex Corp., Austin, Teksas, USA) wyposażonego w trzy lasery (405 nm, 488 nm i 642 nm), pięć kanałów fluorescencyjnych (akwizycja przez wielokanałową kamerę CCD) i detektor rozproszenia bocznego (SSC). Analizę danych po przejęciu przeprowadzono przy użyciu oprogramowania IDEAS (Luminex Corp., Austin, Teksas, USA). Żywotność i aktywność komórek bakterii probiotycznych w próbkach oznaczono poprzez barwienie fluorescencyjne za pomocą zestawu BacLight™ RedoxSensor™ Green Vitality Kit (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA), zawierającego RedoxSensorTM Green i jodek propidyny (PI). Próbki do analizy przygotowano poprzez odwirowanie, a następnie zawieszono w 1% buforze PBS w rozcieńczeniu 1:200. Następnie do próbek dodano następujące barwniki: 1,6 µl RedoxSensorTM Green i 1,2 µl PI. Komórki ze wszystkich grup następnie posortowano przy użyciu sortera komórek BD FACS

Aria™ III (Becton Dickinson, USA) do oddzielnych punktów na płytkach Petriego lub oddzielnych dołków na płytkach 96-dołkowych. Wykorzystano następującą konfigurację sortera: cztery lasery (375 nm, 405 nm, 488 nm i 633 nm), jedenaście detektorów fluorescencji, detektory FSC i detektory SSC; Dysza 70 μm i ciśnienie płynu osłonowego 70 psi (0,483 MPa). W przypadku wzrostu na płytkach Petriego jednostki tworzące kolonie zliczano po 48 i 72 godzinach inkubacji w temperaturze 30°C. W przypadku płytek 96-dołkowych gęstość optyczną przy 600 nm mierzono we wszystkich dołkach po 48 i 72 godzinach.

3.11. DSC i TG/DTA

Temperaturę przemiany szklistej opisuje się jako właściwość materiałów amorficznych, które powstają między innymi przez usunięcie ośrodka rozpraszającego. Zjawisko to występuje w procesie suszenia w złożu fluidalnym, gdzie woda (medium dyspergujące) jest usuwana, a czas suszenia jest niewystarczający do uzyskania krystalizacji, więc wysuszony materiał pozostaje w stanie amorficznym. Po przekroczeniu temperatury krytycznej (temperatury przemiany szklistej) wysuszony materiał zacznie zmieniać swoją strukturę na drodze przejścia fazowego do uzyskania tak zwanego stanu gumowatego. Taka zmiana strukturalna może wpływać na właściwości fizykochemiczne produktu, a także na żywotność suszonych probiotyków. Do analizy zjawiska przemiany szklistej wykorzystano różnicowy kalorymetr skaningowy DSC 8500 (PerkinElmer Inc., Waltham, MA, USA). Urządzenie, wyposażone dodatkowo w Intracooler II i działające pod oprogramowaniem Pyris 10.1, zostało skalibrowane przy użyciu wzorców indu (Tm = 156,60 °C, ΔH = 28,45 J/g, PerkinElmer Inc.) i n-dodekanu (czystość 99,8, Tm = -9,65°C, Merck). Próbki (około 5–6 mg) odważono do 20 μl aluminiowych szalek (PerkinElmer, nr 0219–0062, Waltham, MA, USA) i hermetycznie zamknięto. Analiza temperatury przemiany szklistej obejmowała następujące etapy: (1) przetrzymanie przez 1 min w temperaturze 30°C; oraz (2) ogrzewanie od 30°C do 300°C z szybkością 5°C/min. Odniesieniem było puste, hermetycznie zamknięte naczynko z aluminium. Przemiana szklista, jako przejście fazowe drugiego rzędu, została zidentyfikowana na podstawie zmiany w linii bazowej krzywej pomiarowej i zarejestrowane jako zmiana pojemności cieplnej (ΔCp, J/g °C) w funkcji temperatury. Na podstawie punktu przegięcia krzywej wyznaczono parametr temperatury przemiany szklistej (Tg,°C). Analizę TG/DTA przeprowadzono przy użyciu aparatu STA 449 F5 Jupiter (Netzsch, Selb, Niemcy). W komorze grzewczej najpierw umieszczano 30 mg próbki, którą następnie ogrzewano do temperatury 200°C z szybkością 5°C/min. W trakcie pomiaru rejestrowano zależność między ubytkiem masy a wzrostem temperatury.

3.12. Symulowane trawienie w przewodzie pokarmowym

W celu symulacji warunków występujących w przewodzie pokarmowym i zbadania ich wpływu na suszone fluidalnie preparaty probiotyczne zastosowano badanie z wykorzystaniem modelu przewodu pokarmowego *in vitro*. W oparciu o metodę opisaną przez Minekusa i in. (Minekus et al., 2014) trawienie w symulowanym układzie trawiennym podzielono na fazę żołądkową i jelitową. Nie wykorzystano fazy trawienia w jamie ustnej, gdyż autorzy tej metody uznali ją za opcjonalną. Symulowany płyn żołądkowy (SGF) i symulowany płyn jelitowy (SIF) przygotowano zgodnie z zaleceniami Minekusa i in. z dodatkiem enzymów trawiennych i CaCl2. Zastosowano 1M NaOH i 1M HCl do regulacji pH w obu płynach trawiennych. Do etapu trawienia żołądkowego łączono próbkę po rehydratacji i płyn żołądkowy w stosunku 50:50 (v/v) do końcowej objętości 40 ml. Próbki w trakcie trawienia inkubowano w temperaturze 37°C przez 2 godziny. Po tym etapie próbkę po fazie trawienia w żołądku (40 ml) łączono z 40 ml płynu jelitowego i inkubowano w temperaturze 37°C przez 2 godziny

3.13. Linia komórkowa Caco-2

Caco-2 to linia komórek nabłonkowych wyizolowana z gruczolakoraka okrężnicy. Jedną z unikalnych właściwości linii komórkowej Caco-2 jest jej zdolność do tworzenia otoczki szczoteczkowej z mikrokosmkami. Linię ludzkich komórek nabłonka jelitowego Caco-2 (HTB-37™) uzyskano z American Type Culture Collection (ATCC, Manassas, VA, USA). Komórki hodowano na pożywce Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA) z dodatkiem gentamycyny, 1% endogennych aminokwasów (100X NEAA, Sigma-Aldrich) i 20% płodowej surowicy bydlęcej (FBS, Gibco BRL, Grand Island, Nowy Jork, USA) i utrzymywano w temperaturze 37°C w atmosferze o stałej wilgotności przekraczającej 95% i zawierającej 5% CO2. Komórki Caco-2 umieszczono na membranach PET (wkładki do hodowli komórek Millicell®, średnica 24 mm, wielkość porów 0,4 μm; Millipore, Burlington, MA, USA, Merck Group) przy początkowej gęstości 4×10^5 komórek/cm2 i hodowano przez 21 dni, zmieniając pożywkę trzy razy w tygodniu. Integralność monowarstw komórek Caco-2 monitorowano na podstawie pomiarów przeznabłonkowego oporu elektrycznego (TEER) przy użyciu systemu Millicell Electrical Resistance System (ERS-2, Millipore). W doświadczeniach adhezji bakterii wykorzystano hodowle komórkowe Caco-2 o wartościach TEER $\geq 600\ \Omega \times cm^2$.

3.14. Analiza adhezji

Przed analizą adhezji monowarstwę komórek Caco-2 przemyto dwukrotnie PBS. Następnie dodano pożywkę DMEM (bez czerwieni fenolowej) zawierającą komórki bakteryjne. Tak przygotowane komórki inkubowano przez 2 godziny w temperaturze 37°C. Po inkubacji pożywkę usuwano z komórek nabłonkowych, a monowarstwę komórek delikatnie przemywano PBS celem odmycia nieadherującej frakcji komórek bakterii. Liczbę komórek bakteryjnych określono za pomocą IFC i posiewów na płytkach Petriego.

3.15. Komputerowa symulacja procesu wytwarzania preparatów probiotycznych

Symulację procesu produkcji suszonego fluidalnie preparatu probiotycznego w skali przemysłowej przeprowadzono w programie SuperPro Designer v13 (Intelligen, Scotch Plains, NJ, USA). Kluczowe dla procesu parametry ustalono na podstawie analiz w skali laboratoryjnej. Ustalono również czynniki ekonomiczne, takie jak prognozowany kapitał, koszty operacyjne, koszty surowców i odczynników oraz aparatury. Na podstawie poczynionych założeń przeprowadzono także analizę ekonomiczną procesu.

3.16. Analiza statystyczna

Do opracowania statystycznego oraz wizualizacji wyników wykorzystano język programowania R (wersja 4.3.1.) w środowisku RStudio (Posit, Boston, MA, USA). Wyniki przedstawiane są jako średnie z trzech niezależnych powtórzeń wraz z odchyleniami standardowymi. Do zbadania istotności różnic między próbami wykorzystano jednokierunkową analizę wariancji (ANOVA) oraz porównanie *post hoc* testem Tukeya. Przyjęto poziom istotności $\alpha = 0{,}05$. Dla symulowanego procesu produkcji preparatu probiotycznego dokonano analizy czułości kluczowych czynników (koszt trehalozy, HPMC oraz pożywki i czasy procesów fermentacji, suszenia i powlekania) w programie Crystal Ball w wersji 11.1.2.4 (Oracle, Austin, TX, USA). Założono zakres wahań na poziomie 20% od bazowej wartości dla wymienionych parametrów.

4. Omówienie wyników

4.1. Opracowanie metody oceny jakości preparatów probiotycznych

Cytometria przepływowa z obrazowaniem (IFC) to zaawansowana technika cytometrii przepływowej, która umożliwia analizę morfologii i fizjologii komórek na podstawie cyfrowo przetworzonych obrazów. IFC zapewnia znacznie więcej informacji niż konwencjonalna cytometria przepływowa, ponieważ oprócz intensywności sygnału fluorescencyjnego mierzy również parametry takie jak wielkość komórek, kształt i tekstura. IFC jest szczególnie przydatna do analizy bakterii w preparatach probiotycznych, ponieważ pozwala na dokładne zliczanie bakterii żywych i martwych, a także o pośredniej aktywności metabolicznej i na ocenę ich stanu fizjologicznego. IFC jest również szybsza i bardziej uniwersalna niż liczenie komórek na płytkach, ponieważ nie wymaga stosowania wielu selektywnych podłoży i rozcieńczeń. Cytometria przepływowa pozwala na obserwację komórek *viable but nonculturable* VBNC w preparatach komercyjnych i może być wykorzystana do uzyskania dokładniejszych informacji znacznie szybciej niż klasyczne metody mikrobiologiczne, które wymagają długiego czasu inkubacji. Inne metody stosowane jako alternatywa dla liczenia komórek na płytkach, takie jak fluorescencyjna hybrydyzacja *in situ* i metody zliczania oparte na kwasach nukleinowych, takie jak PCR z odwrotną transkryptazą (RT-PCR) i ilościowa PCR w czasie rzeczywistym (qPCR), są użytecznymi narzędziami do badań i prób klinicznych, nie nadają się jednak do wykorzystania w celu kontroli jakości dla przemysłu. Na tym polega wszechstronność cytometrii przepływowej, którą można wykorzystać do wieloparametrycznej analizy komórek w różnych zastosowaniach, zarówno badawczych, jak i przemysłowych. Publikacja II podsumowuje wyniki analiz cytometrii przepływowej w połączeniu z sortowaniem komórek. Obrazowa cytometria przepływowa (IFC) pozwala na zwiększenie dokładności powszechnie stosowanej cytometrii przepływowej, łącząc jej moc statystyczną z obrazowaniem mikroskopowym w jednym układzie. Tradycyjne cystometria przepływowe zbierają wyłącznie sygnał fluorescencji, rejestrując jego intensywność. IFC zapewnia więcej parametrów, korelując dane dotyczące zmierzonych parametrów światła fluorescencyjnego z danymi cyfrowych obrazów analizowanych komórek. Zastosowanie IFC połączono z wysoce specjalistyczna metodą barwienia z wykorzystaniem RedoxSensorTM Green i jodku propidyny, który okazał się alternatywą dla standardowego barwienia LIVE/DEAD (Żywe/martwe) (Kalyuzhnaya et al., 2008). Pozwoliło to na wykrycie komórek o obniżonej aktywności metabolicznej (komórki viable but nonculturable – VBNC) jako osobnych subpopulacji. Pozwoliło to na oznaczenie większej ilości subpopulacji niż ograniczona

rutynowa dyskryminacja tylko żywych i martwych komórek – oprócz tych dwóch subpopulacji wykryto także subpopulacje mid-active I i mid-active II o różnym poziomie uszkodzenia błony komórkowej i zróżnicowanej aktywności metabolicznej. Subpopulacje o średniej aktywności metabolicznej i pewnym stopniu uszkodzenia komórek odpowiadają opisowi komórek VBNC. Wyniki potwierdzono poprzez sortowanie komórek. Pojedyncze komórki uzyskane z 4 wyznaczonych subpopulacji sortowano do płynnej (bulion MRS) i stałej pożywki (MRS-Agar), a następnie obserwowano zdolność komórek do wzrostu. Umożliwiło to analizę pojedynczej komórka z określonej subpopulacji, a nie całej subpopulacji lub próbki. Potwierdzono również, że czynniki stresowe odpowiadają za różny stopień uszkodzenia błon komórkowych – np. w próbkach po szoku cieplnym (30 min w 50°C) inny był rozkład subpopulacji: 23,87-64,73% komórek aktywnych, 6,12-26,50% komórek martwych, 21,46-33,86% komórek mid-active II i 0,09-1,03% komórek mid-active I (w zależności od testowanego szczepu), niż w próbkach po szoku pH (pH 2,5 przez 30 min), gdzie subpopulacje składały się z: 2,91-9,29% komórek aktywnych, 65,05–83,86% komórek martwych, 8,47–16,54% komórek mid-active II i 1,03–6,63% mid-active I (w zależności od testowanego szczepu). Zarówno subpopulacje mid-active I (o niskiej aktywności metabolicznej i wysokim stopniu uszkodzeń błony komórkowej), jak i mid-active II (o wysokiej średniej aktywności metabolicznej i niskim stopniu uszkodzeń błony komórkowej) wykazywały zdolność do ponownego wzrostu zarówno na podłożu płynnym. Dodatkowo komórki mid-active II wykazywały zdolność do ponownego wzrostu na podłożu stałym. Wyniki cytometrii przepływowej porównano także z wynikami zliczania komórek bakteryjnych metodą płytkową. Opracowana w Publikacji II metoda okazała się przydatna także do oceny uszkodzeń komórek i aktywności metabolicznej w innych próbkach (po adhezji w liniach komórkowych lub symulowanym przejściu przez przewód pokarmowy) i stanowi nowatorski sposób oceny żywotności bakterii probiotycznych.

Na podstawie badań potwierdzono hipotezy:

H.1: Cytometria przepływowa z obrazowaniem pozwala na podział komórek na subpopulacje o zróżnicowanym stanie fizjologicznym (aktywność metaboliczna) i morfologii (stopień uszkodzeń błony komórkowej).

H.2.: Suszenie fluidalne pozwala na uzyskanie preparatu zawierającego rekomendowaną dawkę komórek probiotycznych – na minimalnym poziomie 10^6 komórek na gram preparatu.

Opisano na podstawie Publikacji II.

4.2. Testy przechowalnicze i analiza adhezji

W przedstawionych badaniach opisano również wyniki analizy adhezji komórek probiotycznych w linii komórkowej Caco-2, jak również analizy trwałości preparatów probiotycznych suszonych w złożu fluidalnym i przechowywanych przez 12 miesięcy. Dodatkowo analizujemy za pomocą DSC i TG/DTA właściwości termiczne (temperaturę przemiany szklistej - Tg) substancji stosowanych w powlekaniu w złożu fluidalnym oraz temperatury denaturacji komórek bakterii probiotycznych. Analizy te są poparte obrazową cytometrią przepływową. Komórki, które doznały uszkodzeń błony, podzielono na trzy subpopulacje (mid-active I, mid-active II i martwe). Określono czwartą grupę, zawierającą komórki bez uszkodzeń, i oznaczono ją jako aktywną. Tylko komórki aktywne wykazywały zdolność przylegania do linii komórek nabłonkowych Caco-2, co potwierdzono za pomocą IFC. Wyniki te pokazują, że każde, nawet minimalne uszkodzenie błony komórkowej negatywnie wpływa na zdolność komórki do przylegania do nabłonka. Dzieje się tak nawet wtedy, gdy uszkodzenie błony komórkowej nie prowadzi do śmierci komórki, a komórka zachowuje wysoką aktywność metaboliczną i jest zdolna do regeneracji na tyle, aby utrzymać zdolność do podziału komórek. Do określenia temperatury, w której rozpoczęła się denaturacja w badanych próbkach, która wynosiła od 65 do 70°C, wykorzystano analizę DSC. W przypadku preparatów suszonych analizy termofizyczne wykazały, że substancją powlekającą o najwyższej Tg była HPMC, a najdłuższy okres przydatności do spożycia, bo 12 miesięcy, miały próbki przechowywane w temperaturze -20°C i w modyfikowanej atmosferze. Próbki przechowywane w temperaturze -4 °C zawierały minimalną liczbę mikroorganizmów na poziomie 10^6 komórek na gram preparatu przez 6–9 miesięcy, a jednocześnie były łatwiejsze w przechowywaniu, co potencjalnie czyniło je lepszym wyborem do celów komercyjnych. Porównanie wyników IFC i posiewów na szalkach Petriego wykazało również, że subpopulacja mid-active I nie była wykrywana metodą posiewów. Komórki o średniej aktywności i niskim poziomie uszkodzeń komórkowych mogą nadal wywierać korzystne skutki zdrowotne, pomimo ograniczonej zdolności do adhezji. Powszechnie stosowane klasyczne metody mikrobiologiczne nie są jednak w stanie wykryć tej ważnej subpopulacji, którą można uwzględnić w ogólnej liczbie żywych komórek w celu oceny probiotyków.

Na podstawie badań potwierdzono hipotezy:

H.1: Cytometria przepływowa z obrazowaniem pozwala na podział komórek na subpopulacje o zróżnicowanym stanie fizjologicznym (aktywność metaboliczna) i morfologii (stopień uszkodzeń błony komórkowej).

H.3.: Stopień uszkodzeń błony komórkowej istotnie wpływa na zdolność adhezji bakterii probiotycznych do komórek nabłonka jelitowego.

H.4: Zastosowanie warunków stresowych w trakcie hodowli bakterii pozwala na zwiększenie przeżywalności komórek w preparacie probiotycznym w trakcie jego utrwalania i przechowywania.

H.5: Proces technologiczny i warunki przechowywania wpływają na trwałość preparatu w trakcie długotrwałego przechowywania.

Opisano na podstawie Publikacji III.

4.3. Trawienie *in vitro*, projekt technologiczny i analiza ekonomiczna

Dalszy ciąg badań skupia się na trawieniu *in vitro* suszonych w złożu fluidalnym i powlekanych preparatów probiotycznych, również po obróbce stresem cieplnym i pH w fazie hodowli. Analizy te są poparte obrazową cytometrią przepływową. Wyniki tych analiz wykorzystywano do zaprojektowania i symulacji procesu suszenia przy użyciu oprogramowania SuperPro Designer. Projekt zapewnia zwięzły opis procesu suszenia w złożu fluidalnym probiotyków. Wprowadza także nową metodę oceny probiotyków, opartą na rzeczywistej liczbie biologicznie użytecznych komórek. Współczynnik ten służy do oceny i ekonomicznego uzasadnienia wprowadzenia procedur technologicznych, takich jak subletalne stresy w trakcie hodowli i powlekanie. Projektowany proces obejmuje trzy główne etapy produkcji probiotyków: przygotowanie pożywki, inokulacja i namnażanie biomasy oraz suszenie i powlekanie w złożu fluidalnym. Obliczenia ekonomiczne przeprowadzane dla procesu uwzględniają koszt surowców, utylizację odpadów, zużycie energii i nośników ciepła, koszt sprzętu oraz bezpośredni kapitał trwały. Publikacja IV skupia się na ocenie procesów technologicznych, takich jak powlekanie i dodatkowe stresy, w oparciu nie tylko o ogólną liczbę komórek probiotycznych w produkcie końcowym, ale przede wszystkim w oparciu o liczbę komórek, które zachowują właściwości probiotyczne nawet po trawieniu *in vitro* lub długotrwałym (12 miesięcy) przechowywaniu.

Na podstawie badań potwierdzono hipotezy:

H.4: Zastosowanie warunków stresowych w trakcie hodowli bakterii pozwala na zwiększenie przeżywalności komórek w preparacie probiotycznym w trakcie jego utrwalania i przechowywania.

H.6: Realne oszacowanie kosztu wytworzenia preparatu probiotycznego wymaga uwzględnienia trawienia.

Opisano na podstawie Publikacji IV.

5. Wnioski

- Suszenie fluidalne jest metodą pozwalającą na uzyskanie kapsułkowanych preparatów probiotycznych zawierających co najmniej 10^6 komórek na gram.

- Wprowadzenie szoku (termicznego lub kwasowego) ma korzystny wpływ na przeżywalność komórek bakteryjnych na dalszych etapach procesu technologicznego, a także podczas symulowanego trawienia *in vitro*.

- Powlekanie ma istotny wpływ na stabilność uzyskanych preparatów i przeżywalność komórek po procesie suszenia.

- W preparacie probiotycznym wyróżnia się cztery subpopulacje komórek o zróżnicowanym poziomie uszkodzeń błony komórkowej i aktywności metabolicznej, mianowicie żywe – o dużej aktywności metabolicznej, mid-active I – o niskiej aktywności metabolicznej i znacznym stopniu uszkodzeń błony komórekowej, mid-active II – o dużej aktywności metabolicznej i niskim stopniu uszkodzeń błony komórkowej, oraz martwe – bez aktywności metabolicznej i o znacznym stopniu uszkodzeń. Różnice między subpopulacjami są wynikiem stresów na etapie hodowli oraz występujących w trakcie procesu technologicznego.

- Cytometria przepływowa z obrazowaniem jest metodą umożliwiającą wykrycie komórek VBNC w preparatach probiotycznych.

- Zastosowanie cytometrii przepływowej pozwala na dokładne oznaczenie liczby bakterii w preparacie probiotycznym, z uwzględnieniem komórek VBNC i o pośredniej aktywności metabolicznej.

- Barwienie z wykorzystaniem RedoxSensor Green i jodku propidyny pozwala na dokonanie oceny morfologii oraz fizjologii Gram-dodatnich komórek bakteryjnych.

- Sortowane pojedynczo komórki, należące do subpopulacji o pośredniej aktywności metabolicznej wykazują zdolność do regeneracji i wznowienia wzrostu w optymalnych warunkach zarówno w podłożu płynnym jak i stałym.

- Uszkodzenia komórek ograniczają zdolność adhezji badanych bakterii probiotycznych do komórek linii Caco-2.

- Przechowywanie preparatów w temperaturze -20°C i w zmodyfikowanej atmosferze ochronnej zapewniało ich najlepszą trwałość w trakcie długotrwałego (do 12 miesięcy) przechowywania.

- Komórki o pośredniej aktywności stanowią najbardziej liczną subpopulację w preparatach po procesie suszenia oraz symulowanym trawieniu *in vitro*.

6. Źródła finansowania

Badania zrealizowane w ramach niniejszej pracy doktorskiej oraz powstałe w ich wyniku publikacje były współfinansowane ze środków:

- statutowych Katedry Biotechnologii i Mikrobiologii Żywności Uniwersytetu Przyrodniczego w Poznaniu;
- programu Ministerstwa Nauki i Szkolnictwa Wyższego „Regionalna Inicjatywa Doskonałości" (nr 005/RID/2018/19);
- „Zintegrowanego Programu Uniwersytetu Przyrodniczego w Poznaniu na rzecz Innowacyjnej Wielkopolski" (nr POWR.03.05.00- 00-ZR42/18);
- projektu Młoda Kadra Uniwersytetu Przyrodniczego w Poznaniu

7. Spis literatury źródłowej

Ananta, E., Volkert, M., & Knorr, D. (2005). Cellular injuries and storage stability of spray-dried Lactobacillus rhamnosus GG. *International Dairy Journal*, *15*(4), 399–409. https://doi.org/10.1016/j.idairyj.2004.08.004

Arepally, D., & Goswami, T. K. (2019). Effect of inlet air temperature and gum Arabic concentration on encapsulation of probiotics by spray drying. *LWT*, *99*, 583–593. https://doi.org/10.1016/j.lwt.2018.10.022

Assari, M. R., Basirat Tabrizi, H., & Najafpour, E. (2013). Energy and exergy analysis of fluidized bed dryer based on two-fluid modeling. *International Journal of Thermal Sciences*, *64*, 213–219. https://doi.org/10.1016/j.ijthermalsci.2012.08.020

Avila-Reyes, S. V., Garcia-Suarez, F. J., Jiménez, M. T., San Martín-Gonzalez, M. F., & Bello-Perez, L. A. (2014). Protection of L. rhamnosus by spray-drying using two prebiotics colloids to enhance the viability. *Carbohydrate Polymers*, *102*, 423–430. https://doi.org/10.1016/j.carbpol.2013.11.033

Broeckx, G., Vandenheuvel, D., Claes, I. J. J., Lebeer, S., & Kiekens, F. (2016). Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. *International Journal of Pharmaceutics*, *505*(1–2), 303–318. https://doi.org/10.1016/j.ijpharm.2016.04.002

Bron, P. A., Kleerebezem, M., Brummer, R.-J., Cani, P. D., Mercenier, A., MacDonald, T. T., Garcia-Ródenas, C. L., & Wells, J. M. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *British Journal of Nutrition*, *117*(1), 93–107. https://doi.org/10.1017/S0007114516004037

Bucka-Kolendo, J., & Sokołowska, B. (2017). Lactic acid bacteria stress response to preservation processes in the beverage and juice industry. *Acta Biochimica Polonica*, *64*(3), 459–464. https://doi.org/10.18388/abp.2017_1496

Chang, Y. H., Jeong, C. H., Cheng, W. N., Choi, Y., Shin, D. M., Lee, S., & Han, S. G. (2021). Quality characteristics of yogurts fermented with short-chain fatty acid-producing probiotics and their effects on mucin production and probiotic adhesion onto human colon epithelial cells. *Journal of Dairy Science*, *104*(7), 7415–7425. https://doi.org/10.3168/jds.2020-19820

Fiore, W., Arioli, S., & Guglielmetti, S. (2020). The Neglected Microbial Components of Commercial Probiotic Formulations. *Microorganisms*, *8*(8), 1177. https://doi.org/10.3390/microorganisms8081177

Fu, N., & Chen, X. D. (2011). Towards a maximal cell survival in convective thermal drying processes. *Food Research International*, *44*(5), 1127–1149. https://doi.org/10.1016/j.foodres.2011.03.053

Gill, H., & Prasad, J. (2008). Probiotics, Immunomodulation, and Health Benefits. *Bioactive Components of Milk*, *606*, 423–454. https://doi.org/10.1007/978-0-387-74087-4_17

Gorreja, F., & Walker, W. A. (2022). The potential role of adherence factors in probiotic function in the gastrointestinal tract of adults and pediatrics: A narrative review of experimental and human studies. *Gut Microbes*, *14*(1), 2149214. https://doi.org/10.1080/19490976.2022.2149214

Hathi, Z., Mettu, S., Priya, A., Athukoralalage, S., Lam, T. N., Choudhury, N. R., Dutta, N. K., El-Omar, E. M., Gong, L., Mohan, G., & Lin, C. S. K. (2021). Methodological advances and challenges in probiotic bacteria production: Ongoing strategies and future perspectives. *Biochemical Engineering Journal*, *176*, 108199. https://doi.org/10.1016/j.bej.2021.108199

Huang, S., Vignolles, M.-L., Chen, X. D., Le Loir, Y., Jan, G., Schuck, P., & Jeantet, R. (2017). Spray drying of probiotics and other food-grade bacteria: A review. *Trends in Food Science & Technology*, *63*, 1–17. https://doi.org/10.1016/j.tifs.2017.02.007

Jessie Lau, L. Y., & Chye, F. Y. (2018). Antagonistic effects of Lactobacillus plantarum 0612 on the adhesion of selected foodborne enteropathogens in various colonic environments. *Food Control*, *91*, 237–247. https://doi.org/10.1016/j.foodcont.2018.04.001

Joint FAO WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food (Ed.). (2006). *Probiotics in food: Health and nutritional properties and guidelines for evaluation ; report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina, 1 - 4 October 2001 ; report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, 30 April - 1 May 2002*. Food and Agriculture Organization of the United Nations [u.a].

Kalyuzhnaya, M. G., Lidstrom, M. E., & Chistoserdova, L. (2008). Real-time detection of actively metabolizing microbes by redox sensing as applied to methylotroph populations in Lake Washington. *The ISME Journal*, *2*(7), 696–706. https://doi.org/10.1038/ismej.2008.32

Kerry, G. R., Patra, J. K., Gouda, S., Park, Y., Shin, H.-S., & Das, G. (2018). Benefaction of probiotics for human health: A review. *Journal of Food and Drug Analysis*, *26*(3), 927–939. https://doi.org/10.1016/j.jfda.2018.01.002

Kiepś, J., & Dembczyński, R. (2022). *Current Trends in the Production of Probiotic Formulations*. 20.

Kiepś, J., Juzwa, W., & Dembczyński, R. (2023). Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups. *International Journal of Molecular Sciences*, *24*(7), Article 7. https://doi.org/10.3390/ijms24076841

Kiepś, J., Juzwa, W., Olejnik, A., Sip, A., Tomaszewska-Gras, J., & Dembczyński, R. (2023). The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line. *Nutrients*, *15*(15), 3484. https://doi.org/10.3390/nu15153484

Lopez-Santamarina, A., Gonzalez, E. G., Lamas, A., Mondragon, A. del C., Regal, P., & Miranda, J. M. (2021). Probiotics as a Possible Strategy for the Prevention and Treatment of Allergies. A Narrative Review. *Foods*, *10*(4), 701. https://doi.org/10.3390/foods10040701

Mehanna, M. M., & Abla, K. K. (2022). Recent advances in freeze-drying: Variables, cycle optimization, and innovative techniques. *Pharmaceutical Development and Technology*, *27*(8), 904–923. https://doi.org/10.1080/10837450.2022.2129385

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., … Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food Funct.*, *5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Mohanty, D., Panda, S., Kumar, S., & Ray, P. (2019). In vitro evaluation of adherence and anti-infective property of probiotic Lactobacillus plantarum DM 69 against Salmonella enterica. *Microbial Pathogenesis*, *126*, 212–217. https://doi.org/10.1016/j.micpath.2018.11.014

Mohideen Batcha, M. F., Amirnordin, S. H., & Md Yudin, A. S. (2023). 4—Fluidized bed dryers. In S. M. Jafari & N. Malekjani (Eds.), *Drying Technology in Food Processing* (pp. 67–122). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-819895-7.00006-7

Monteagudo-Mera, A., Rastall, R. A., Gibson, G. R., Charalampopoulos, D., & Chatzifragkou, A. (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. *Applied Microbiology and Biotechnology*, *103*(16), 6463–6472. https://doi.org/10.1007/s00253-019-09978-7

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods*, *66*(2), 183–193. https://doi.org/10.1016/j.mimet.2006.02.017

Perdana, J., Fox, M. B., Siwei, C., Boom, R. M., & Schutyser, M. A. I. (2014). Interactions between formulation and spray drying conditions related to survival of Lactobacillus plantarum WCFS1. *Food Research International*, *56*, 9–17. https://doi.org/10.1016/j.foodres.2013.12.007

Safeer Abbas, M., Afzaal, M., Saeed, F., Asghar, A., Jianfeng, L., Ahmad, A., Ullah, Q., Elahi, S., Ateeq, H., Shah, Y. A., Nouman, M., & Shah, M. A. (2023). Probiotic viability as affected by encapsulation materials: Recent updates and perspectives. *International Journal of Food Properties*, *26*(1), 1324–1350. https://doi.org/10.1080/10942912.2023.2213408

Sanders, M. E., Merenstein, D., Merrifield, C. A., & Hutkins, R. (2018). Probiotics for human use. *Nutrition Bulletin*, *43*(3), 212–225. https://doi.org/10.1111/nbu.12334

Santivarangkna, C., Kulozik, U., & Foerst, P. (2008). Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. *Journal of Applied Microbiology*, *105*(1), 1–13. https://doi.org/10.1111/j.1365-2672.2008.03744.x

Walsham, A. D. S., MacKenzie, D. A., Cook, V., Wemyss-Holden, S., Hews, C. L., Juge, N., & Schüller, S. (2016). Lactobacillus reuteri Inhibition of Enteropathogenic Escherichia coli Adherence to Human Intestinal Epithelium. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.00244

Wang, T., Teng, K., Liu, G., Liu, Y., Zhang, J., Zhang, X., Zhang, M., Tao, Y., & Zhong, J. (2018). Lactobacillus reuteri HCM2 protects mice against Enterotoxigenic Escherichia coli through modulation of gut microbiota. *Scientific Reports*, *8*(1), Article 1. https://doi.org/10.1038/s41598-018-35702-y

Yang, X., Wang, C., Wang, Q., Zhang, Z., Nie, W., & Shang, L. (2023). Armored probiotics for oral delivery. *Smart Medicine*. https://doi.org/10.1002/SMMD.20230019

8. Załączniki

Oświadczenie o współautorstwie

Niniejszym oświadczamy, że jesteśmy współautorami pracy „Current Trends in the Production of Probiotic Formulations" opublikowanej w czasopiśmie *Foods*.

Jednocześnie wyrażamy zgodę na wykorzystanie przez mgr inż. Jakuba Kiepś ww. publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk rolniczych w dyscyplinie technologia żywności i żywienia.

Indywidualny udział w powstawaniu pracy polegał na:

Mgr inż. Jakub Kiepś: autorstwo koncepcji; opracowanie przeglądu literatury; opracowanie grafik; opracowanie manuskryptu; pozyskanie finansowania	67%	..... (podpis)
Dr hab. Radosław Dembczyński: autorstwo koncepcji; pozyskanie finansowania; nadzór	33%	..... (podpis)

Oświadczenie o współautorstwie

Niniejszym oświadczamy, że jesteśmy współautorami pracy „Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups" opublikowanej w czasopiśmie *International Journal of Molecular Sciences*.

Jednocześnie wyrażamy zgodę na wykorzystanie przez mgr inż. Jakuba Kiepś ww. publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk rolniczych w dyscyplinie technologia żywności i żywienia.

Indywidualny udział w powstawaniu pracy polegał na:

Mgr inż. Jakub Kiepś: autorstwo koncepcji; opracowanie metodyki; przeprowadzenie badań; analiza wyników; zarządzanie danymi; interpretacja wyników i sformułowanie wniosków; przygotowanie manuskryptu; poprawki i edycja; opracowanie grafik; pozyskanie finansowania	67%	J. Kiepś ..... (podpis)
Prof. UPP, dr hab. inż. Wojciech Juzwa: autorstwo koncepcji; opracowanie metodyki	13%	..... (podpis)
Dr hab. Radosław Dembczyński: autorstwo koncepcji; opracowanie metodyki; nadzór	20%	..... (podpis)

Oświadczenie o współautorstwie

Niniejszym oświadczamy, że jesteśmy współautorami pracy „The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line" opublikowanej w czasopiśmie *Nutrients*.

Jednocześnie wyrażamy zgodę na wykorzystanie przez mgr inż. Jakuba Kiepś ww. publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk rolniczych w dyscyplinie technologia żywności i żywienia.

Indywidualny udział w powstawaniu pracy polegał na:

Mgr inż. Jakub Kiepś: autorstwo koncepcji; opracowanie metodyki; przeprowadzenie badań; analiza wyników; zarządzanie danymi; interpretacja wyników i sformułowanie wniosków; przygotowanie manuskryptu; poprawki i edycja; opracowanie grafik	60%	..... (podpis)
Prof. UPP, dr hab. inż. Wojciech Juzwa: opracowanie metodyki	6%	..... (podpis)
Prof. UPP, dr hab. Anna Olejnik: opracowanie metodyki	7%	..... (podpis)
Prof. UPP, dr hab. Anna Sip: opracowanie metodyki	7%	..... (podpis)
Prof. UPP, dr hab. Jolanta Tomaszewska-Gras: opracowanie metodyki	7%	..... (podpis)
Dr hab. Radosław Dembczyński: opracowanie metodyki; nadzór	13%	..... (podpis)

Oświadczenie o współautorstwie

Niniejszym oświadczamy, że jesteśmy współautorami pracy „Economic Analysis of the Production Process of Probiotics Based on the Biological and Physiological Parameters of the Cells" opublikowanej w czasopiśmie *Applied Sciences*.

Jednocześnie wyrażamy zgodę na wykorzystanie przez mgr inż. Jakuba Kiepś ww. publikacji w postępowaniu o nadanie stopnia doktora w dziedzinie nauk rolniczych w dyscyplinie technologia żywności i żywienia.

Indywidualny udział w powstawaniu pracy polegał na:

Mgr inż. Jakub Kiepś: autorstwo koncepcji; opracowanie metodyki; przeprowadzenie badań; analiza wyników; zarządzanie danymi; interpretacja wyników i sformułowanie wniosków; przygotowanie manuskryptu; poprawki i edycja; opracowanie grafik	60%	J. Kiep ..... (podpis)
Prof. UPP, dr hab. Anna Olejnik: opracowanie metodyki; materiały i odczynniki	13%	A Olejnik ..... (podpis)
Prof. UPP, dr hab. inż. Wojciech Juzwa: opracowanie metodyki; materiały i odczynniki	13%	..... (podpis)
Dr hab. Radosław Dembczyński: opracowanie metodyki; nadzór	14%	..... (podpis)

foods

MDPI

Review

Current Trends in the Production of Probiotic Formulations

Jakub Kiepś and Radosław Dembczyński *

Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland
* Correspondence: radoslaw.dembczynski@up.poznan.pl

Abstract: Preparations containing probiotic strains of bacteria have a beneficial effect on human and animal health. The benefits of probiotics translate into an increased interest in techniques for the preservation of microorganisms. This review compares different drying methods and their improvements, with specific reference to processing conditions, microorganisms, and protective substances. It also highlights some factors that may influence the quality and stability of the final probiotic preparations, including thermal, osmotic, oxidative, and acidic stresses, as well as dehydration and shear forces. Processing and storage result in the loss of viability and stability in probiotic formulations. Herein, the addition of protective substances, the optimization of process parameters, and the adaptation of cells to stress factors before drying are described as countermeasures to these challenges. The latest trends and developments in the fields of drying technologies and probiotic production are also discussed. These developments include novel application methods, controlled release, the use of food matrices, and the use of analytical methods to determine the viability of probiotic bacteria.

Keywords: lactic acid bacteria; spray drying; freeze drying; vacuum drying; fluid bed drying; viability; shelf-life; stress factors; protectants

Citation: Kiepś, J.; Dembczyński, R. Current Trends in the Production of Probiotic Formulations. *Foods* **2022**, *11*, 2330. https://doi.org/10.3390/foods11152330

Academic Editors: Celia C. G. Silva and Susana C. Ribeiro

Received: 27 June 2022
Accepted: 2 August 2022
Published: 4 August 2022

1. Introduction

Concentrated probiotic bacteria used in animal nutrition and consumed by humans most commonly occur in the form of dried biomass. Most probiotic bacteria belong to the group of lactic acid bacteria with GRAS status. Probiotic preparations come in various forms: capsules, suspensions, powders, and combined into probiotic food. All of these forms could be further improved as they share the issue of a loss of viability during both processing and storage. For example, fluid suspensions, however relatively easy to produce, are the least stable form of probiotics with the shortest shelf-life [1]. Probiotics in solid forms, such as capsules and powders, are more stable and can be stored for a longer period of time than fluid suspensions. Their viability and shelf-life could be further improved by the addition of protective substances or by the introduction of stress factors to probiotic bacteria prior to their drying. All probiotics contain live bacteria, mainly from the genera *Lactobacillus* and *Bifidobacterium* [2]. The results of clinical trials show the positive effects of taking probiotics on diseases of the gastrointestinal tract, including irritable bowel syndrome, diarrhea, enteritis, and allergic conditions, such as atopic dermatitis. Probiotics have also been shown to increase the body's immune resistance through immunomodulation [3]. As described by the WHO, to have a beneficial health effect, probiotic preparations should contain a minimum number of live bacteria (colony-forming units), i.e., at least 10^6 cfu/g. The qualitative parameters of dry cells (the number of live cells and their biological activity) usually depend on the applied drying method. These methods include spray drying, freeze drying, vacuum drying, and fluid bed drying. The obtained probiotic formulations can then be used in a variety of novel formulations, such as nasal sprays, creams, and lotions [4,5]. They are also used in food matrices to enrich

their beneficial health effects. Some food matrices, e.g., ice cream, are also used to improve the stability and shelf-life of probiotics [6].

2. Drying Methods

Cryopreservation is one of the most commonly used methods of preserving and storing live cultures of microorganisms for a long time; this approach is used in microbiological laboratories. From a commercial point of view, this method has disadvantages, which include high levels of energy consumption and the need to maintain and transport the samples at temperatures below zero. In addition, freezing and thawing the cells of microorganisms can damage them. When large amounts of probiotic cultures are produced, it is preferred to use other methods of preservation, such as different drying techniques [7]. The basic principles of these drying techniques are summarized in Figure 1. Examples of the viability of some probiotic strains dried by different methods are shown in Table 1, and the protective effects of different materials are summarized in Table 2.

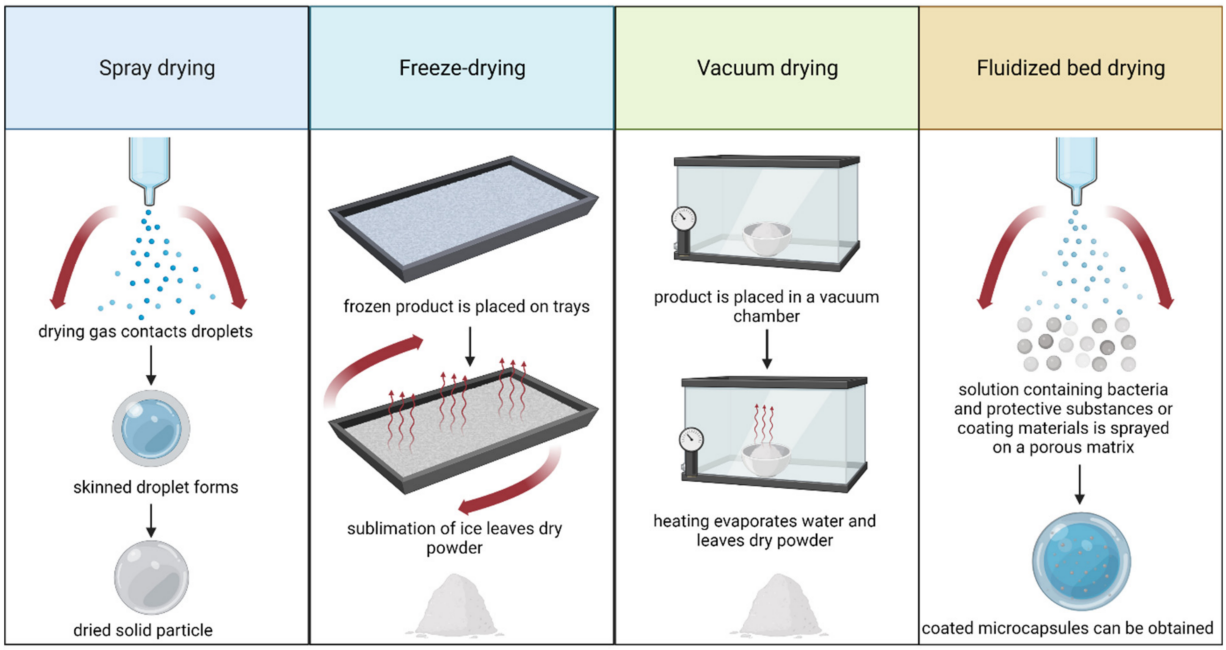


Figure 1. Summary of the basic principles for the most commonly applied drying techniques. Created with Biorender.com (accessed on 27 July 2022).

2.1. Traditional Drying Methods

2.1.1. Spray Drying

Spray drying is a fast and relatively inexpensive technique that makes it possible to obtain dry, mostly spherical powder particles with good flow properties, uniform shape, and particle size distribution [8]. The drying process occurs in four stages. In the first stage, the microorganism suspension is sprayed into small droplets. Then, the droplets are carried by hot air, with three different methods for the air to contact the droplets: co-current, counter-current, and mixed [9]. Because probiotic bacteria are sensitive to high temperatures, the co-current flow is usually applied. In this way, drops with a high water content contact the high-temperature inlet air, and the dry particles contact the lowest-temperature exhaust air, which reduces the risk of damage to microorganisms. For example, in previous works, inlet air temperatures for the spray drying of *L. rhamnosus* were 130–150 °C [10]; for probiotic almond milk powder containing *L. plantarum*, temperatures of 170–190 °C were used [11]. The third stage of the spray-drying process is the drying of the droplets and the formation of dry particles. It is at this stage that the microorganisms are most susceptible to thermal inactivation [12].

In comparison with other methods, spray drying has several advantages. These include a short drying time, the capacity for continuous operation, and low cost; these factors translate into the possibility of drying large volumes of suspension in a relatively short time. Additionally, it is possible to influence the characteristics of the powder, and the process is relatively easy to scale up [13].

Spray freeze drying is a method that combines the advantages of spray drying and freeze drying. It is conducted in three stages: the atomization of a cell suspension in a spray dryer, freezing (over liquid nitrogen), and freeze drying. In this process, the cells are first atomized with the addition of a protective material (such as WPI) over liquid nitrogen, which allows the droplets to quickly freeze. They are then additionally freeze dried. Studies conducted using spray freeze drying showed an encapsulation efficiency of 88–95% for *L. plantarum* MTCC 5422 with various wall materials. The overall encapsulation efficiency was, however, lower than in regular freeze drying due to the additional stress factors that occurred during atomization and freezing [14].

2.1.2. Freeze Drying

Freeze drying, or lyophilization, is a common method of removing water from probiotic bacterial cells to ensure their storage stability. The dryer consists of a vacuum chamber with a freezing system, a system for removing water vapor, and heating elements that are necessary to supply heat for sublimation. The freeze-drying process occurs in three stages: the freezing of the cell culture, sublimation, and final drying, the first of which is often carried out outside the dryer. In the next stage of the process, the frozen water is removed in the sublimation process under reduced pressure, and in the last stage, non-frozen water is removed in the process of desorption in order to attain the final water content [15]). Because the conditions of the freeze-drying process are milder than those of the spray-drying process, probiotic cultures dried by this method usually show higher rates of survival [16].

Despite the frequent use of this method of drying microorganisms, including probiotics, freeze drying has several disadvantages. It is an expensive and lengthy batch process, and the final product is often compact and hard. Regardless of this, freeze drying is a useful and widely used drying technique with several strategies already developed to maximize the viability of probiotic cultures. Among such modifications is pulse-spouted microwave freeze drying [17,18]. It aims to shorten the drying time in comparison with the traditional variant of freeze drying.

2.1.3. Vacuum Drying

A vacuum dryer consists of a chamber in which heated shelves are located. Trays containing wet biomass are placed on the shelves, and the water vapor is removed using a vacuum pump and condensed in a condenser. During freeze drying, the cells are frozen before the water is removed, while in vacuum drying, they remain in liquid form. Moreover, vacuum dryers operate at a higher temperature and pressure, and the energy consumption is 40% lower compared to freeze drying [19]. Typical pressures for vacuum drying are above 30–60 mbar, which corresponds to a boiling point of water of 25–30 °C; for freeze drying, the pressure is lower than 6 mbar [7,20]. The main disadvantage of vacuum drying, compared to spray or fluid bed drying, is the long processing time, ranging from 20 to 100 h [1].

New developments in the field of vacuum drying include the use of pulse-spouted microwave vacuum drying (PSMVD) [17]. Banana cubes dried by PSMVD showed an expansion trend, resulting in a better structure and rehydration ratio. PSMVD-dried cubes also provided better nutritional value as measured by the content of ascorbic acid, which reached 7.96 mg 100 g^{-1} (compared to 4.23 mg 100 g^{-1} for the traditional variant of vacuum drying).

2.1.4. Fluidized Bed Drying

Fluidized bed drying is a process in which heated gas, usually air, flows at a certain speed through a layer of solid particles, causing them to reach a fluidized flow state.

Because the fluidization process has very good heat and mass exchange conditions, water is quickly evaporated from the dried material. The time required for fluidized bed drying (1 min to 2 h) is shorter than that of freeze drying and comparable to that of spray drying. The relatively low drying temperature does not cause thermal stress [1]. The cell biomass is not dried on its own but mixed with additional material that acts as a carrier or matrix to which the cells adhere. In practice, many loose and powdered materials have been used for this purpose, such as wheat flour, skimmed milk powder, casein, maltodextrin, starch, microcrystalline cellulose, inulin, and NaCl [1,21–23]. Usually, the matrix material is first placed in the dryer chamber and fluidized; then, the bacterial suspension is sprayed onto the fluidized matrix via a nozzle. Another method is to prepare the granulate first; after mixing the wet biomass with the matrix material and forming the granules using a sieve, pellet mill, or drum granulator, they are then dried in a fluidized bed [24,25]. It is important to consider the purpose of the dried bacterial product when selecting the matrix material as it can have variable technological characteristics or provide additional properties (e.g., prebiotic inulin) [21].

2.2. Novel Immobilization Methods

New methods of probiotic cell immobilization are also emerging as an alternative to drying processes and microencapsulation. Škrlec et al. [26]. have developed two types of electrospun nanofibers (Figure 2): monolithic poly(ethylene oxide) and composite poly(ethylene oxide)/lyoprotectant. *L. plantarum* cells were applied to these nanofibers and achieved high cell concentrations (up to 7.6×10^8 cfu/mg). Moreover, their survival during storage at 25 °C was promising, with a 1.83 log decrease in viability over 24 weeks (from 8.51 to 6.68 cfu/mg). For comparison, lyophilized samples showed a 1.70 log decrease in viability over the same period (from 8.97 to 7.27 cfu/mg). The differences between nanofibers and lyophilized samples stored at 4 °C were also minimal. The release time was also measured. Nearly all (>90%) *L. plantarum* cells were released from the nanofibers within the first 30 min of the experiment. This was confirmed by both plate cell counts and fluorescence measurements of the mCherry protein and provided insight into possible applications of probiotic-loaded nanofibers. The 30 min release period allowed for the controlled delivery of probiotic bacteria and was sufficient for their adhesion to the intestinal epithelium and mucosa.

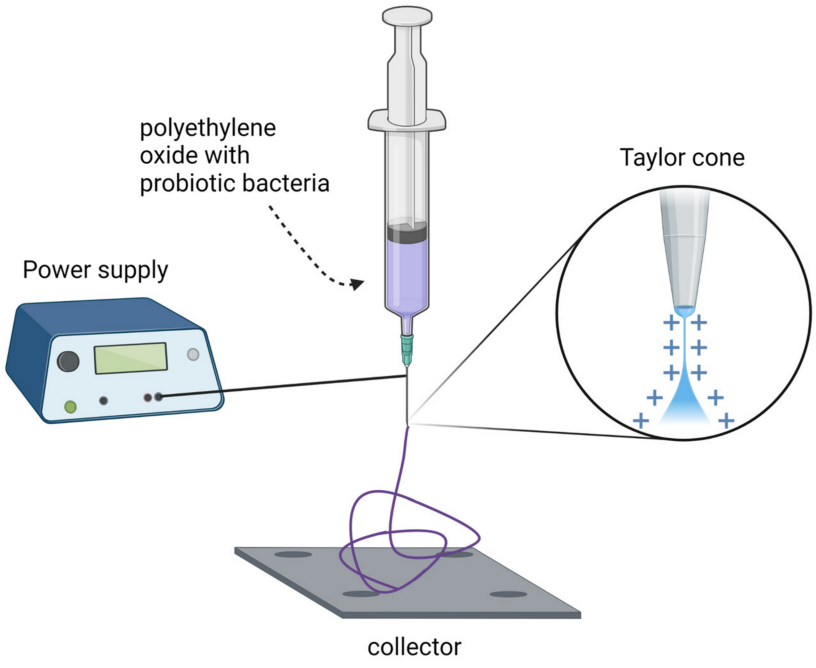


Figure 2. Principal elements of nanofiber fabrication by electrospinning. Created with Biorender.com (accessed on 1 June 2022).

3D printing is a novel method used in food industry applications that allows for the design of customized products. Yoha et al. [27] studied the effects of the 3D printing of probiotic encapsulates on their viability. To prepare the base for the 3D printing of *L. plantarum* (NCIM 2083), cells were dried using four encapsulation techniques (spray drying, freeze drying, spray freeze drying, and refractance window drying) with the addition of a prebiotic matrix (fructooligosaccharide, whey protein, and maltodextrin in a ratio 4:1:1, respectively). Dried microcapsules were then used for 3D printing with a composite flour formulation using the food 3D printer CARK [28]. Yoha et al. (2021) reported that the 3D printing process did not lower the viability of probiotic bacteria. They also determined that freeze drying yielded the highest level of cell viability, i.e., 8.23 ± 0.21 $\log_{10}$ CFU/g, followed by spray freeze drying (8.18 ± 0.16 $\log_{10}$ CFU/g). Under in vitro digestion, freeze-dried probiotics showed a lower level of viability (6.12 ± 0.29 $\log_{10}$ CFU/mL) than spray-freeze-dried samples (6.43 ± 0.29 $\log_{10}$ CFU/mL).

2.3. Auxiliary Methods

The standard drying methods, by themselves, are well-established and optimized. They can be, however, improved upon by the introduction of auxiliary methods, such as fluid bed coating. The combination of two different techniques allows the use of the most popular methods, such as spray drying or freeze drying, in the first step; then, the stability of the preparation is improved upon by fluid bed coating with different protective substances.

In their research, Jacobsen et al. [24] showed that the viability of freeze-dried probiotics after granulation and fluid bed coating was only slightly reduced. Freeze-dried *L. reuteri* LR92 was used to produce probiotic pellets by granulation, extrusion, and spheronization. The obtained probiotic pellets were then subjected to fluid coating with the Eudragit S100 and Eudragit FS30D coating suspensions to achieve delayed release. To evaluate the targeted delivery of coated probiotic pellets, an in vitro model simulating the conditions of the human gastric system, duodenum/jejunum, and ileum was developed. The release of active substances from the coated pellets was determined by the quantification of the released marker riboflavin with HPLC. The results show that the coated probiotic pellets achieved the desired release profile (release in the ileum) based on the release of riboflavin.

Fluidized bed drying was also used to prepare dried probiotic apple snacks. The apple cubes were first dried in a fluidized bed at 50 °C until they reached a level of water activity lower than 0.5 and a moisture content below 15%. Then, the dried apple cubes were coated with a solution of hydroxyethyl cellulose and polyethylene glycol mixed with washed *Bacillus coagulans* spores. The optimal ratio of coating substances was established at 0.125 g of HEC to 11.7 uL of PEG. Coating with the optimal coating mixture resulted in achieving a 77.7% coverage of the sample area. The achieved product was microbiologically stable during storage at room temperature for 90 days and was able to maintain at least an 8 log CFU/30 g portion. Reductions in enzymatic activity, specifically the activity of polyphenol oxidase (by 86%) and of peroxidase (by 92%), represented an additional improvement [29].

2.4. Factors Affecting the Viability of Probiotics during Drying

During drying, probiotic microorganisms are exposed to various stress factors, such as excessive dehydration and thermal, mechanical, osmotic, and oxidative stresses [30]. Probiotic microorganisms belong to the group of products with low thermal stability; at the same time, there is a certain critical water content that must be maintained. A reduction in water content below this critical value may cause the dehydration of the cells and, therefore, their inactivation. Thermal stress and dehydration are considered the main causes of losses of the viability of probiotic bacteria during spray drying. Stress factors that affect probiotic bacteria during different processing stages are presented in Figure 3.

Figure 3. Stress factors affecting probiotics during different stages of their preparation and administration. Created with Biorender.com (accessed on 27 July 2022).

Thermal stress, i.e., the heat inactivation of microorganisms, is a significant risk in the second stage of drying, according to various authors. In this phase of the process, microorganisms can reach the temperature of the drying air, especially since the dried particles often remain in the dryer until the entire process is completed. Not all bacteria are equally prone to thermal inactivation. For example, *L. acidophilus* has shown better survival rates under various drying temperatures than *E. coli* K12. This can be explained by differences in the thickness of the cell wall, which is thicker in the case of Gram-positive bacteria (e.g., *L. acidophilus)*. Moreover, drying in a medium containing nutrient broth yielded better survival rates than drying in a medium without broth components [31].

High temperatures can denature intracellular proteins and destabilize cell membranes, which in turn leads to cell death. At the same time, higher temperatures cause a decrease in the water activity of the dried samples, which translates into increased storage stability. Therefore, when choosing spray drying parameters for probiotic microorganisms, it is important to determine an optimal outlet air temperature that is high enough for the dried samples to have low water activity and, on the other hand, low enough to prevent cell damage [20]. Air temperature also significantly affects the bulk density of dried probiotic powders. As the temperature increases, evaporation rates also increase. The powder dries to a more porous structure and is more prone to forming hollow particles [32].

The inactivation of microorganisms caused by dehydration often occurs simultaneously with heat damage. During drying, water molecules are removed from the cells, which limits chemical reactions and metabolic activity. Because water is essential for the stabilization of various components of the cell, its removal may result in a loss of cell integrity, changes in cell structure, and damage to the enzyme system [7]. This applies to, among other things, changes in the lipid bilayer of the cell membrane that can cause the leakage of intracellular fluid and, consequently, cell death [33]. In experiments on drying single droplets of an *L. plantarum* suspension, it was shown that at an outlet air temperature below 45 °C, inactivation due to dehydration was dominant, while above this temperature, dehydration and temperature stress occurred simultaneously [34]. The authors of the study also believed that the longer the drying time, the more cells would undergo dehydrative inactivation.

Osmotic stress during drying occurs as a result of cells losing water to the environment, which increases the molarity of the intracellular solution and reduces the volume of the cytoplasm. A loss of cellular turgor occurs, and the cell undergoes plasmolysis, which, as a consequence, leads to a loss of viability [7].

Oxidative stress is caused by oxygen contained in the air and dissolved in an aqueous suspension of microorganisms [35]. Probiotic bacterial tolerance to oxygen is varied, with most *Bifidobacterium* species requiring strict anaerobic conditions and many strains of lactic acid bacteria tolerating oxygen. Oxidative stress is caused by reactive oxygen particles interacting with proteins, nucleic acids, and lipids. As a result, protein denaturation and lipid oxidation occur, leading to cell membrane damage and cell death [30].

The cells of probiotic microorganisms can also be inactivated by shear forces as their suspension is sprayed into the dryer head. Several studies have shown a relationship between the pressure of the suspension administered through atomizing nozzles and the survival of probiotic bacteria [35].

Similar to other methods of drying probiotic bacteria, in fluidized bed drying, certain factors cause losses of cell viability, mainly by osmotic stress, excessive dehydration, and oxidative stress [20]. It is believed that the threat of thermal shock at the temperatures used in the fluidized bed drying of microorganisms is insignificant up to a material moisture level of 15% and increases as the water activity of the dried material decreases [7]. Additionally, the pressure in the atomizing nozzle can affect the viability of the cells. An increase in nozzle pressure above 1.5 bar reduced the viability of *Enterococcus faecium* cells by 4 log cycles [23].

In the process of freeze drying, microorganisms are exposed to various stress factors caused by freezing and the sublimation of ice, leading to changes in the cell. These include deformation, mechanical damage to the cellular structure by ice crystals formed during the process, the loss of semipermeable properties of cell membranes, changes in the structure of membrane lipids, and the denaturation of protein components due to an increase in the concentration of intracellular compounds [7].

Ice crystals form at the biomass-freezing stage. The crystal growth depends on the freezing rate and temperature. Rapid freezing is recommended because the ice crystals reach small dimensions and do not damage the microbial cells. In addition, slowly lowering the temperature leads to ice forming mainly outside the cells, which leads to excessive dehydration. The formation of ice crystals is not the only threat to cell viability. During ice crystallization, the intracellular solution thickens, which can lead to osmotic stress. Moreover, the removal of water bound to the cells may damage surface proteins, the cell wall, and the cytoplasmic membrane. The lipid fraction of the bilayer cell membrane, where the structure of polar phospholipid parts may change, is particularly vulnerable to damage during dehydration [33].

Because vacuum drying occurs at a temperature higher than freeze drying but lower than spray drying, it is a milder process in terms of the effects of high or low temperature on the cells of microorganisms. Furthermore, the lack of oxygen in the drying environment can reduce oxidative stress, especially when drying oxygen-sensitive bacteria, e.g., *Bifidobacteria* [30]. Dehydration stress, however, is considered a major threat to cell viability during this process. For example, during vacuum drying, cell damage is observed mainly in the cell membrane [36].

The described stress factors, when introduced in a controlled manner in the culturing stage, can also be used to prevent the loss of viability during drying and storage. Research by Hernández et al. [37] confirms that fermentation parameters, such as pH and temperature, influence the stress resilience of *L. reuteri* DSM 17,938 during freeze drying. Simultaneous exposure to mild heat (50 °C) and osmotic stress (0.6 M of NaCl) also significantly improved the storage stability of *L. casei* CRL 431 when compared with bacteria exposed to just one of those stress factors [38].

2.5. Prevention of Stress Factors

The prevention of stress factors in the spray drying of probiotic microorganisms can be achieved by careful selection of the appropriate drying strategy [7,13]:

- The addition of protective substances;
- The proper selection of process parameters;
- The adaptation of cells to stress factors before drying.

These preventative measures affect the survival of probiotic bacteria directly during drying, as well as after drying during storage. The following substances are considered protectants and additives used to improve the survival rate of probiotics during spray drying: saccharides, skimmed milk, whey proteins, inulin, trehalose, and oligosaccharides, as well as polymers, such as gum arabic [19,32,33,39,40]. Some examples are also summarized in Table 2. In the scientific literature, several hypotheses have been put forward explaining the protective effect of these substances on the cell membrane and intracellular proteins. These include the theory of vitrification, the hypothesis about the exchange of water molecules in the hydration layer of proteins and the cell membrane with a protective substance (the "water replacement hypothesis"), and the hypothesis of hydration force (the "hydration force hypothesis") [7].

The activities aimed at optimizing the spray-drying process parameters for probiotic bacteria include, first and foremost, the correct selection of the chamber inlet and outlet air temperatures, the appropriate spray nozzle configuration, the atomizing pressure, and the flow volume of the suspension fed to the dryer [1,19]. A significant improvement in the viability of spray-dried *L. lactis* after replacing air with nitrogen has also been demonstrated [35].

To increase the survival of probiotic cells during spray drying, they can also be exposed to stress conditions during culturing. These stress conditions include exposure to low pH, thermal shock, culturing microorganisms without additional nutrition, exposure to sodium chloride and monosodium glutamate, and culturing with the addition of saccharides, such as mannose and sucrose. In general, it is also believed that cellular biomass derived from the stationary culture phase has better survival rates than that derived from the logarithmic growth phase [20].

There are numerous measures that were developed to improve the viability of freeze-dried bacteria. These include the addition of protective substances to bacterial suspensions, the appropriate control of process parameters during bacteria cultivation, and the adaptation of cells to stress factors before the drying process. The effectiveness of these operations may vary depending on the species of the microorganism [7]. These actions are, in many cases, similar to those used for spray drying.

The most common way to prevent stress factors in freeze-dried probiotic bacteria is to use cryo- and lyoprotectants. Cryoprotectants are water-soluble chemical compounds that lower the melting point of ice. When ice crystals form in the first stage of the process, bacterial cells cluster in the non-frozen fraction. The addition of cryoprotectants increases the volume of the non-frozen fraction of the solution, which increases the space occupied by cells, which in turn reduces cell damage as a result of mechanical and osmotic stresses. These cryoprotectants include polyols, polysaccharides, mono- and disaccharides, amino acids, proteins, minerals, organic acid salts, and complex vitamins [15,41]. In turn, lyoprotectants protect probiotic bacteria at the stage of water removal from the cell. The types and mechanisms of action of lyoprotectants are similar to those described for spray drying. Some sugars, such as sucrose and trehalose, act as both cryo- and lyoprotectants, which translates into their high effectiveness in ensuring the survival of probiotics after freeze drying [22].

The main strategies for protecting probiotic bacteria against stress factors in vacuum drying include the use of protective substances and the selection of process parameters [7]. Among the protective substances most commonly used in the vacuum drying of probiotics are sugars and polyalcohols, such as trehalose and sorbitol. The protective mechanism of these substances is the same as for spray and freeze drying [20].

The relatively low temperature of vacuum drying enables the dehydration of biomass prepared in a semisolid state, such as pellets mixed with a protective substance. The drying efficiency is increased compared to other methods because less water is removed; therefore, a smaller quantity of protective substances can be used [42]. This was proved by vacuum drying (100 mbar, 43 °C, 12 h) *L. bulgaricus* bacteria in the form of a pellet with the addition of powdered lactose, sorbitol, inulin, and xanthan gum [43]. An improvement in the rate

of cell survival was found only for samples with 1% sorbitol as the protective substance. The protective effects of sorbitol are due to its ability to lower membrane phase transition temperatures via the interaction with phosphate groups in the membrane [44].

Drying time and temperature are the most important process parameters to be taken into account when optimizing the drying process. The shorter the drying time and the lower the temperature, the higher the survival rate of the dried cells [7]. For example, for *L. delbrueckii* subsp. *bulgaricus* dried at 30, 45, and 70 °C (13.3 mbar, 10 min), damage to the cell membrane was higher with decreases in water activity and increases in drying temperature [42].

The countermeasures used against threats to the viability of fluid-dried bacteria are essentially the same as those used in other drying techniques and include the addition of protective substances, control of process parameters, and the adaptation of cells to stress factors before drying.

The addition of various protective substances to dried probiotic bacteria is the most commonly used protective method. The viability of fluidized-bed-dried probiotic bacteria is also highly dependent on their final humidity. The authors of [1] believed that the critical level of humidity that threatens the viability of *L. helveticus* cells was 1–3%. It was indicated that this might depend on the bacterial species; for *L. salivarius*, for example, the critical humidity was in the range of 5–6%.

There are also certain factors that can influence the viability and survival of probiotic bacteria during storage. To increase the shelf-life of dried probiotics, various protective measures can be used. These include the addition of antioxidants, such as 0.5% (*w*/*w*) vitamin E; they protect the final formulation against oxidative stress [38]. Storage at a lower temperature, i.e., 4 °C, can also result in an improvement in the survival rate of dried probiotic powder compared to the results when stored at 22 °C or 35 °C [22]. Examples of shelf-life analyses and viability measurements during storage are summarized in Table 3.

Table 1. Comparison of cell concentrations after different drying methods.

Reduction [log cfu/g]	Microorganism	Growth Parameters	Cell Concentration before Drying [log cfu/g]	Cell Concentration after Drying [log cfu/g]	Drying Method	Reference
<1	*Lactiplantibacillus plantarum 299v*	MRS broth, 37 °C	10.3	11.3	Freeze drying	[16]
	Pediococcus acidilactici HA-6111-2	MRS broth, 37 °C	10.5	11.2	Freeze drying	[16]
	Lactiplantibacillus plantarum 299v	MRS broth, 37 °C	9.4	9.5	Spray drying	[16]
	Pediococcus acidilactici HA-6111-2	MRS broth, 37 °C	9.0	9.4	Spray drying	[16]
<1	*Lactobacillus kefir* CIDCA 8348	MRS broth, 30 °C	8.8	8.2	Spray drying	[45]
	Lactobacillus plantarum CIDCA 83114	MRS broth, 30 °C	9.9	9.8	Spray drying	[45]
	Lactobacillus kefir CIDCA 8321	MRS broth, 30 °C	8.4	8.1	Spray drying	[45]
	Lactobacillus rhamnosus LGG	MRS broth, 37 °C	11.0	10.2	Spray drying	[46]
	Lactobacillus casei AMBR2	MRS broth, 37 °C	11.0	10.3	Spray drying	[46]

Table 1. *Cont.*

Reduction [log cfu/g]	Microorganism	Growth Parameters	Cell Concentration before Drying [log cfu/g]	Cell Concentration after Drying [log cfu/g]	Drying Method	Reference
>1	*Lactobacillus acidophilus* NCDC016	MRS broth, 37 °C	11.2	10.0	Spray drying	[32]
	Escherichia Coli K12	TSB, 30 °C	10.7–10.9	7.9	Spray drying	[31]
>1	*Lactobacillus reuteri* DSM 20016	MRS broth, 37 °C	8.7–9.7	7.7	Fluidized bed drying	[47]

Table 2. Protective effects of different materials.

Reduction Post-Drying [log cfu/g]	Microorganism	Drying Method	Protective Substances	Cell Concentration before Drying [log cfu/g]	Cell Concentration after Drying [log cfu/g]	Survivability [%]	Reference
<1	*Bifidobacterium bifidum*	Spray drying (double layered)	Gum arabic 9%, 1% β-cyclodextrin, 1% lecithin	6.93	6.18	89.22	[48]
	Bifidobacterium bifidum	Spray chilling (double layered)	Hydrogenated palm oil, 2% Tween 80	6.12	6.01	98.25	[48]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Gelatin 10%	9.95	9.06	91.55	[49]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Whey protein concentrate 20%	9.65	8.86	91.81	[49]
	Lactobacillus rhamnosus	Spray drying	Native rice starch 10%	9.26	8.98	53.24	[40]
	Lactobacillus rhamnosus	Spray drying	Inulin 15%	9.18	8.91	53.55	[40]
	Lactobacillus brevis WK12	Freeze drying	Soy powder solution 10%	11.30	11.26	90.00	[50]
	Lactococcus lactis WK11	Freeze drying	Soy powder solution 10%	11.30	11.27	94.00	[50]
>1	*Bifidobacterium bifidum*	Spray drying	Gum arabic 9%, 1% β-cyclodextrin	10.12	7.57	74.81	[48]
	Bifidobacterium bifidum	Spray chilling	Hydrogenated palm oil, 2% Tween 80	9.51	8.25	86.79	[48]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Modified starch 20%	9.65	8.64	89.53	[49]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Maltodextrin 20%	9.65	8.61	89.24	[49]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Pea protein isolate 10%	9.95	8.55	86.52	[49]
	Saccharomyces cerevisiae var. *boulardii*	Spray drying	Gum Arabic 20%	9.65	8.17	84.69	[49]

Table 3. Shelf-life and viability of probiotics.

Microorganism	Preparation Method and Matrix	Storage Conditions	Initial Cell Concentration [log cfu/g]	Cell Concentration after Storage [log cfu/g]	Monitored Parameters	Reference
Bacillus coagulans	Fluid-bed-dried apple snacks	90 days at 25 °C	7.89	6.78	viable cell counts, water activity and moisture content, enzyme activity, total phenolic content, antioxidant capacity, vitamin E concentration	[29]
Lactiplantibacillus plantarum 299v	Spray drying in orange juice	12 months, 25 °C, a_w = 0.03 hermetic glass flasks with silica gel	7.90	6.30	viable cell counts, water activity	[51]
Pediococcus acidilactici HA-6111-2	Spray drying in orange juice	12 months, 25 °C, a_w = 0.03 hermetic glass flasks with silica gel	8.70	8.00	viable cell counts, water activity	[51]
Lactiplantibacillus plantarum Lp 115-400b	coconut water oatmeal with inulin (1 g/100 mL)	4 °C, 49 days	7.06 (9.12 at day 7)	7.23	viable cell counts, pH, lactic acid content, rheological parameters	[52]
Lactiplantibacillus plantarum Lp 115-400b	coconut water oatmeal	4 °C, 49 days	6.99 (9.01 at day 7)	6.41	viable cell counts, pH, lactic acid content, rheological parameters	[52]

3. New Trends in the Drying and Application of Probiotics

Until recently, the standard methods of preserving probiotic bacteria consisted of the four types of drying presented above (spray drying, freeze drying, vacuum drying, and fluid bed drying). As new technologies and needs emerge, we observe the development of new trends in the drying and formulation of probiotics. These include new application methods that combine the existing methods, research on new matrices for probiotic bacteria, and new properties of probiotic strains.

3.1. Various Application Methods

New developments in the formulation of dried probiotics include different forms of administration, such as nasal sprays and creams or lotions. The development of new application methods for probiotics, besides orally administered tablets and microcapsules, allows for better use of their wide range of health-promoting properties. It also allows them to be better adapted to the needs of patients.

Probiotic formulations have been found to have a beneficial effect on the upper respiratory tract (URT), preventing acute respiratory tract infections. *L. casei* AMBR2, isolated from the human URT, was chosen for the preparation of a probiotic nasal spray by spray drying [4]. For the preparation of the drying solution, different combinations of saccharides and polymers were used. The following combinations provided the highest shelf-life stability for *L. casei* AMBR2 while also maintaining a stable formulation: 2.5% (*w*/*v*) sucrose and 0.4% (*w*/*v*) xanthan gum, 2.5% (*w*/*v*) isomalt and 0.4% (*w*/*v*) xanthan gum, 2.5% (*w*/*v*) trehalose and 1% (*w*/*v*) HPMC (hydroxypropyl methylcellulose), and 2.5% (*w*/*v*) lactose and 1% (*w*/*v*) HPMC. Shelf-life viability was measured during 28 weeks of storage under refrigerated conditions, and all mentioned formulations showed a viability level higher than 5×10^9 CFU/g. During the spray tests, after the resuspension of the powder, no significant viability changes were noticed. In the course of the functionality tests,

adherence to the Calu-3 cell line was measured. For the formulations containing HPMC, adherence was unchanged in comparison to the adherence of fresh *L. casei* AMBR2 cells (>10%). Formulations containing xanthan gum had significantly lower levels of adherence (up to 5%). The antimicrobial effects of *L. casei* AMBR2 against *S. aureus*, *M. catarrhalis*, and *H. influenzae* (URT pathogens) were also confirmed for both fresh and dried cells, with lower inhibition zones in some assays with dried samples.

Topically applied probiotics are the focus of many studies as the combination of probiotic and antimicrobial properties allows for the effective treatment of different skin disorders, including atopic dermatitis and acne. Atopic dermatitis is a chronic, inflammatory skin condition traditionally treated with histamines, corticosteroids, biopharmaceuticals, and antimicrobials. Other non-pharmaceutical methods of treatment are being researched because, for some patients, the currently used treatments are ineffective or associated with side effects. A lotion containing the heat-treated probiotic strain *Lactobacillus johnsonii* NCC 533 was applied for 3 weeks in a group of patients with atopic dermatitis [5]. The results obtained after the treatment showed that the probiotic lotion controlled *Staphylococcus aureus* colonization (which is one of the causes of atopic dermatitis, especially in the acute phase). Additionally, a local clinical improvement was found in patients who used the lotion over 3 weeks according to SCORAD (the SCORing Atopic Dermatitis tool).

Antimicrobial activity is also important in the treatment of other skin disorders, such as acne. The skin adhesion and antimicrobial activity of different probiotic strains were studied by Lopes et al. [53]. The use of probiotics allows for the equilibration of the skin microbiota and modulates the immune system. Additionally, the bacteriocins produced by certain probiotic strains affect pathogenic microorganisms by inhibiting their growth. Cell-free culture supernatants (CFCS) were used for evaluation of this phenomenon and showed antimicrobial activity towards *Escherichia coli*, *Cutibacterium acnes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Their antimicrobial activity was confirmed by measuring the inhibition zones. The observed effect was attributed to the production of organic acids by lactic acid bacteria, which were able to inhibit the growth of pathogenic microorganisms by decreasing the pH of the medium. In tests with neutralized cell-free culture supernatant, no antimicrobial activity was observed; this result further proved that, in this case, the antimicrobial effect was linked with the production of organic acids and not with the production of bacteriocins. Most of the used strains also displayed the ability to prevent biofilm formation by reducing the ability of pathogenic cells to attach to and create a biofilm. Among seven tested probiotic strains, three were able to prevent biofilm formation by *E. coli*, five of them prevented biofilm formation by *S. aureus*, six prevented biofilm formation by *P. aeruginosa*, and all strains showed the ability to decrease biofilm formation by *C. acnes*.

3.2. Controlled Release

Controlled release is one of the main focus points in the current development of drugs, biopharmaceuticals, and supplements. In addition, for probiotics, it is key to prepare formulations that are capable of withstanding the acidic environment and various enzymes found in the gastrointestinal tract while retaining their properties and viability for release in the intestine.

Sánchez-Portilla et al. [21] used polymethacrylate-based copolymers (Eudragit) to prepare microencapsulated *Bifidobacterium* by fluid bed drying. Two matrices were used in this process: microcrystalline cellulose (Avicel) and prebiotic inulin. During preparation, the viability of the cells decreased from the initial concentration of 10^9 by 0.99 $\log_{10}$ cfu/g for the formulation with Avicel and by 1.33 $\log_{10}$ cfu/g for the formulation with inulin. During long-term storage, viability was maintained at 6.6 $\log_{10}$ cfu/g for the first 3 months and remained at over 4.5 $\log_{10}$ cfu/g for both formulations after 2 years of storage. During the resistance tests, both products resisted stomach acidic conditions of pH 3. The main difference between the two products was in the mechanism of the release of *Bifidobacteria*. In the product with inulin as a matrix, due to the water solubility of inulin, release was

dependent only on the protective polymer used for coating. Meanwhile, in the product with microcrystalline cellulose as a matrix, release was dependent on both the coating layer and the matrix particles, which retained the bacteria and provided resistance even under alkaline conditions.

Targeted release was also the goal of the study conducted by Huang et al. [54]. Alginate, sucrose, whey protein isolate, and shellac were used as encapsulating materials in the external emulsification process, after which the microcapsules were freeze-dried. *L. reuteri* TMW 1.656 was exposed to different stress conditions (digestive and gastric juices as well as heating) after microencapsulation and drying; then, it was stored under ambient conditions to measure the inactivation of the cells. Probiotic stability was higher for the alginate capsules with added shellac under all conditions, which was explained by the decrease in hygroscopicity. The addition of whey protein isolate to alginate and shellac resulted in a further increase in viability (especially for gastric juice exposure and heat shock), and this finding was related to the protective effects of whey protein. The combination of modified rice protein and shellac was used by Wang et al. (2021) [55] to prepare an enteric coating for probiotic microcapsules. The addition of modified rice protein was able to modify the properties of shellac-based coatings. The results showed that the version with the addition of rice protein was superior at preserving probiotic viability during storage and digestion. Similarly, microcapsules composed of sporopollenin exine and coated with calcium alginate/carboxymethylpachymaran shell were confirmed to enhance the storage stability of probiotics while providing sustained release in the gastrointestinal tract [56].

Interpenetrating polymer network (IPN) hydrogels obtained by the enzymatic method were studied by Yan et al. [57] as potential carriers for probiotics. The hydrogels were prepared using a combination of biopolymers, namely soy protein isolate and sugar beet pectin. The concentrations of soy protein isolate in the final solution varied from 4% to 10%, and the sugar beet pectin concentration varied from 2.5% to 5%. Afterwards, a probiotic suspension containing about 10 log cfu/mL *L. paracasei LS14* was added and mixed with the hydrogel solution. To improve hydrogel formation, the enzyme laccase was also added in different concentrations (2–14 U/g). Hydrogel containing 10% SPI and 3.5% SBP and induced by 10 U/g laccase was highlighted as yielding the highest probiotic viability (~7.5 cfu/mL). It was also the least sensitive to simulated gastric conditions, showing no decrease in probiotic cell viability after 2 h of incubation.

3.3. Probiotics in Food Matrices

Probiotics are available in a wide array of commercial products, but most of them are offered in the form of dietary supplements. However, there is a large group of products that contain natural probiotics, such as naturally fermented yogurts, kefir, kimchi, and sauerkraut [58]. In addition, non-fermented and, more importantly, non-dairy food products can provide a matrix for probiotics. The use of different food matrices allows for the preparation of healthy probiotic food, taking into account allergies, nutritional preferences, taste, and the aroma of the final product. Probiotic food products of non-dairy origin, such as cereals, fruit, and vegetable- and meat-based products, are also rich sources of protein, minerals, vitamins, dietary fiber, antioxidants, and other bioactive substances that may provide extra health benefits [59]. Another advantage of using food matrices is their potential to improve the storage stability of probiotics.

Chocolate is one of the most versatile functional foods, and it can be enriched with probiotics without the loss of sensory attributes [60]. It can also be prepared in various forms, and it can combine different beneficial effects. For example, chocolate and hazelnut spreads were developed that are not only enriched with probiotics but also contain less fat, which was replaced by healthy triacylglycerols [61]. There are, however, some challenges to overcome, such as processing, storage, and gastrointestinal conditions. Formulations containing cocoa powder and sodium alginate were used in the encapsulation of probiotics by emulsion-based freeze drying, with an encapsulation efficiency of up to 95% [62]. The use of encapsulated probiotics allowed researchers to maintain the viability of the probiotics

in the product at over 7.5 log cfu/g after 90 days of storage at 25 °C; they also recorded a high level of viability (8.0 log cfu/g) during in vitro gastrointestinal digestion.

Among the various products with probiotic properties is butiá (*Butia odorata)* ice cream supplemented with *Bifidobacterium lactis* (BI-04). Ice cream ensured the viability of probiotic bacteria during 90 days of storage at −18 °C. Additionally, butiá was able to maintain its bioactive components during storage. The authors also conducted a market analysis that showed high acceptance of novel functional food products among panelists [6].

Banana powder is an excellent probiotic matrix due to its porous structure, nutritious properties, low cost, and overall availability. Freeze-dried banana powder was prepared after mixing banana paste with different probiotic formulations. These formulations included *Lactobacillus acidophilus* and *Lactobacillus casei* microencapsulated with whey protein isolate, fructooligosaccharides, and a combination of both at a 1:1 ratio. After the incorporation into banana paste, the probiotic formulations were then refrigerated for 72 h at −32 °C and transferred to a freeze dryer for 24 h at −40 °C. Afterwards, the obtained product was ground and filtered into a fine powder. The results show that the use of microspheres combining whey protein isolate and fructooligosaccharides resulted in the highest encapsulation yield (98%). The obtained product was also stable during storage at the temperature of 4 °C and increased the viability of bacteria after 90 min of exposure to gastrointestinal conditions (7.85 log CFU/g and 7.52 log CFU/g for the microencapsulated cells of *L. acidophilus* and *L. casei*, respectively) compared with free cells (a decrease of 4.69 log CFU/g for *L. acidophilus* and 5.64 log CFU/g for *L. casei*) [63].

The survival of microencapsulated *Lactococcus lactis* Subsp. *lactis* R7 was measured for various food matrices by Rosolen et al. [64]. A quantity of 1 g of spray-dried bacteria microcapsules containing approximately 12 log CFU g^{-1} was added to 100 mL of milk, milk cream, and blueberry juice. These samples were then stored for 28 days at 4 °C and compared with samples containing free, non-encapsulated bacteria. The results showed that the acidic pH of blueberry juice had damaging effects on free cells, resulting in their viability falling below the minimum value to be considered probiotic after 14 days of storage. The microencapsulated sample had better viability in the blueberry juice, showing a loss of viability after 21 days. In both milk and milk cream, the microencapsulated bacteria showed higher levels of viability after 28 days of storage than the samples with free cells. Additionally, the free cells promoted higher acidification in milk, which proves that the probiotic bacteria were successfully trapped inside the microcapsules during encapsulation.

4. Assessment of Strain Suitability and Viability

While almost all probiotic formulations use lactic acid bacteria with GRAS status, there are some additional requirements to be met when preparing a probiotic product. Different strains can vary in terms of their environmental resistance, growth characteristics, production of metabolites, and their effects on human health, which can make some more suitable for certain applications. This assessment should be conducted using both classic microbiological methods and more advanced tools, such as flow cytometry, while also including a thorough analysis of gene expression and proteomics. Table 4 lists methods used in recent studies to assess probiotic bacteria.

4.1. Microbiological Analysis

The basic procedure in the assessment of probiotics is the use of plate cell counts for the enumeration of viable cells. It can be divided into the following steps [65]:

1. Sample preparation: This depends on the matrix (frozen, dried, liquid, or free cells).
2. Dilution: This includes the prior homogenization or rehydration and the use of a dilution medium containing peptone, NaCl, or phosphate salts, as well as the addition of antioxidants for oxygen-sensitive strains.
3. Plating: This is performed with a strain-specific plating medium.

4. Incubation: This takes into consideration the optimum temperature (mostly 37 °C as many probiotics naturally inhabit the gastrointestinal tract) and the aerobic/anaerobic conditions preferred by the specific strain.

One of the issues with relying solely on plate counts for viability assessments of probiotics is that such counts are only limited to the viable cells growing on the plate medium. There are, however, other groups of cells, such as those that are viable but not culturable (VBNC), that should be considered in the assessment of probiotics [66].

4.2. Flow Cytometry

Flow cytometry can be used to expand the assessment conducted using conventional microbiological methods. The viability of probiotic cells can be analyzed using dual staining with SYBR Green I and propidium iodide (PI) dyes. SYBR Green I allows the detection of viable and active cells by binding to DNA and emitting green light, while PI indicates cellular damage in dead cells because it is membrane impermeable and only binds to DNA in cells with damaged membranes, emitting red light. Such a protocol enabled the identification of viable and dead cells in *L. plantarum* [67] and allowed for a much faster analysis compared to conventional microbiological methods while providing a higher number of observations and, thus, increasing statistical certainty.

4.3. Gene Expression and Proteomic Analysis

Despite the wide application of probiotic bacteria and their extensive use, some mechanisms, especially those linked with stress responses, remain to be addressed. Researching the proteins and gene expression involved in these processes can allow for a better understanding of the stress responses to drying and environmental conditions, allowing for better cell survival and industrial process optimization.

The protein expression patterns of *Oenococcus oeni* SD-2a that was subjected to freeze-drying stress were analyzed by Yang et al. [68]. Biofilm formation is the main method of adaptation of *O. oeni* against stress and is linked with different potential signals (QS autoinducers, peptides, and volatile and organic compounds), matrix composition (polysaccharides), and energy supply (carbon starvation). The HSP20 protein and Clp proteases were also linked with improved resistance to stress induced by the addition of monosodium glutamate (MSG), which improved cell integrity during freeze drying.

Leuconostoc mesenteroides BD3749 forms cell aggregates as a reaction to oxidative stress through the upregulation of the glucansucrase-encoding gene Gsy. *L. mesenteroides* BD3749 cells synthesize large amounts of insoluble exopolysaccharides in response to oxidative stress, which reduces the accumulation of reactive oxygen species in bacterial cells, improving their survival. In a relevant study, Gsy and its upregulation following exposure to oxygen were responsible for the synthesis of those insoluble exopolysaccharides that mediated the aggregation processes [69].

Table 4. Methods for the assessment of probiotics.

Method	Key Applications	Reference
Plate counting	viable cell enumeration	[65]
Flow cytometry	cell integrity, membrane damage (live/dead staining)	[46,67]
Scanning electron microscopy	cell morphology, surface characteristics	[46,68]
Laser diffraction	particle size	[46]
Two-dimensional gel electrophoresis (2-DE)	protein pattern analysis (proteins involved in biofilm formation, quorum sensing, volatile compounds production, stress response)	[68]
Mass spectrometry	peptide mass fingerprinting	[68]
Bioinformatics	protein identification, prediction of protein interaction, subcellular localization	[68]
RT-qPCR	gene expression	[69]

5. Future Challenges and Concluding Remarks

Drying techniques: Among the methods described, freeze drying guarantees the best viability of dried bacteria. It is, however, the most expensive method, and the process has a long duration. In those two categories, both fluid bed drying and spray drying are better, with fluid bed drying being less expensive and faster than spray drying. Fluid bed drying also has additional potential as a secondary method, i.e., it can be combined with other drying techniques to coat the product in a fluid bed after drying using a different method, further improving its stability during storage. Newly developed methods and modifications of known techniques, such as electrospinning and spray freeze drying, offer opportunities for further technological development and could potentially replace the techniques used up until now.

Response to stress conditions: It has been pointed out that the influence of different stresses associated with drying on the viability of the cells can be decreased by habituating those cells to stress factors in the culturing stage. Such methods have not yet been thoroughly studied in relation to gene expression. Knowledge of the expression of specific genes in response to targeted stress may be beneficial for the production of stable and viable probiotics with longer shelf-life.

Multiparametric analysis: Most studies focus on the survival of bacterial cells during drying. While this is an important parameter, it is not ideal for the assessment of the quality of the obtained product. It does not take into consideration the activity of the probiotics in the gastrointestinal tract, which may be influenced by the type of drying, the drying conditions, the use of protectants, and the stresses experienced by cells in the digestive tract. Other parameters worth researching are cell activity (and the activity of viable but nonculturable cells), cell adhesion to the intestinal epithelium, and the expression of shock proteins.

Strain viability: It should also be noted that the vast majority of research currently focuses on a poorly diversified group of bacteria, mostly lactobacilli. Finding new diverse strains with probiotic activity should be a focus of future studies. The differentiation of probiotics could lead to the development of new formulations targeting various health issues at once. It could also help to develop probiotics as food additives with strains conferring positive effects both in terms of their probiotic activity and possibly also antimicrobial activity. There is also an increasing tendency in the market to develop multi-strain probiotics. Such products offer a broader spectrum of beneficial and synergistic effects. However, such effects are not yet well studied and need future work [70].

New applications: Probiotics have taken many new and varied forms. The development of products suitable for use in different applications should not be focused only on dietary supplements and dairy probiotic food, which have been the point of focus of many studies conducted on probiotics. New applications are being sought in fields such as cosmetics, functional foods, non-dairy probiotic foods, nutraceuticals, and medicine [46,59,71].

Target groups: Following global trends should be a priority when selecting target groups for probiotics. For example, allergies are an increasing public health concern; therefore, measures to mitigate or eliminate their effects are being researched. The beneficial effects of probiotics have been proven in rhinitis, asthma, and atopic dermatitis [72]. Another global trend, especially pronounced in developed countries, is the aging of society. Probiotics enhance the immune response, which is especially important in elderly individuals. Additionally, probiotics may improve the effectiveness of vaccination [73].

Author Contributions: Conceptualization, J.K. and R.D.; writing, J.K.; figures, J.K.; supervision, R.D.; funding acquisition, J.K. and R.D. All authors have read and agreed to the published version of the manuscript.

Funding: This publication was financed within the framework of the "Regional Initiative Excellence" program of the Polish Ministry of Science and Higher Education in the years 2019–2022 (No. 005/RID/2018/19), with a total financing amount of 12,000,000.00 PLN.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no competing interest.

References

1. Santivarangkna, C.; Kulozik, U.; Foerst, P. Alternative Drying Processes for the Industrial Preservation of Lactic Acid Starter Cultures. *Biotechnol. Prog.* **2007**, *23*, 302–315. [CrossRef] [PubMed]
2. Niamah, A.K.; Gddoa Al-Sahlany, S.T.; Ibrahim, S.A.; Verma, D.K.; Thakur, M.; Singh, S.; Patel, A.R.; Aguilar, C.N.; Utama, G.L. Electro-Hydrodynamic Processing for Encapsulation of Probiotics: A Review on Recent Trends, Technological Development, Challenges and Future Prospect. *Food Biosci.* **2021**, *44*, 101458. [CrossRef]
3. Gill, H.; Prasad, J. Probiotics, Immunomodulation, and Health Benefits. In *Bioactive Components of Milk*; Advances in Experimental Medicine and Biology Series; Springer: New York, NY, USA, 2008; Volume 606, pp. 423–454. [CrossRef]
4. Jokicevic, K.; Kiekens, S.; Byl, E.; De Boeck, I.; Cauwenberghs, E.; Lebeer, S.; Kiekens, F. Probiotic Nasal Spray Development by Spray Drying. *Eur. J. Pharm. Biopharm.* **2021**, *159*, 211–220. [CrossRef] [PubMed]
5. Blanchet-Réthoré, S.; Bourdès, V.; Mercenier, A.; Haddar, C.H.; Verhoeven, P.O.; Andres, P. Effect of a Lotion Containing the Heat-Treated Probiotic Strain Lactobacillus Johnsonii NCC 533 on Staphylococcus Aureus Colonization in Atopic Dermatitis. *Clin. Cosmet. Investig. Dermatol.* **2017**, *10*, 249–257. [CrossRef]
6. dos Santos Cruxen, C.E.; Hoffmann, J.F.; Zandoná, G.P.; Fiorentini, Â.M.; Rombaldi, C.V.; Chaves, F.C. Probiotic Butiá (Butia Odorata) Ice Cream: Development, Characterization, Stability of Bioactive Compounds, and Viability of Bifidobacterium Lactis during Storage. *LWT* **2017**, *75*, 379–385. [CrossRef]
7. Broeckx, G.; Vandenheuvel, D.; Claes, I.J.J.; Lebeer, S.; Kiekens, F. Drying Techniques of Probiotic Bacteria as an Important Step towards the Development of Novel Pharmabiotics. *Int. J. Pharm.* **2016**, *505*, 303–318. [CrossRef]
8. dos Santos, R.C.S.; Finkler, L.; Finkler, C.L.L. Microencapsulation of *Lactobacillus Casei* by Spray Drying. *J. Microencapsul.* **2014**, *31*, 759–767. [CrossRef]
9. Sosnik, A.; Seremeta, K. Advantages and Challenges of the Spray-Drying Technology for the Production of Pure Drug Particles and Drug-Loaded Polymeric Carriers. *Adv. Colloid Interface Sci.* **2015**, *223*, 40–54. [CrossRef]
10. Arepally, D.; Reddy, R.S.; Goswami, T.K. Studies on Survivability, Storage Stability of Encapsulated Spray Dried Probiotic Powder. *Curr. Res. Food Sci.* **2020**, *3*, 235–242. [CrossRef]
11. Lipan, L.; Rusu, B.; Sendra, E.; Hernández, F.; Vázquez-Araújo, L.; Vodnar, D.C.; Carbonell-Barrachina, Á.A. Spray Drying and Storage of Probiotic-enriched Almond Milk: Probiotic Survival and Physicochemical Properties. *J. Sci. Food Agric.* **2020**, *100*, 3697–3708. [CrossRef]
12. Golman, B.; Julklang, W. Analysis of Drying Kinetics of a Slurry Droplet in the Falling Rate Period of Spray Drying. *Int. J. Chem. Mol. Eng.* **2013**, *7*, 5.
13. Huang, S.; Vignolles, M.-L.; Chen, X.D.; Le Loir, Y.; Jan, G.; Schuck, P.; Jeantet, R. Spray Drying of Probiotics and Other Food-Grade Bacteria: A Review. *Trends Food Sci. Technol.* **2017**, *63*, 1–17. [CrossRef]
14. Rajam, R.; Anandharamakrishnan, C. Spray Freeze Drying Method for Microencapsulation of Lactobacillus Plantarum. *J. Food Eng.* **2015**, *166*, 95–103. [CrossRef]
15. Gaidhani, K.A.; Harwalkar, M.; Bhambere, D.; Nirgude, P.S. Lyophilization/Freeze Drying—A Review. *World J. Pharm. Res.* **2015**, *4*, 29.
16. Barbosa, J.; Borges, S.; Amorim, M.; Pereira, M.J.; Oliveira, A.; Pintado, M.E.; Teixeira, P. Comparison of Spray Drying, Freeze Drying and Convective Hot Air Drying for the Production of a Probiotic Orange Powder. *J. Funct. Foods* **2015**, *17*, 340–351. [CrossRef]
17. Jiang, H.; Zhang, M.; Mujumdar, A.S.; Lim, R.-X. Comparison of Drying Characteristic and Uniformity of Banana Cubes Dried by Pulse-Spouted Microwave Vacuum Drying, Freeze Drying and Microwave Freeze Drying: Comparison of Banana Cubes Dried by Different Drying Method. *J. Sci. Food Agric.* **2014**, *94*, 1827–1834. [CrossRef]
18. Liu, W.; Zhang, M.; Bhandari, B.; Yu, D. A Novel Combination of LF-NMR and NIR to Intelligent Control in Pulse-Spouted Microwave Freeze Drying of Blueberry. *LWT* **2021**, *137*, 110455. [CrossRef]
19. Morgan, C.A.; Herman, N.; White, P.A.; Vesey, G. Preservation of Micro-Organisms by Drying; A Review. *J. Microbiol. Methods* **2006**, *66*, 183–193. [CrossRef]
20. Fu, N.; Chen, X.D. Towards a Maximal Cell Survival in Convective Thermal Drying Processes. *Food Res. Int.* **2011**, *44*, 1127–1149. [CrossRef]
21. Sánchez-Portilla, Z.; Melgoza-Contreras, L.M.; Reynoso-Camacho, R.; Pérez-Carreón, J.I.; Gutiérrez-Nava, A. Incorporation of Bifidobacterium Sp. into Powder Products through a Fluidized Bed Process for Enteric Targeted Release. *J. Dairy Sci.* **2020**, *103*, 11129–11137. [CrossRef]
22. Strasser, S.; Neureiter, M.; Geppl, M.; Braun, R.; Danner, H. Influence of Lyophilization, Fluidized Bed Drying, Addition of Protectants, and Storage on the Viability of Lactic Acid Bacteria. *J. Appl. Microbiol.* **2009**, *107*, 167–177. [CrossRef] [PubMed]
23. Stummer, S.; Toegel, S.; Rabenreither, M.-C.; Unger, F.M.; Wirth, M.; Viernstein, H.; Salar-Behzadi, S. Fluidized-Bed Drying as a Feasible Method for Dehydration of Enterococcus Faecium M74. *J. Food Eng.* **2012**, *111*, 156–165. [CrossRef]
24. Jacobsen, N.M.Y.; Caglayan, I.; Caglayan, A.; Bar-Shalom, D.; Müllertz, A. Achieving Delayed Release of Freeze-Dried Probiotic Strains by Extrusion, Spheronization and Fluid Bed Coating—Evaluated Using a Three-Step in Vitro Model. *Int. J. Pharm.* **2020**, *591*, 120022. [CrossRef] [PubMed]

25. Wirunpan, M.; Savedboworn, W.; Wanchaitanawong, P. Survival and Shelf Life of Lactobacillus Lactis 1464 in Shrimp Feed Pellet after Fluidized Bed Drying. *Agric. Nat. Resour.* **2016**, *50*, 1–7. [CrossRef]
26. Škrlec, K.; Zupančič, Š.; Prpar Mihevc, S.; Kocbek, P.; Kristl, J.; Berlec, A. Development of Electrospun Nanofibers That Enable High Loading and Long-Term Viability of Probiotics. *Eur. J. Pharm. Biopharm.* **2019**, *136*, 108–119. [CrossRef]
27. Yoha, K.S.; Anukiruthika, T.; Anila, W.; Moses, J.A.; Anandharamakrishnan, C. 3D Printing of Encapsulated Probiotics: Effect of Different Post-Processing Methods on the Stability of Lactiplantibacillus Plantarum (NCIM 2083) under Static in Vitro Digestion Conditions and during Storage. *LWT* **2021**, *146*, 111461. [CrossRef]
28. Anukiruthika, T.; Moses, J.A.; Anandharamakrishnan, C. 3D Printing of Egg Yolk and White with Rice Flour Blends. *J. Food Eng.* **2020**, *265*, 109691. [CrossRef]
29. Galvão, A.M.M.T.; Rodrigues, S.; Fernandes, F.A.N. Probiotic Dried Apple Snacks: Development of Probiotic Coating and Shelf-life Studies. *J. Food Process. Preserv.* **2020**, *44*, e14974. [CrossRef]
30. Santivarangkna, C.; Kulozik, U.; Foerst, P. Inactivation Mechanisms of Lactic Acid Starter Cultures Preserved by Drying Processes. *J. Appl. Microbiol.* **2008**, *105*, 1–13. [CrossRef]
31. Pispan, S.; Hewitt, C.J.; Stapley, A.G.F. Comparison of Cell Survival Rates of E. Coli K12 and L. Acidophilus Undergoing Spray Drying. *Food Bioprod. Process.* **2013**, *91*, 362–369. [CrossRef]
32. Arepally, D.; Goswami, T.K. Effect of Inlet Air Temperature and Gum Arabic Concentration on Encapsulation of Probiotics by Spray Drying. *LWT* **2019**, *99*, 583–593. [CrossRef]
33. Perdana, J.; Fox, M.B.; Siwei, C.; Boom, R.M.; Schutyser, M.A.I. Interactions between Formulation and Spray Drying Conditions Related to Survival of Lactobacillus Plantarum WCFS1. *Food Res. Int.* **2014**, *56*, 9–17. [CrossRef]
34. Perdana, J.; Fox, M.B.; Schutyser, M.A.I.; Boom, R.M. Mimicking Spray Drying by Drying of Single Droplets Deposited on a Flat Surface. *Food Bioprocess Technol.* **2013**, *6*, 964–977. [CrossRef]
35. Ghandi, A.; Powell, I.B.; Chen, X.D.; Adhikari, B. The Effect of Dryer Inlet and Outlet Air Temperatures and Protectant Solids on the Survival of *Lactococcus Lactis* during Spray Drying. *Dry. Technol.* **2012**, *30*, 1649–1657. [CrossRef]
36. Bauer, S.A.W.; Kulozik, U.; Foerst, P. Drying Kinetics and Survival of Bacteria Suspensions of *L. paracasei* F19 in Low-Temperature Vacuum Drying. *Dry. Technol.* **2013**, *31*, 1497–1503. [CrossRef]
37. Hernández, A.; Larsson, C.U.; Sawicki, R.; van Niel, E.W.J.; Roos, S.; Håkansson, S. Impact of the Fermentation Parameters PH and Temperature on Stress Resilience of Lactobacillus Reuteri DSM 17938. *AMB Express* **2019**, *9*, 66. [CrossRef]
38. Nag, A.; Das, S. Improving Ambient Temperature Stability of Probiotics with Stress Adaptation and Fluidized Bed Drying. *J. Funct. Foods* **2013**, *5*, 170–177. [CrossRef]
39. Ananta, E.; Volkert, M.; Knorr, D. Cellular Injuries and Storage Stability of Spray-Dried Lactobacillus Rhamnosus GG. *Int. Dairy J.* **2005**, *15*, 399–409. [CrossRef]
40. Avila-Reyes, S.V.; Garcia-Suarez, F.J.; Jiménez, M.T.; San Martín-Gonzalez, M.F.; Bello-Perez, L.A. Protection of L. Rhamnosus by Spray-Drying Using Two Prebiotics Colloids to Enhance the Viability. *Carbohydr. Polym.* **2014**, *102*, 423–430. [CrossRef]
41. Zhao, G.; Zhang, G. Effect of Protective Agents, Freezing Temperature, Rehydration Media on Viability of Malolactic Bacteria Subjected to Freeze-Drying. *J. Appl. Microbiol.* **2005**, *99*, 333–338. [CrossRef]
42. Tymczyszyn, E.E.; Díaz, R.; Pataro, A.; Sandonato, N.; Gómez-Zavaglia, A.; Disalvo, E.A. Critical Water Activity for the Preservation of Lactobacillus Bulgaricus by Vacuum Drying. *Int. J. Food Microbiol.* **2008**, *128*, 342–347. [CrossRef] [PubMed]
43. Santivarangkna, C.; Kulozik, U.; Foerst, P. Effect of Carbohydrates on the Survival of *Lactobacillus helveticus* during Vacuum Drying. *Lett. Appl. Microbiol.* **2006**, *42*, 271–276. [CrossRef] [PubMed]
44. Santivarangkna, C.; Naumann, D.; Kulozik, U.; Foerst, P. Protective Effects of Sorbitol during the Vacuum Drying of Lactobacillus Helveticus: An FT-IR Study. *Ann. Microbiol.* **2010**, *60*, 235–242. [CrossRef]
45. Golowczyc, M.A.; Silva, J.; Teixeira, P.; De Antoni, G.L.; Abraham, A.G. Cellular Injuries of Spray-Dried Lactobacillus Spp. Isolated from Kefir and Their Impact on Probiotic Properties. *Int. J. Food Microbiol.* **2011**, *144*, 556–560. [CrossRef] [PubMed]
46. Jokicevic, K.; Broeckx, G.; Vandenheuvel, D.; Boeck, I.D.; Allonsius, C.N.; Lebeer, S.; Kiekens, F. Processing of Potential Upper Respiratory Tract Probiotics by Spray Drying. *Dry. Technol.* **2020**, *39*, 2179–2193. [CrossRef]
47. Schell, D.; Beermann, C. Fluidized Bed Microencapsulation of *Lactobacillus reuteri* with Sweet Whey and Shellac for Improved Acid Resistance and In-Vitro Gastro-Intestinal Survival. *Food Res. Int.* **2014**, *62*, 308–314. [CrossRef]
48. Arslan-Tontul, S.; Erbas, M. Single and Double Layered Microencapsulation of Probiotics by Spray Drying and Spray Chilling. *LWT Food Sci. Technol.* **2017**, *81*, 160–169. [CrossRef]
49. Arslan, S.; Erbas, M.; Tontul, I.; Topuz, A. Microencapsulation of Probiotic Saccharomyces Cerevisiae Var. Boulardii with Different Wall Materials by Spray Drying. *LWT Food Sci. Technol.* **2015**, *63*, 685–690. [CrossRef]
50. Gwak, H.J.; Lee, J.-H.; Kim, T.-W.; Choi, H.-J.; Jang, J.-Y.; Lee, S.I.; Park, H.W. Protective Effect of Soy Powder and Microencapsulation on Freeze-Dried Lactobacillus Brevis WK12 and Lactococcus Lactis WK11 during Storage. *Food Sci. Biotechnol.* **2015**, *24*, 2155–2160. [CrossRef]
51. Barbosa, J.; Borges, S.; Teixeira, P. Effect of Different Conditions of Growth and Storage on the Cell Counts of Two Lactic Acid Bacteria after Spray Drying in Orange Juice. *Beverages* **2016**, *2*, 8. [CrossRef]
52. Dharmasena, M.; Barron, F.; Fraser, A.; Jiang, X. Refrigerated Shelf Life of a Coconut Water-Oatmeal Mix and the Viability of Lactobacillus Plantarum Lp 115-400B. *Foods* **2015**, *4*, 328–337. [CrossRef]

53. Lopes, E.G.; Moreira, D.A.; Gullón, P.; Gullón, B.; Cardelle-Cobas, A.; Tavaria, F.K. Topical Application of Probiotics in Skin: Adhesion, Antimicrobial and Antibiofilm in Vitro Assays. *J. Appl. Microbiol.* **2017**, *122*, 450–461. [CrossRef]
54. Huang, X.; Gänzle, M.; Zhang, H.; Zhao, M.; Fang, Y.; Nishinari, K. Microencapsulation of Probiotic Lactobacilli with Shellac as Moisture Barrier and to Allow Controlled Release. *J. Sci. Food Agric.* **2021**, *101*, 726–734. [CrossRef]
55. Wang, A.; Lin, J.; Zhong, Q. Enteric Rice Protein-Shellac Composite Coating to Enhance the Viability of Probiotic Lactobacillus Salivarius NRRL B-30514. *Food Hydrocoll.* **2021**, *113*, 106469. [CrossRef]
56. Deng, Z.; Li, J.; Song, R.; Zhou, B.; Li, B.; Liang, H. Carboxymethylpachymaran/Alginate Gel Entrapping of Natural Pollen Capsules for the Encapsulation, Protection and Delivery of Probiotics with Enhanced Viability. *Food Hydrocoll.* **2021**, *120*, 106855. [CrossRef]
57. Yan, W.; Jia, X.; Zhang, Q.; Chen, H.; Zhu, Q.; Yin, L. Interpenetrating Polymer Network Hydrogels of Soy Protein Isolate and Sugar Beet Pectin as a Potential Carrier for Probiotics. *Food Hydrocoll.* **2021**, *113*, 106453. [CrossRef]
58. Behera, S.S.; Panda, S.K. Ethnic and Industrial Probiotic Foods and Beverages: Efficacy and Acceptance. *Curr. Opin. Food Sci.* **2020**, *32*, 29–36. [CrossRef]
59. Min, M.; Bunt, C.R.; Mason, S.L.; Hussain, M.A. Non-Dairy Probiotic Food Products: An Emerging Group of Functional Foods. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 2626–2641. [CrossRef]
60. Islam, M.Z.; Masum, A.K.M.; Harun-ur-Rashid, M. Milk Chocolate Matrix as a Carrier of Novel Lactobacillus Acidophilus LDMB-01: Physicochemical Analysis, Probiotic Storage Stability and in Vitro Gastrointestinal Digestion. *J. Agric. Food Res.* **2022**, *7*, 100263. [CrossRef]
61. de Souza Correia Cozentino, I.; Veloso de Paula, A.; Augusto Ribeiro, C.; Duran Alonso, J.; Grimaldi, R.; Luccas, V.; Taranto, M.P.; Cardoso Umbelino Cavallini, D. Development of a Potentially Functional Chocolate Spread Containing Probiotics and Structured Triglycerides. *LWT* **2022**, *154*, 112746. [CrossRef]
62. Hossain, M.N.; Ranadheera, C.S.; Fang, Z.; Ajlouni, S. Impact of Encapsulating Probiotics with Cocoa Powder on the Viability of Probiotics during Chocolate Processing, Storage, and in Vitro Gastrointestinal Digestion. *J. Food Sci.* **2021**, *86*, 1629–1641. [CrossRef] [PubMed]
63. Massounga Bora, A.F.; Li, X.; Zhu, Y.; Du, L. Improved Viability of Microencapsulated Probiotics in a Freeze-Dried Banana Powder During Storage and Under Simulated Gastrointestinal Tract. *Probiotics Antimicrob. Proteins* **2019**, *11*, 1330–1339. [CrossRef] [PubMed]
64. Rosolen, M.D.; Bordini, F.W.; de Quardos da Luz, G.; Diaz, P.S.; Conceição, F.R.; Fiorentini, Â.M.; da Silva, W.P.; Pieniz, S. Survival of Microencapsulated Lactococcus Lactis Subsp. Lactis R7 Applied in Different Food Matrices. *Appl. Biochem. Biotechnol.* **2022**, *194*, 2135–2150. [CrossRef] [PubMed]
65. Champagne, C.P.; Ross, R.P.; Saarela, M.; Hansen, K.F.; Charalampopoulos, D. Recommendations for the Viability Assessment of Probiotics as Concentrated Cultures and in Food Matrices. *Int. J. Food Microbiol.* **2011**, *149*, 185–193. [CrossRef]
66. Fiore, W.; Arioli, S.; Guglielmetti, S. The Neglected Microbial Components of Commercial Probiotic Formulations. *Microorganisms* **2020**, *8*, 1177. [CrossRef]
67. Bensch, G.; Rüger, M.; Wassermann, M.; Weinholz, S.; Reichl, U.; Cordes, C. Flow Cytometric Viability Assessment of Lactic Acid Bacteria Starter Cultures Produced by Fluidized Bed Drying. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 4897–4909. [CrossRef]
68. Yang, K.; Liu, M.; Wang, J.; Hassan, H.; Zhang, J.; Qi, Y.; Wei, X.; Fan, M.; Zhang, G. Surface Characteristics and Proteomic Analysis Insights on the Response of Oenococcus Oeni SD-2a to Freeze-Drying Stress. *Food Chem.* **2018**, *264*, 377–385. [CrossRef]
69. Yan, M.; Han, J.; Xu, X.; Liu, L.; Gao, C.; Zheng, H.; Chen, Y.; Tao, Y.; Zhou, H.; Li, Y.; et al. Gsy, a Novel Glucansucrase from *Leuconostoc mesenteroides*, Mediates the Formation of Cell Aggregates in Response to Oxidative Stress. *Sci. Rep.* **2016**, *6*, 38122. [CrossRef]
70. Ouwehand, A.C.; Invernici, M.M.; Furlaneto, F.A.C.; Messora, M.R. Effectiveness of Multi-Strain Versus Single-Strain Probiotics: Current Status and Recommendations for the Future. *J. Clin. Gastroenterol.* **2018**, *52*, S35–S40. [CrossRef]
71. Reque, P.M.; Brandelli, A. Encapsulation of Probiotics and Nutraceuticals: Applications in Functional Food Industry. *Trends Food Sci. Technol.* **2021**, *114*, 1–10. [CrossRef]
72. Lopez-Santamarina, A.; Gonzalez, E.G.; Lamas, A.; Mondragon, A.d.C.; Regal, P.; Miranda, J.M. Probiotics as a Possible Strategy for the Prevention and Treatment of Allergies. A Narrative Review. *Foods* **2021**, *10*, 701. [CrossRef]
73. Akatsu, H. Exploring the Effect of Probiotics, Prebiotics, and Postbiotics in Strengthening Immune Activity in the Elderly. *Vaccines* **2021**, *9*, 136. [CrossRef]

International Journal of
Molecular Sciences

MDPI

Article

Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups

Jakub Kiepś *, Wojciech Juzwa and Radosław Dembczyński *

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland; wojciech.juzwa@up.poznan.pl
* Correspondence: jakub.kieps@up.poznan.pl (J.K.); radoslaw.dembczynski@up.poznan.pl (R.D.)

Abstract: Probiotic bacteria can be introduced to stresses during the culturing phase as an alternative to the use of protectants and coating substances during drying. Accurate enumeration of the bacterial count in a probiotic formulation can be provided using imaging flow cytometry (IFC). IFC overcomes the weak points of conventional, commonly used flow cytometry by combining its statistical power with the imaging content of microscopy in one system. Traditional flow cytometers only collect the fluorescence signal intensities, while IFC provides many more steps as it correlates the data on the measured parameters of fluorescence light with digitally processed images of the analyzed cells. As an alternative to standard methods (plate cell counts and traditional flow cytometry) IFC provides additional insight into the physiology and morphology of the cell. The use of complementary dyes (RedoxSensorTM Green and propidium iodide) allows for the designation of groups based on their metabolic activity and membrane damage. Additionally, cell sorting is incorporated to assess each group in terms of growth on different media (MRS-Agar and MRS broth). Results show that the groups with intermediate metabolic activity and some degree of cellular damage correspond with the description of viable but nonculturable cells.

Keywords: viability; fluid bed drying; lactic acid bacteria; stress factors; quality control; rapid assessment

Citation: Kiepś, J.; Juzwa, W.; Dembczyński, R. Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups. *Int. J. Mol. Sci.* **2023**, *24*, 6841. https://doi.org/10.3390/ijms24076841

Received: 27 February 2023
Revised: 3 April 2023
Accepted: 4 April 2023
Published: 6 April 2023

1. Introduction

Probiotic bacteria, which mainly include lactic acid bacteria (LAB), confer various health benefits. Among them, researchers often describe the stimulation of the immune system providing improvement in immunoinflammatory disorders and allergies, reduced risk of colon cancer and modulation of intestinal microbiota [1]. Multiple health benefits are the main reason for the constant interest in probiotic preparations, their properties and methods of their production, as well as tools for their analysis.

Traditionally, probiotic bacteria were supplied in frozen or liquid form, however these have been superseded by the introduction of more convenient dried formulations. Most lactic acid starter cultures and probiotics are prepared in the form of freeze-dried preparations, but alternative drying techniques such as spray drying, fluid bed drying, vacuum drying, or mixed drying systems are gaining in popularity and are being constantly improved [2]. Their main advantages over freeze-drying are high cost efficiency and lower process duration; on the other hand, methods such as spray drying and fluid bed drying introduce additional stress factors including thermal inactivation, and shearing forces occurring during the atomization of the cell suspension. In both cases, fluid bed drying provides lower air temperature and atomizing pressure, which makes it better suited to use with thermolabile and susceptible materials [3]. The obtained probiotic preparations are secured in many ways to ensure the best possible shelf-life and gastrointestinal survival. Fluid bed drying allows the coating of the preparation with protective and coating substances during the process to ensure their enhanced viability [4]. It can be also used to coat

preparations obtained by different methods and can be applied to obtain controlled release of the final product [5]. Protective substances and selected carriers (trehalose, maltose, maltodextrin, lactose, oligofructose, inulin) are also used during spray drying as thermal protectants and to lower the water activity in the preparation for a longer shelf-life [6,7]. An alternative to the addition of protective substances is to subject the bacteria to various stress conditions during their culturing. Bacterial cells adapt after their exposure to stress factors (e.g., heat shock or osmotic stress) which can improve their stability over time and their survival during the drying process. Osmotically stressed *L. acidophilus* ATCC 4356 cells showed better shelf life during long term ambient storage after fluid bed drying in comparison with unstressed fluid bed dried cells [8]. During drying and processing, the probiotic cells are exposed to different stresses. Mechanical stress, mainly shearing forces, occurs during atomization of the microbial solution, osmotic stress is present during dehydration, and thermal stress during the drying stages. More precise analytical methods that can recognize both cellular damage and the metabolic activity of cells are a valuable tool for the production and assessment of probiotics. The viability assessment of probiotics is most commonly conducted by the plate count method—an established and trusted method that lacks, however, the recognition of non-growing cells, and cannot differentiate between the different levels of metabolic activity in the cell. While critical in measuring the number of viable cells in dried probiotic products, it tends to underestimate the cell numbers, especially because of differences in sample preparation, rehydration and dilution preparation [9]. It is also unable to recognize the viable but nonculturable cells (VBNC), which can also show beneficial probiotic effects [10]. Single cell analysis using flow cytometry with fluorescence staining can be used to establish an alternative viability assay for probiotic preparations [11]. For the simultaneous assessment of cell membrane permeability and overall cell viability, a dual staining approach with propidium iodide and RedoxSensor^{TM} Green can be applied [12]. Cell sorting was also introduced to confirm the results obtained by flow cytometry. This allows to sort a selected number of cells (from a single cell up to 10^6) onto either a 96-well plate with liquid medium or a Petri dish with agar. Such analysis was conducted for the cells subjected to process stresses (pH shock and uncontrolled pH during culturing, heat shock during drying) to show the metabolic and physiological differences between the analyzed preparations and to demonstrate the potential of the applied method.

2. Results and Discussion

For cellular staining, two dyes were used: RedoxSensor^{TM} Green and propidium iodide. Each of these allows for the evaluation of different parameters. RedoxSensor^{TM} Green is used to assess the metabolic activity in cells. It is membrane permeable and binds to the reductase enzymes, forming a green fluorescent (520 nm) product. PI in turn is not membrane permeable, which allows for the assessment of membrane integrity in the analyzed cells. Damaged cells absorb the dye, which then binds with DNA and emits a red fluorescence (635 nm). Using simultaneous staining with the mentioned dyes with complex samples presented a distribution indicating four physiologically different subpopulations (Figure 1). The active group shows high values for RSG, indicating high metabolic activity, while the PI signal levels demonstrate almost no cellular damage. The second group, described as dead cells, shows exactly the opposite—high signal values for PI and almost none for RSG. There are also two additional groups, marked mid-active I and mid-active II. In these groups both RSG and PI simultaneously emit their signal, which means that cells in those groups are both damaged and metabolically active (Figure 2). Additionally, the growth of these cells after sorting into liquid and agar media is significantly hindered. Such cells suit the descriptions of VBNC (viable but non-culturable) cells and are omitted in plate cell counting on pour plates [13]. Their recognition is especially important, since due to their metabolic activity these cells could still bring potential health benefits in probiotics.

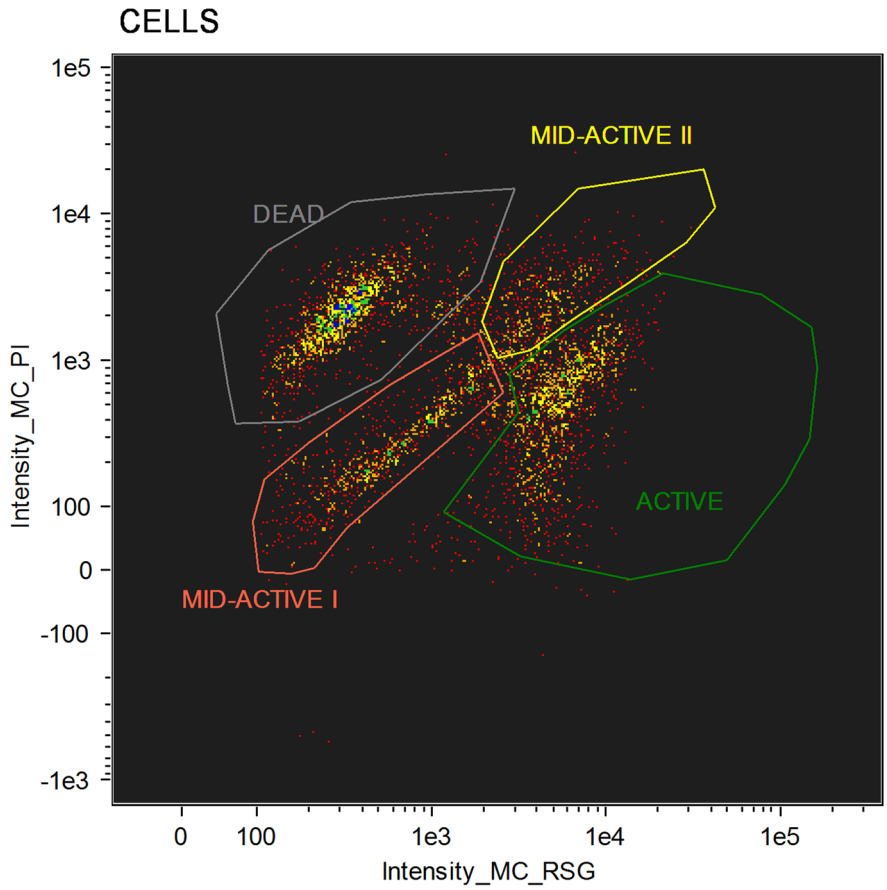


Figure 1. Group distribution after flow cytometry with RedoxSensor^{TM} Green and propidium iodide staining.

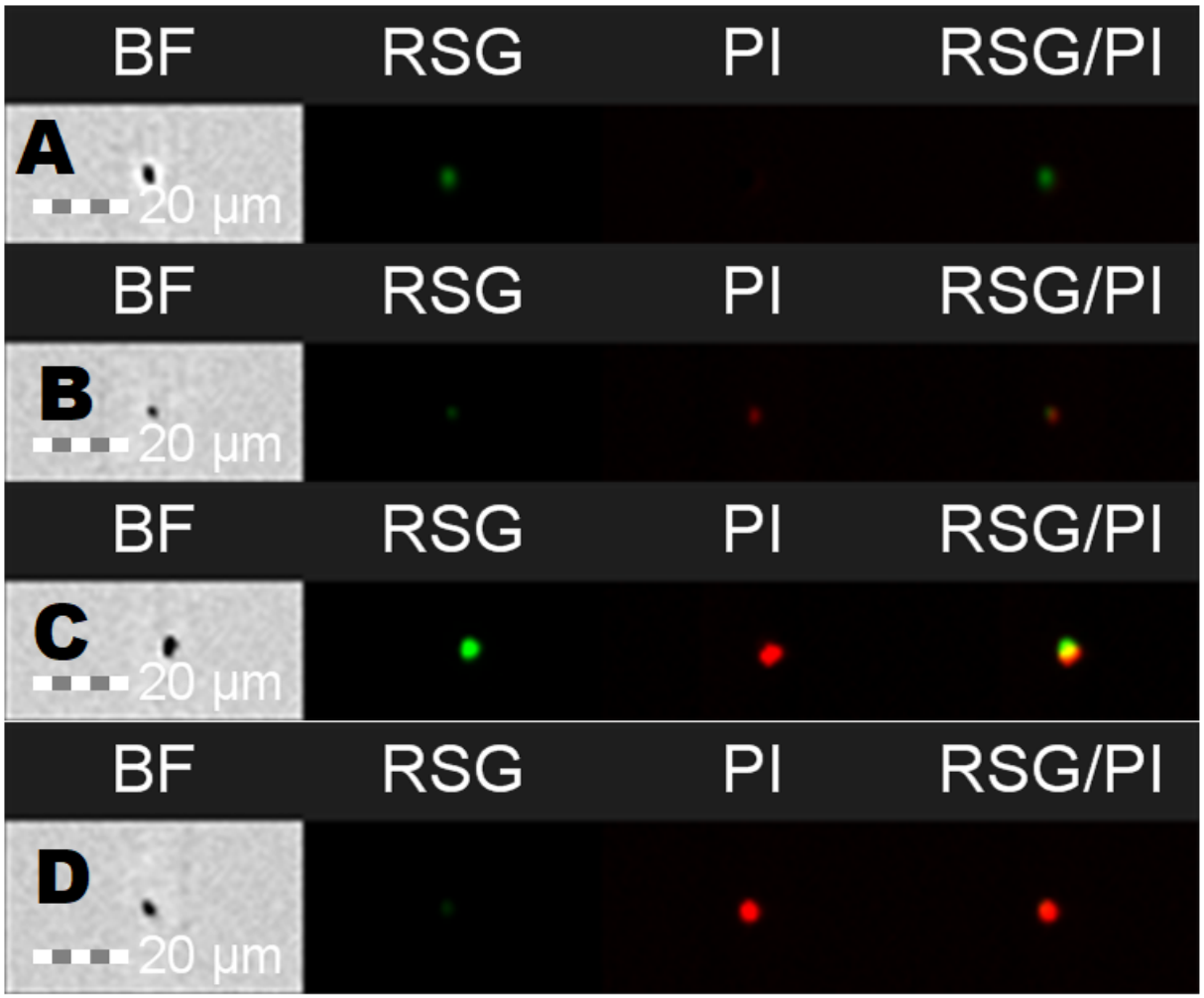


Figure 2. Flow cytometric images of cells for different channels: BF—brightfield, RSG—RedoxSensor^{TM} Green, PI—propidium iodide, RSG/PI—mixed signals for both dyes. Samples represent different subpopulations: A—active, B—mid-active 1, C—mid-active 2, D—dead.

2.1. Viability and Activity of Treated Strains

2.1.1. Control

Cells in the control samples were grown in optimal conditions. This resulted (Figure 3) in most of the cells belonging to the active subpopulation (62.9% ± 0.22 for *E. faecium*, 77.5% ± 1.20 for *L. mesenteroides* and 69.7% ± 0.35 for *C. divergens).* The dead cells accounted for no more than 10% of the total number of cells (9.15% ± 0.62 for *E. Faecium,* 8.02% ± 0.60 for *L. mesenteroides* and 8.07% ± 0.37 for *C. divergens).* The mid-active II subpopulation constituted 17.93% ± 0.26 for *E. faecium*, 6.21% ± 0.73 for *L. mesenteroides* and 15.35% ± 0.27 for *C. divergens.* The mid-active I subpopulation occurred the least, with 0.84% ± 0.07 for *E. faecium*, 0.39% ± 0.10 for *L. mesenteroides* and 0.21% ± 0.08 for *C. divergens.*

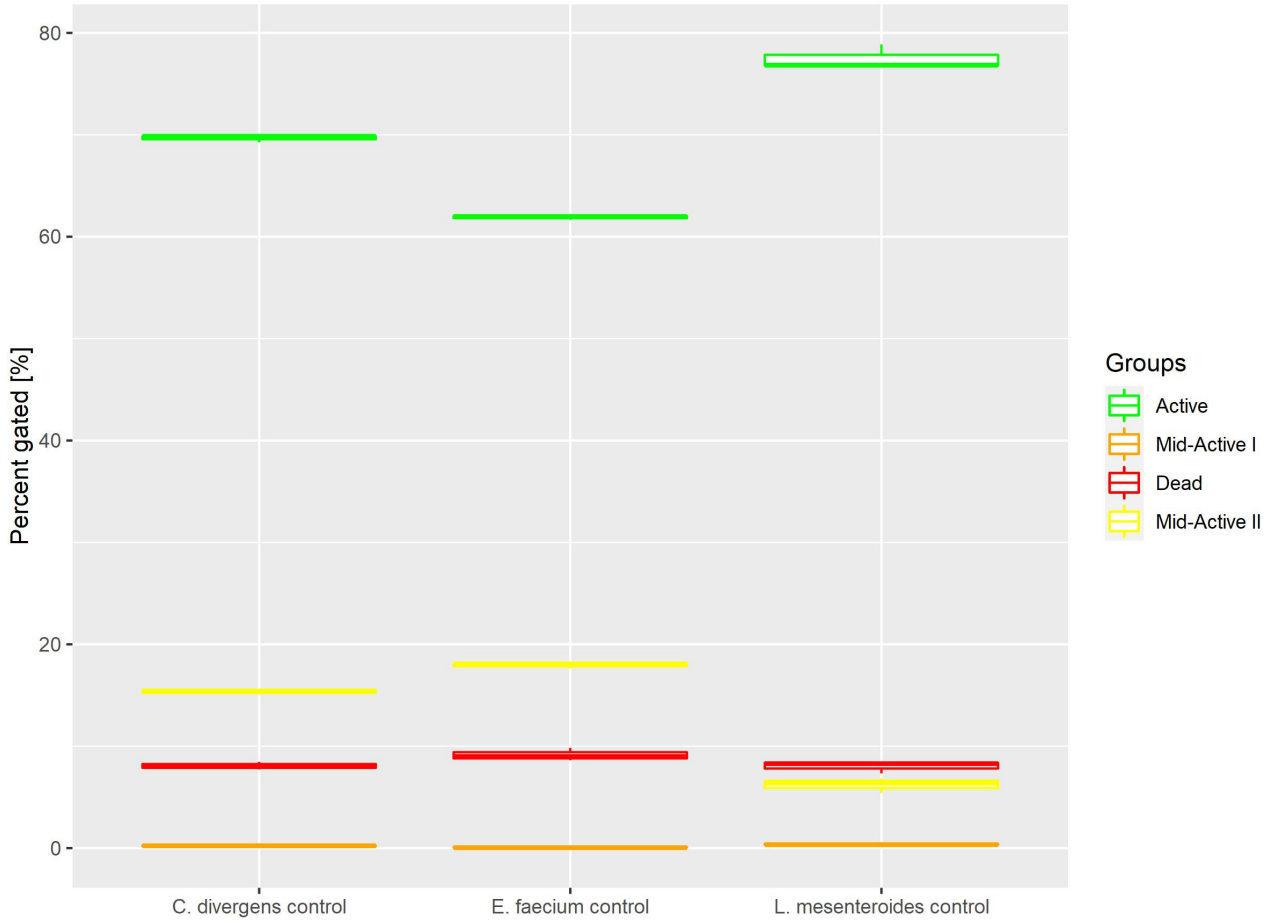


Figure 3. Cell subpopulation percentages in control samples. The distribution of cells between different groups was measured after culturing in optimal conditions.

2.1.2. Heat Shock

Heat shock samples presented differences between the tested strains (Figure 4). For *E. faecium* and *C. divergens*, most of the cells belonged to the active subpopulation (63.79% ± 1.12 for *E. faecium*, 25.23% ± 1.20 for *L. mesenteroides* and 57.87% ± 1.50 for *C. divergens).* Meanwhile, *L. mesenteroides* proved to be less resistant to heat stress, and the dead cells were the most numerous subpopulation for that strain (6.12% ± 0.68 for *E. faecium*, 26.5% ± 0.88 for *L. mesenteroides* and 10.50% ± 0.37 for *C. divergens).* The mid-active II subpopulation reached values of 21.60% ± 1.24 for *E. faecium*, 33.86% ± 0.18 for *L. mesenteroides* and 21.46% ± 1.17 for *C. divergens.* The mid-active I subpopulation occurred the least, with 0.09% ± 0.06 for *E. faecium*, 1.03% ± 0.17 for *L. mesenteroides* and 0.41% ± 0.14 for *C. divergens.*

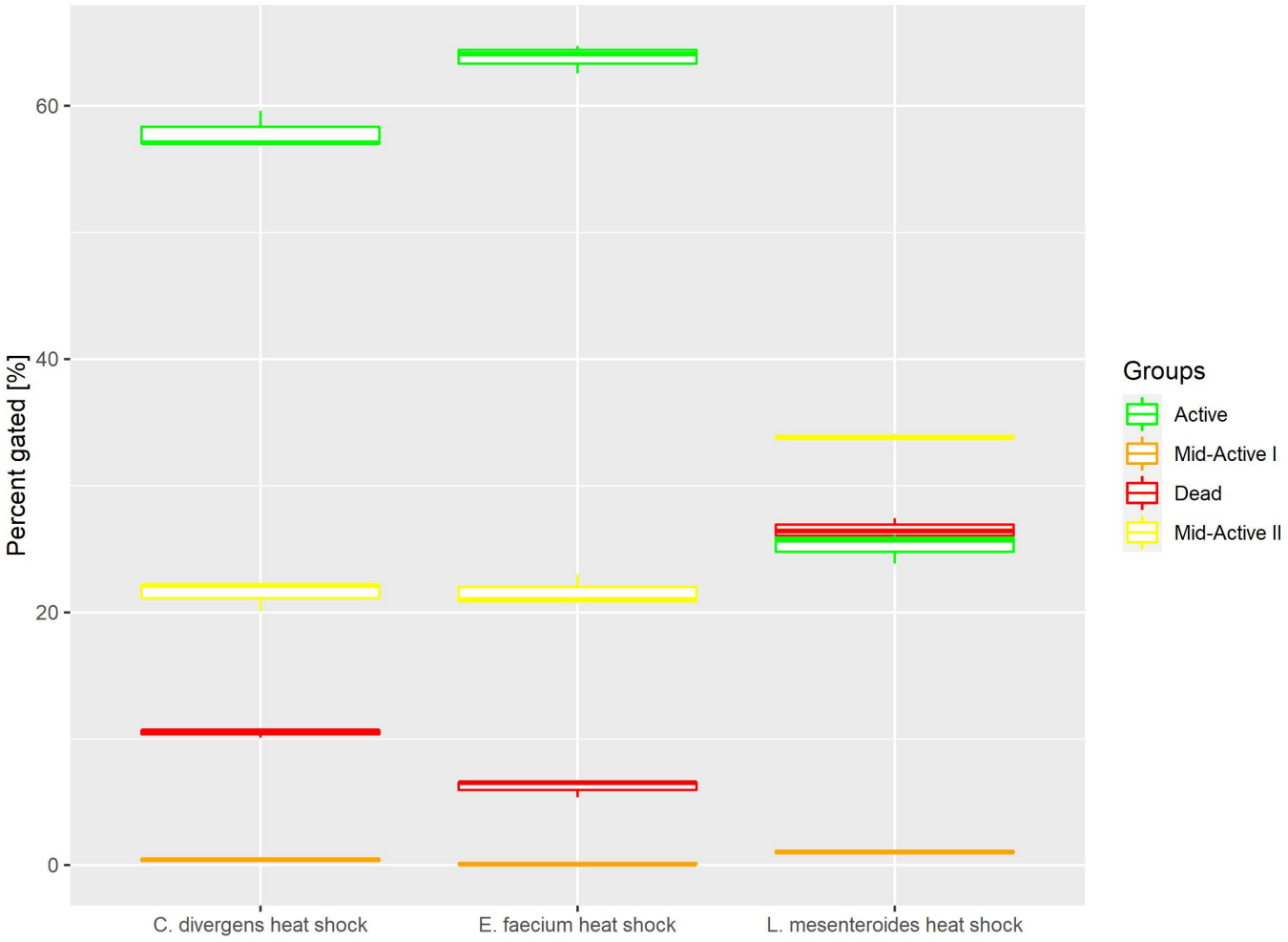


Figure 4. Cell subpopulation percentages in samples after heat shock. The distribution of cells between different groups was measured after incubating cells at 50 °C for 30 min.

2.1.3. pH Shock

Cells in the control samples were exposed to a pH shock. This resulted (Figure 5) in most of the cells being dead (71.68% ± 1.23 for *E. faecium*, 83.86% ± 0.53 for *L. mesenteroides* and 65.05% ± 0.65 for *C. divergens).* The active cells accounted for no more than 10% of the total number of cells (3.17% ± 0.68 for *E. faecium*, 3.04% ± 0.12 for *L. mesenteroides* and 9.08% ± 0.26 for *C. divergens).* The mid-active I subpopulation occurred the least, with 2.06% ± 0.27 for *E. faecium*, 1.03% ± 0.10 for *L. mesenteroides* and 6.63% ± 0.91 for *C. divergens.* The mid-active II subpopulation constituted 16.54% ± 1.03 for *E. faecium*, 8.47% ± 0.56 for *L. mesenteroides* and 11.49% ± 0.76 for *C. divergens.*

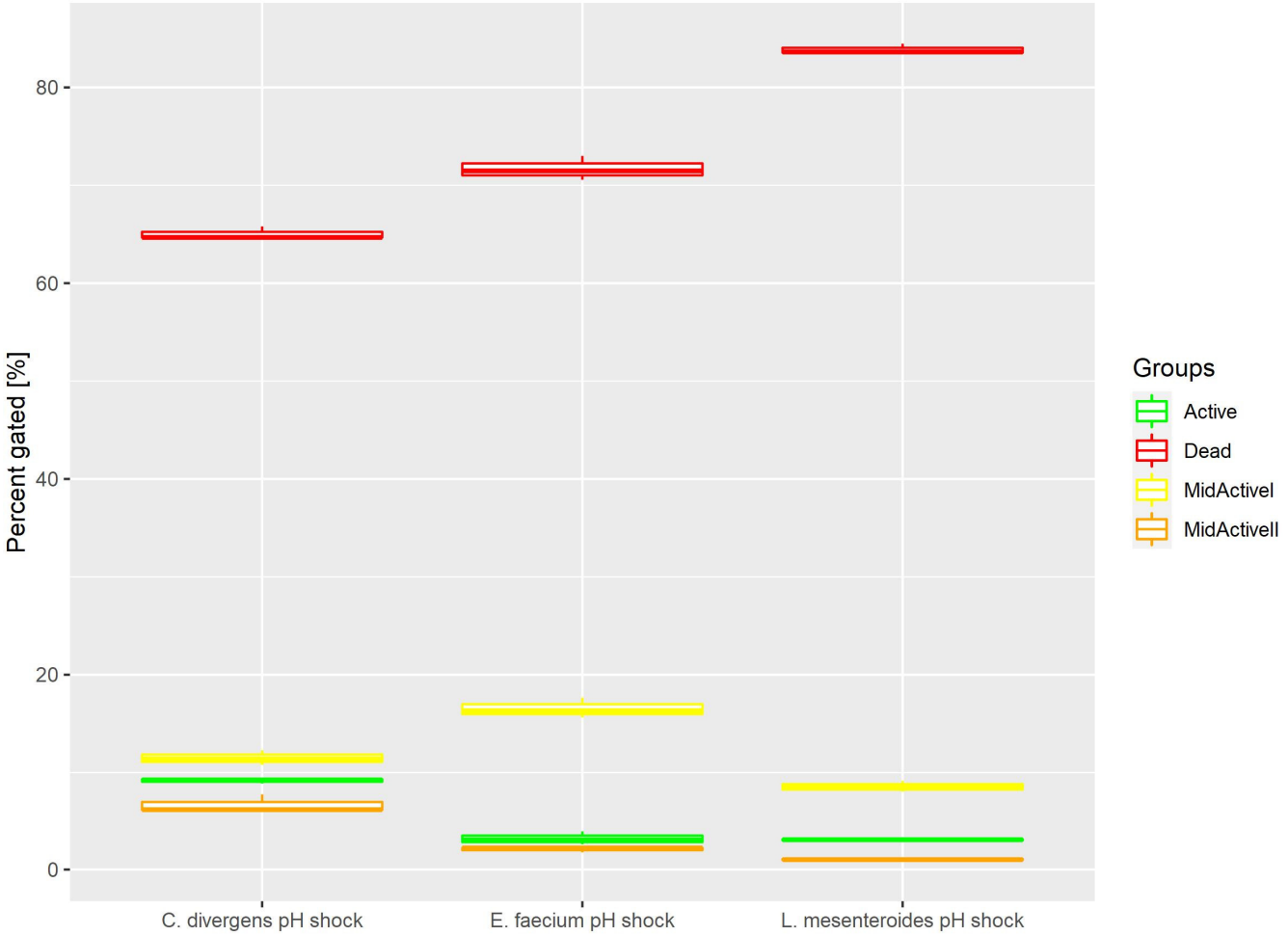


Figure 5. Cell subpopulation percentages in samples after pH shock. The distribution of cells between different groups was measured after exposing cells to pH 2.5 for 30 min.

2.1.4. Uncontrolled pH

Cells in the control samples were grown without pH control. This resulted (Figure 6) in the active and dead cell subpopulations distributing evenly. Active: 32.22% ± 1.00 for *E. faecium*, 32.32% ± 1.85 for *L. mesenteroides* and 29.85% ± 1.39 for *C. divergens*. Dead: 34.59% ± 0.20 for *E. faecium*, 34.25% ± 2.09 for *L. mesenteroides* and 32.88% ± 1.73 for *C. divergens*. The mid-active II subpopulation was calculated at 11.08% ± 0.53 for *E. faecium*, 20.58% ± 0.52 for *L. mesenteroides* and 11.02% ± 0.73 for *C. divergens*. The mid-active I subpopulation was observed in 14.22% ± 0.53 for *E. faecium*, 6.15% ± 0.36 for *L. mesenteroides* and 17.79% ± 0.54 for *C. divergens*.

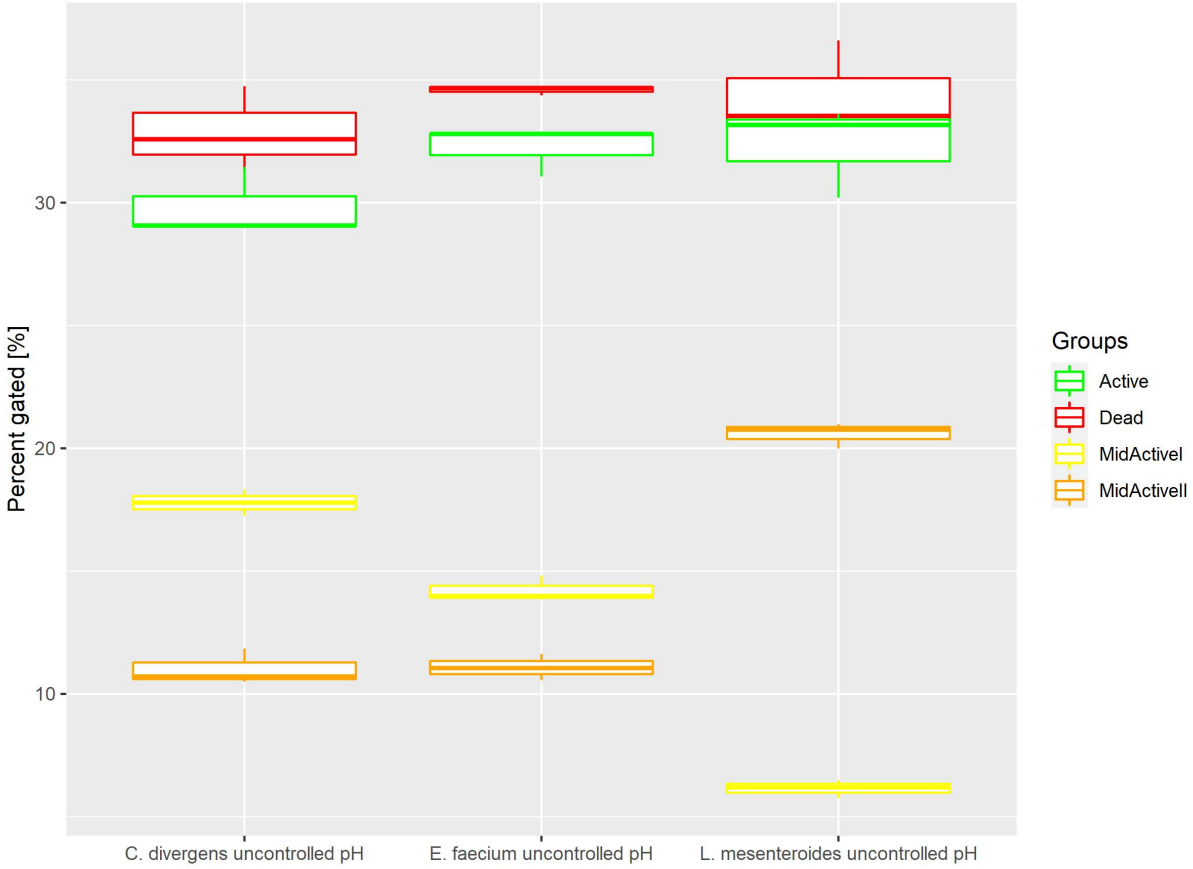


Figure 6. Cell subpopulation percentages in samples cultured without pH control. The distribution of cells between different groups was measured after culturing without pH regulation.

2.1.5. Dried Samples

Cells in the dried samples were exposed to temperature as well as dehydration. This resulted (Figure 7) in the active and dead cell subpopulations distributing evenly. Active: 36.31% ± 0.61 for *E. faecium*, 33.23% ± 0.39 for *L. mesenteroides* and 34.78% ± 0.91 for *C. divergens.* Dead: 6.49% ± 0.31 for *E. faecium,* 10.42% ± 0.42 for *L. mesenteroides* and 9.22% ± 0.43% for *C. divergens.* The mid-active II subpopulation was calculated at 41.77% ± 0.22 for *E. faecium*, 44.61% ± 0.05 for *L. mesenteroides* and 39.45% ± 0.32 for *C. divergens.* The mid-active I subpopulation occurred the least, with 0.69% ± 0.10 for *E. faecium*, 0.41% ± 0.05 for *L. mesenteroides* and 0.68% ± 0.12 for *C. divergens.*

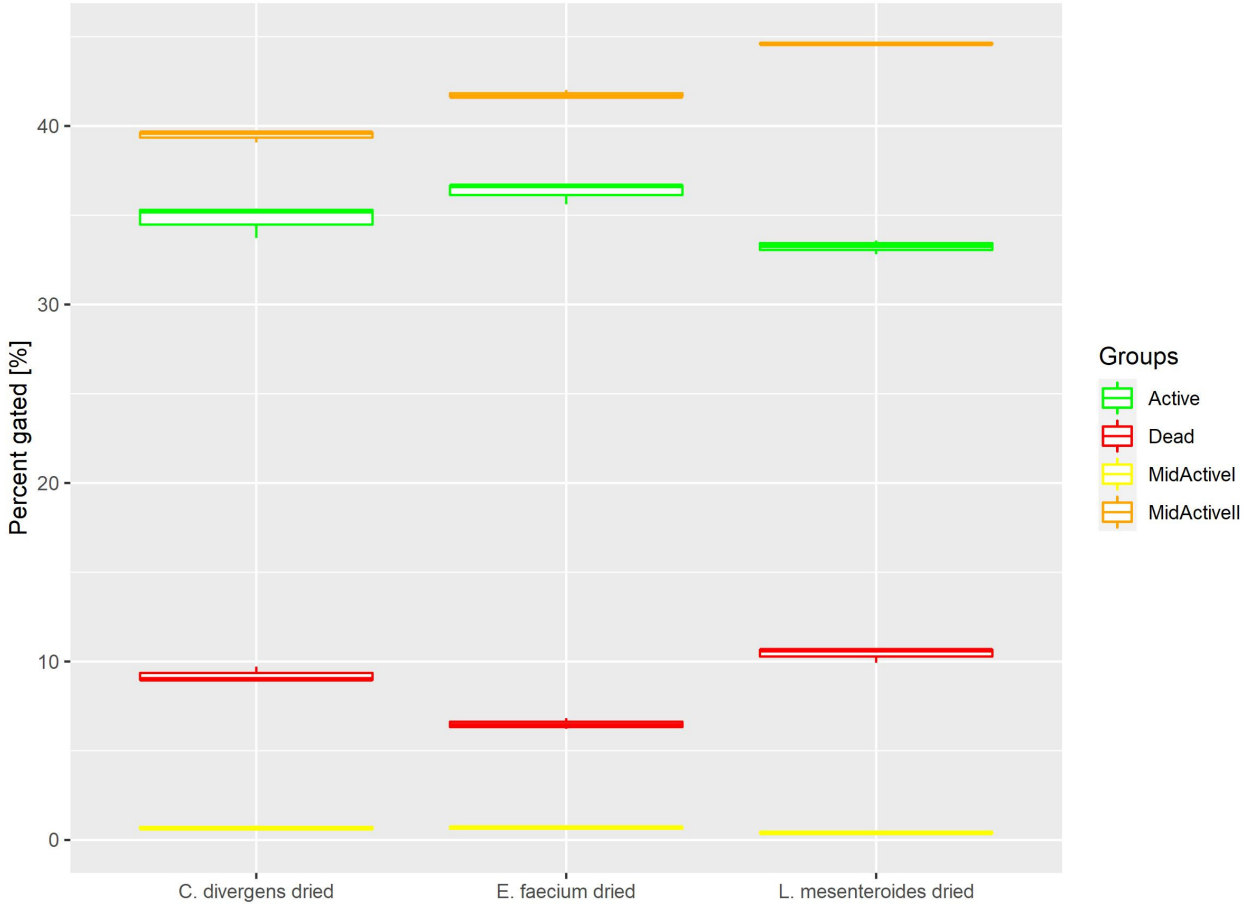


Figure 7. Cell subpopulation percentages in samples after fluid bed drying.

2.1.6. Coated Samples

Cells in the coated samples were exposed to a higher temperature for twice the time of the dried samples. This resulted (Figure 8) in most of the cells being dead (73.53% ± 0.59 for *E. faecium*, 87.05% ± 0.66 for *L. mesenteroides* and 90.06% ± 0.53 for *C. divergens).* The active cells accounted for no more than 1% of the total number of cells (0.99% ± 0.12 for *E. faecium*, 0.43% ± 0.07 for *L. mesenteroides* and 0.32% ± 0.20 for *C. divergens*). The mid-active II subpopulation constituted 8.67% ± 0.46 for *E. faecium*, 1.94% ± 0.30 for *L. mesenteroides* and 1.26% ± 0.15 for *C. divergens.* The mid-active I subpopulation was observed in the following percentages 11.71% ± 0.93 for *E. faecium*, 7.63% ± 0.58 for *L. mesenteroides* and 6.18% ± 0.40 for *C. divergens.*

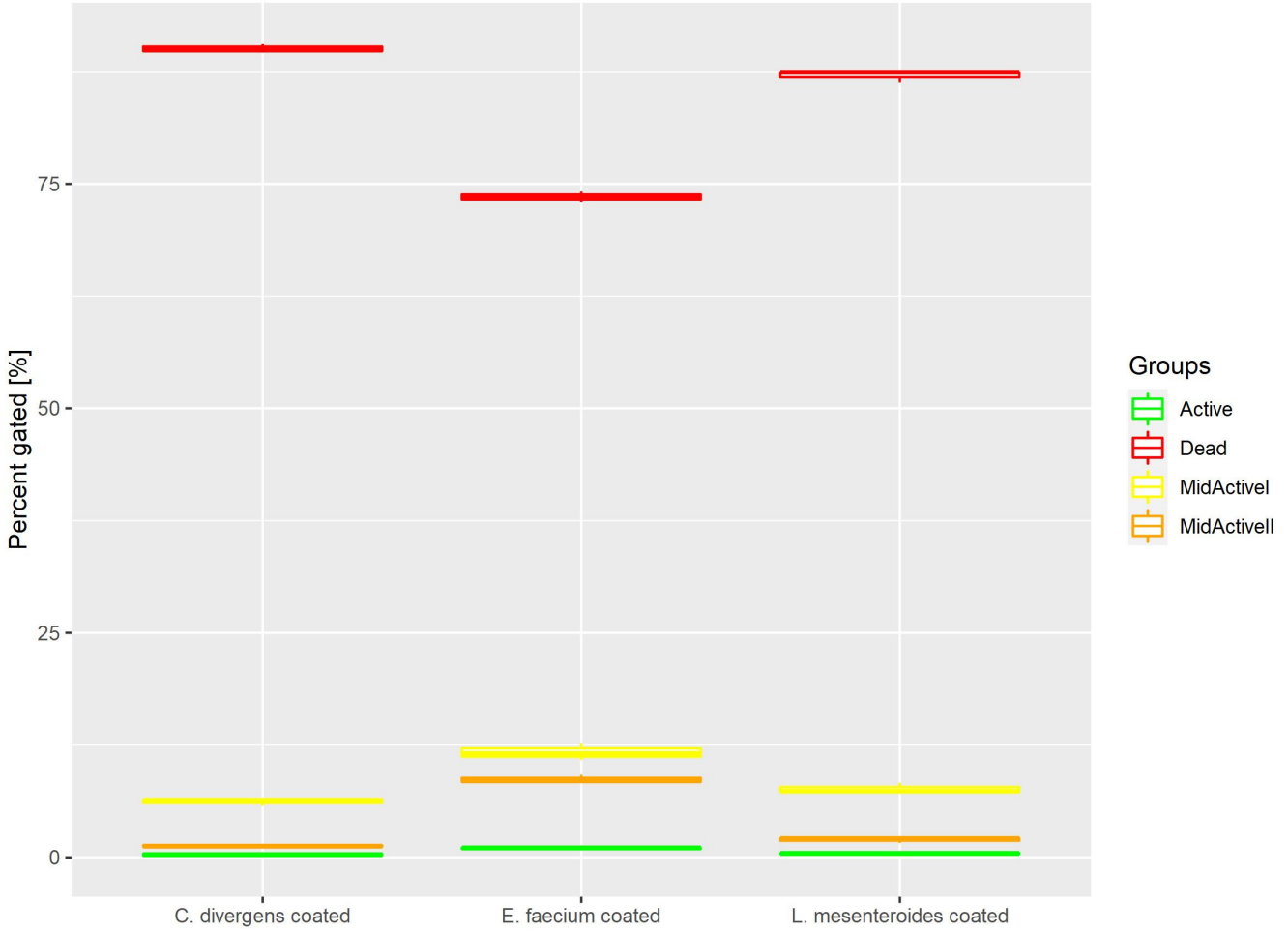


Figure 8. Cell subpopulation percentages in dried samples after coating.

2.2. Comparison of Plate Counts and Flow Cytometric Analysis

Cells were enumerated using plate counts and during flow cytometric analysis (Table 1). While the total cell counts are comparable, the differences in the number of cells counted for the same sample using both methods come mainly from the flow cytometry taking into consideration cells from groups marked as dead and mid-active, while plate counts were able to take only growing cells, marked as active, into account. Other factors affecting the cell counts in flow cytometry are the software gates that were set to eliminate non-cellular debris and other pollutants from the analytic image. Similarly, gates based on aspect ratio and area of observed objects were set to discriminate between single cells and aggregates, which lead to the removal of cell aggregates and therefore lowered the final cell count in comparison with plate cell counts. This is especially notable in the case of strongly aggregating cells, such as *E. faecium* and *L. mesenteroides*, and is more prominent in stressed samples, since the formation of aggregates is one of the defense mechanisms employed by cells undergoing stress [14,15]. The advantage of flow cytometry is its speed (no need for incubation as in plate cell counts) and the ability of universal multiparametric analysis. Plate cell counts require a range of selective media, and no single medium or isolation protocol is viable for all probiotic strains. Reliable enumeration also requires a certain number of colonies on a plate, commonly ranging between 30–300 cfu/plate, which constitutes a relatively narrow range, and the necessity for multiple dilutions further reduces the accuracy of this method [16].

Table 1. Comparison of bacterial cell enumeration using plate cell counts and flow cytometric analysis.

Sample Name	Plate Cell Counts [cfu/mL]	Flow Cytometry [obj/mL]				
		Total	Dead	Mid-Active I	Mid-Active II	Active
L. mesenteroides	2.7×10^8	3.57×10^8	2.16×10^7	1.05×10^6	1.68×10^7	2.10×10^8
L. mesenteroides heat shock	1.29×10^8	1.85×10^8	3.52×10^7	1.36×10^6	4.50×10^7	3.35×10^7
E. faecium	4.05×10^8	2.72×10^8	2.17×10^7	2.02×10^5	4.26×10^7	1.47×10^8
E. faecium heat shock	5.85×10^8	2.53×10^8	1.32×10^7	2.04×10^5	4.65×10^7	1.37×10^8
C. divergens	4.05×10^8	3.40×10^8	2.13×10^7	5.68×10^5	4.05×10^7	1.84×10^8
C. divergens heat shock	9.35×10^7	2.50×10^8	2.13×10^7	8.37×10^5	4.36×10^7	1.18×10^8

2.3. Cell Sorting into Titration Plates and Petri Dishes

The imaging flow cytometry supported the sorting of microbial cells from the defined subpopulations, aimed at the correlation of flow cytometric data (evaluation of the structure and physiology of the bacterial cells) with the growing potential of the defined subpopulations. The potential of this approach has been demonstrated in previous work [17]. This was performed using the cell sorter's single-cell sorting mode. The procedure involved the sorting of single bacterial cells from the defined subpopulations into separate spots on a Petri dish containing MRS-Agar medium. The Petri dish surface was divided into four separate sections corresponding to subpopulations of active, dead, and two discrete subpopulations of mid-active cells: mid-active I and mid-active II (Table 2). Using the cell sorter, 24 single cells from each sample were dropped onto plates. The growth on the Petri dishes was observed as colony forming units and was measured after 72 h of incubation to allow the colonies to form from a single cell. The measurement of growth on the titration plates was conducted using a plate reader. An optical density of 600 nm was used to determine whether growth had occurred in wells. OD600 was first measured for sterile MRS broth and then subtracted from further measurements. In this way, the threshold of OD600 that was used to detect growth was established at 0.1. The ability of the mid-active I and mid-active II groups to grow even while undergoing some cellular damage can point to those cells entering the VBNC state. This is described as a group with lower metabolic activity, unable to grow on routine laboratory media [13], which can be observed for the mid-active I group in coated *L. mesenteroides* samples. The results of the assessment of cell viability using flow cytometry correspond with the results obtained using plate cell counts. The group discrimination established based on flow cytometric data is also confirmed by the results of cell sorting. The mid-active I and mid-active II groups are characterized by differences in metabolic activity (higher for mid-active II), and the mid-active II group was able to grow on plates, while there was little to no growth for samples from the mid-active I group. The effect of incubation time is also noteworthy: differences in growth were observed not only for different media but also after 72 h in comparison with growth after 48h.

Table 2. Growth rates of bacterial cells after sorting on titration plates with solid medium and 96-well plates with liquid medium.

Sample Name	MRS-Agar on Petri Dishes [cfu]				MRS Broth on 96-Well Plates [OD600 > 0.1]			
	Active	Mid-Active I	Mid-Active II	Dead	Active	Mid-Active I	Mid-Active II	Dead
E. faecium dried 48 h/72 h	20.8%/37.5%	0%/0%	8.3%/8.3%	0%/0%	75%/83.3%	0%/0%	8.3%/16.7%	0%/0%
E. faecium coated 48 h/72 h	20.8%/29.2%	0%/0%	25%/29.2%	0%/0%	20.8%/29.2%	0%/0%	12.5%/12.5%	0%/0%
L. mesenteroides dried 48 h/72 h	4.2%/8.3%	0%/0%	8.3%/8.3%	0%/0%	4.2%/8.3%	0%/0%	0%/0%	0%/0%
L. mesenteroides coated 48 h/72 h	20.8%/33.3%	0%/0%	4.2%/12.5%	0%/0%	8.3%/20.8%	4.2%/12.5%	4.2%/4.2%	0%/4.2%

2.4. Imaging Flow Cytometry and Its Use in the Assessment of Diversity within Complex Populations

The uniqueness of the conducted research lies in the prospect of the use of imaging flow cytometry (IFC) in combination with specific fluorescent staining to enable the in-depth characterization of the physiological states of bacterial cells from tested samples. This was due to gaining a higher data resolution and the direct correlation of fluorescence intensity measurement (definition of subpopulation) with cellular morphology. The correlation with cell morphology, based on the digital signal processing of generated images of the analyzed objects (cells), provided a significant step forward with regard to interpretation of the results. Thus, the simultaneous discrimination of bacterial cells from non-cellular debris, e.g., biofilm particles and single cells from aggregates, was facilitated. BacLight™ RedoxSensor™ Green Vitality Kit (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) was used to characterize and distinguish the different physiological states of the bacterial cells, providing the definition of subpopulations of active, dead, and two discrete subpopulations of mid-active cells: mid-active I and mid-active II (Figure 9). Thus, the significant resolution of the applied assay enabled the monitoring of the physiological status of the bacterial cells, revealing functional heterogeneity of microbes within the tested samples at single cell level [18].

The in-depth characterization of the physiological states of bacterial cells from tested samples accomplished using the IFC-based assay is also associated with the concept of the supported sorting approach. This concept assumes the use of the significant resolving power of the processed IFC data to define precise boundaries (gate definition) of microbial subpopulation for cell sorting experiments. This approach implemented in our research improved the unique cellular feature definition to provide more specific isolation of microbial cells from active, dead and two discrete subpopulations of mid-active cells: mid-active I and mid-active II subpopulations. Flow cytometry allows to observe VBNC cells in commercial preparations [19,20] and can be used to provide more accurate information much faster than classical microbiological methods, which require long incubation times. Other methods used as an alternative to plate cell counts, such as fluorescent in-situ hybridization, and nucleic acid-based enumeration methods, such as reverse transcriptase PCR (RT-PCR) and real-time quantitative PCR (qPCR), are useful tools for research and clinical trials yet are not suitable, however, for quality control or industry [16,21]. Herein lies the versatility of flow cytometry, which can be used for the multiparametric analysis of cells in various applications, both for research and industrial alike.

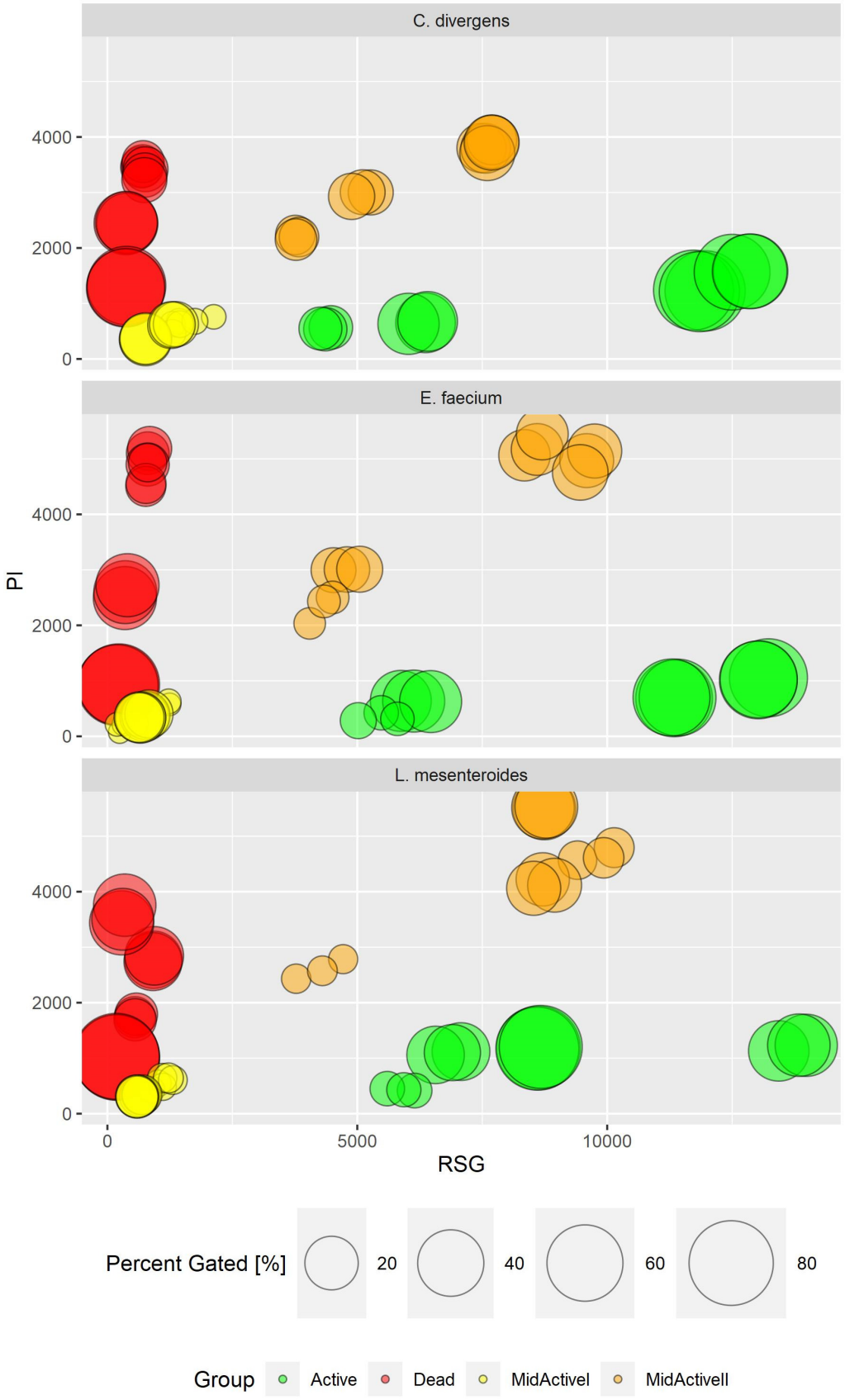


Figure 9. Subpopulation distribution in different strains by PI and RSG signal value (in RFU—relative fluorescence units). Comparison of results for all experiments.

3. Materials and Methods

3.1. Inoculum Preparation

Strains chosen for this experiment were *L. mesenteroides 51* KBiMŻ, *E. faecium 73* KBiMŻ, and *C. divergens* 3 KBiMŻ. All three strains were identified using MALDI-TOF mass spectrometry. MRS broth was chosen for propagation as a medium ensuring optimal growth conditions for lactic acid bacteria. To obtain the highest biomass yield, it is necessary to prepare inoculum in a volume of 10% of the bioreactor culture. Therefore, for a culture volume of 1 L, preparation was conducted in two stages, gradually increasing the volume, which allowed for better adaptation of microorganisms and shortened the resting phase in culture. Preparation of the inoculum was carried out in a laminar chamber to reduce the risk of infection. The first step was to thaw the strain, stored in the freezer, which was carried out on ice to limit cell damage that could occur in case of faster thawing. After the microorganisms had thawed and reached room temperature, they were transferred to a 15 mL Falcon conical tube containing 9 mL MRS broth, which was then sealed with parafilm and incubated for 24 h at 30 °C. After incubation, 10 mL of the inoculum was transferred to a flask containing 90 mL of MRS broth, followed by another 24 h incubation at 30 °C. After these steps, the inoculum was ready to be used to start a culture in the bioreactor.

3.2. Bacterial Cultures

Cell biomass was cultured using Biostat A plus bioreactors [Sartorius]. Before culturing, the pH electrode was calibrated against buffers at pH 4 and pH 9. One liter of MRS broth medium was used for seeding. The bioreactor was subsequently autoclaved at 121 °C with a 20 min exposure time to provide sterile conditions. After sterilization and cooling of the bioreactor, the inoculum was added in a volume of 10% of the volume of the medium (100 mL), while the bioreactor was pressurized with nitrogen to minimize the risk of infection with airborne microorganisms. In the inoculated bioreactor, the culture was grown at 30 °C, with a stirrer speed of 150 RPM and pH set at 6.5 (the optimal value for the selected strains). The pH value was monitored and kept constant by regulation with a 30% NaOH solution. The culture was grown for 24 h, and the end of the exponential growth phase was conferred by a graph of NaOH consumption and pH changes over time. The stabilization of pH at the set level with the simultaneous absence of base consumption indicated the end of microbial growth and inhibition of the production of acidifying metabolites. After completion of the culturing, the culture was pumped into sterilized centrifuge vessels with a spout hose, using a peristaltic pump.

3.3. Stress Factors during Culturing

To introduce stress conditions, certain parameters were changed for different cultures, e.g., short-term (30 min) thermal and acid stress were introduced by increasing the temperature (up to 50 °C) or by changing the pH to 2.5. In another variant the culturing was also conducted without pH control. The purpose of these changes in conditions was to check whether the stress induced on bacterial cells significantly affects their survival during fluidized bed drying.

3.4. Fluid Bed Drying

GEA Strea-1 laboratory fluid bed dryer was used for the drying process. Firstly, the matrix (crystalline microcellulose or starch products) was added to the drum of the fluid bed dryer. Next, the stream of filtered and heated air was introduced through the bottom perforated plate, allowing to keep the matrix in the fluid phase and ensuring even drying in a set temperature range (up to 50 °C). Microorganisms suspended in the solution of a protective substance (5% trehalose) were fed to the dryer by an external peristaltic pump. They entered an atomizing nozzle, supplied with air under the pressure of 2 bar. Drying and coating took approximately 30 min for each step using 100 g of matrix and after completion of the process the obtained samples were packed for storage and further analysis.

3.5. Plate Count Method

The plate counting method was used in combination with flow cytometry to determine the number of live microorganisms present in the samples after drying and in the bioreactor culture. This was carried out under sterile conditions under a laminar chamber, in duplicate, and the decimal dilution method was used to prepare the samples. It involves the preparation of tubes containing 9 mL of solvent (0.9% NaCl), and then pipetting 1 mL of the sample into the first tube, so that a dilution of 10^{-1} is obtained. After thoroughly vortexing the tube to mix the sample evenly, 1 mL of the sample with a dilution of 10^{-1} is transferred to the next tube, and the whole process is repeated until the required order of dilution is obtained. In the case of cell cultures, it was necessary to prepare dilutions of up to 10^{-9} for the resulting samples. For the culture fluid and suspension for drying it was possible to take 1 mL directly for dilution, however, in the case of the finished formulation, i.e., the granulate, it was necessary to rehydrate it. For that purpose, 1 g of sample was weighed into a 99 mL flask with 0.9% NaCl, which was then placed in a 37 °C water bath. After 30 min of shaking in a water bath, further dilutions were prepared using the suspension. 1 mL of diluted samples were applied to the Petri dishes. The samples on the plates were then poured with MRS-Agar medium (previously sterilized and stored at 55 °C to prevent solidification of the medium) cooled to about 45 °C, mixed thoroughly and then allowed to solidify. The Petri dishes were incubated in aerobic conditions in an incubation chamber at 30 °C for 48 h, after which time visible colonies were counted and evaluated. The results of counting visible colonies are considered statistically significant only for plates with the dilution sample for which the number of colonies ranged from 30 to 300.

3.6. Flow Cytometry and Cell Sorting

Flow cytometry is a cell count method that was used as an alternative method to classic plate cultures. Bacterial cells were examined for cellular metabolic activity and viability using imaging flow cytometer Amnis FlowSight™ (Luminex Corp., Austin, TX, USA) equipped with three lasers (405 nm, 488 nm and 642 nm), five fluorescence channels (acquisition by a multi-channel CCD camera), and side scatter detector (SSC). Post-acquisition data analysis was performed using the IDEAS software (Luminex Corp., Austin, TX, USA). Three-step morphological characteristics of the analyzed cells were performed: (i) in the first step the Gradient RMS parameter from brightfield signals (Ch01) was used, which enabled the discrimination of the high resolution cell images, (ii) the second step involved the brightfield digital image processing parameters: Aspect Ratio and Area to characterize shape and size of the analyzed bacterial cells in combination with the discrimination of bacterial cells from non-cellular debris (particles from prebiotic components) and single cells from aggregates. The viability and activity of probiotic bacteria cells in the samples was determined by fluorescent staining with RedoxSensor™ Green (Figure 10) and PI (propidium iodide) (Figure 11). The samples for analysis were prepared by centrifugation and then suspended in a 1% PBS buffer in 1:200 dilution. Then, the following dyes were added to the samples pipetted into Eppendorf tubes in a volume of 500 ul: 1.6 µL of RedoxSensor™ Green and 1.2 µL of PI. The cells in the samples were counted and assessed for morphology (microscopic image), activity (signal for RedoxSensor™ Green) and integrity of the cell membrane (signal for PI). After the mentioned steps, the cells were sorted to isolate and further analyze populations of interest (e.g., cells with reduced metabolic activity—VBNC). The sorter allows to isolate a designated number of cells into titration plates with MRS broth or Petri dishes with MRS-Agar medium. Cell sorting is conducted based on readings obtained using fluorescent staining. Four different cell groups were observed in samples, namely active, mid-active I, mid-active II and dead cells. They were determined by two separate parameters—their metabolic activity (RedoxSensor™ Green) and viability (PI). Cells from all groups were then sorted using the BD FACS Aria™III (Becton Dickinson, USA) cell sorter into separate spots on Petri dishes or separate wells on 96-well titration plates. The configuration of the instrument was as follows: four lasers (375 nm, 405 nm, 488 nm and 633 nm), eleven fluorescence detectors, forward scatter (FSC) and

side scatter (SSC) detectors; 70 µm nozzle and 70 psi (0.483 MPa) sheath fluid pressure. For growth on Petri dishes, the colony forming units were enumerated after 48 h of incubation at 30 °C. For titration plates, the optical density at 600nm was measured in all wells after 48 and 72 h.

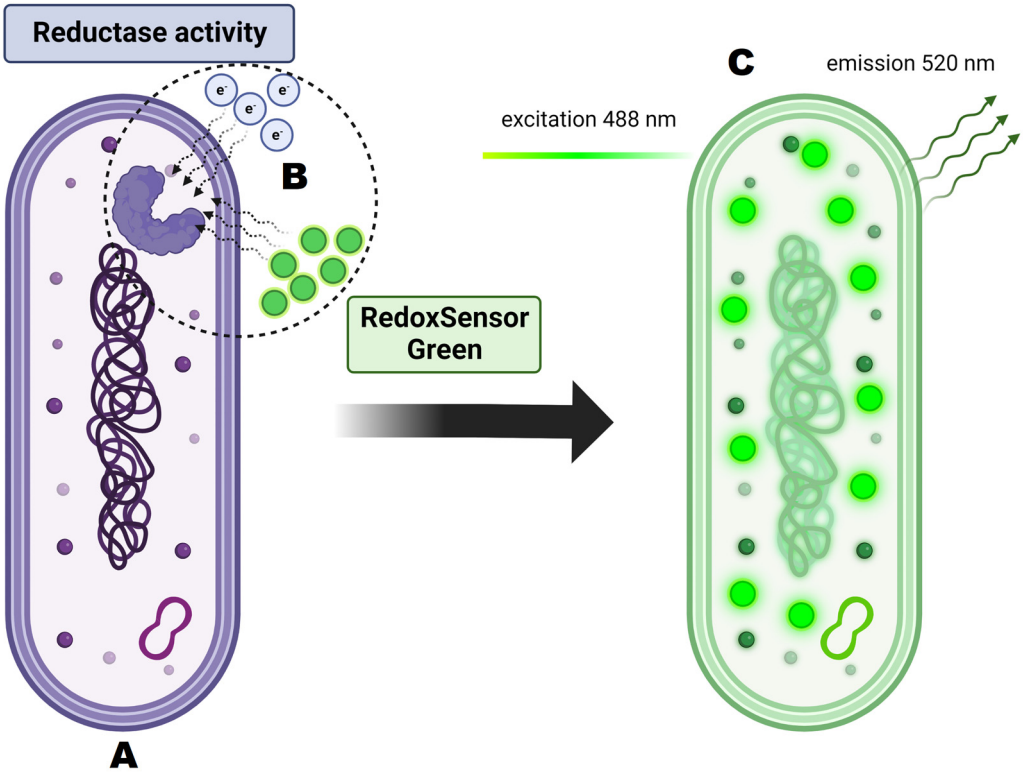


Figure 10. Principles of RedoxSensor™ Green staining and its activity on the cellular level. (**A**) Analyzed cell is dyed with RedoxSensor™ Green. (**B**) Dye molecules permeate the cell membrane and interact with reductases. Reductase activity reflects changes in electron transport chain function and in vitality. (**C**) After excitation at 488 nm the dye emits green-fluorescent signal at 520 nm.

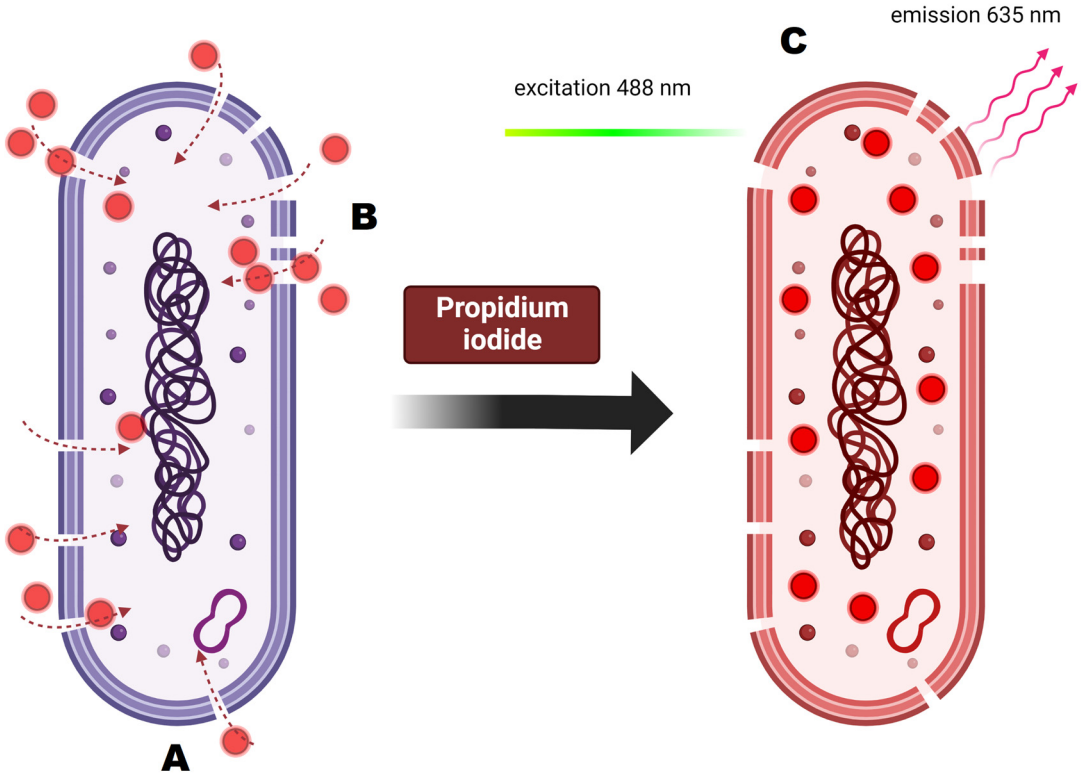


Figure 11. Principles of propidium iodide staining and its activity on the cellular level. (**A**) Analyzed cell is dyed with propidium iodide. (**B**) PI is membrane impermeable so it enters only the cells with damaged cell membrane. (**C**) Once inside the cell, PI binds to DNA by intercalating between the bases with no preference. After binding, the fluorescence is enhanced and excited at 488 nm, resulting in emission maximum at 635.

3.7. Machine-Learning Assisted Discrimination of Cells vs. Debris in Tested Samples

A machine learning (ML)-based protocol was employed to facilitate and improve the discrimination of cells from cellular and non-cellular debris (Figure 12). The Machine Learning (ML) module is incorporated in to the IDEAS® 6.3. software, which was designed to process data acquired by Amnis Flow Sight imaging flow cytometer (Luminex Corp., Austin, TX, USA). After manual selection of the two "truth" populations, the ML algorithm calculated two super features (classifiers) that maximally separated each "truth" population from the others. The implementation of the ML module was described in detail in the work of Konieczny et al. [22]. For every classifier, truth populations between 31 and 32 events were manually tagged and loaded into the ML module. ML generated and tested features of all eight main categories corresponding to the BF and SSC channels. The resulting cells_vs_debris_classifier contains a series of seven differentially weighted features/parameters (Table 3). Both classifiers were plotted into histograms, and events with values higher than zero belong to images that are best represented by their classifier.

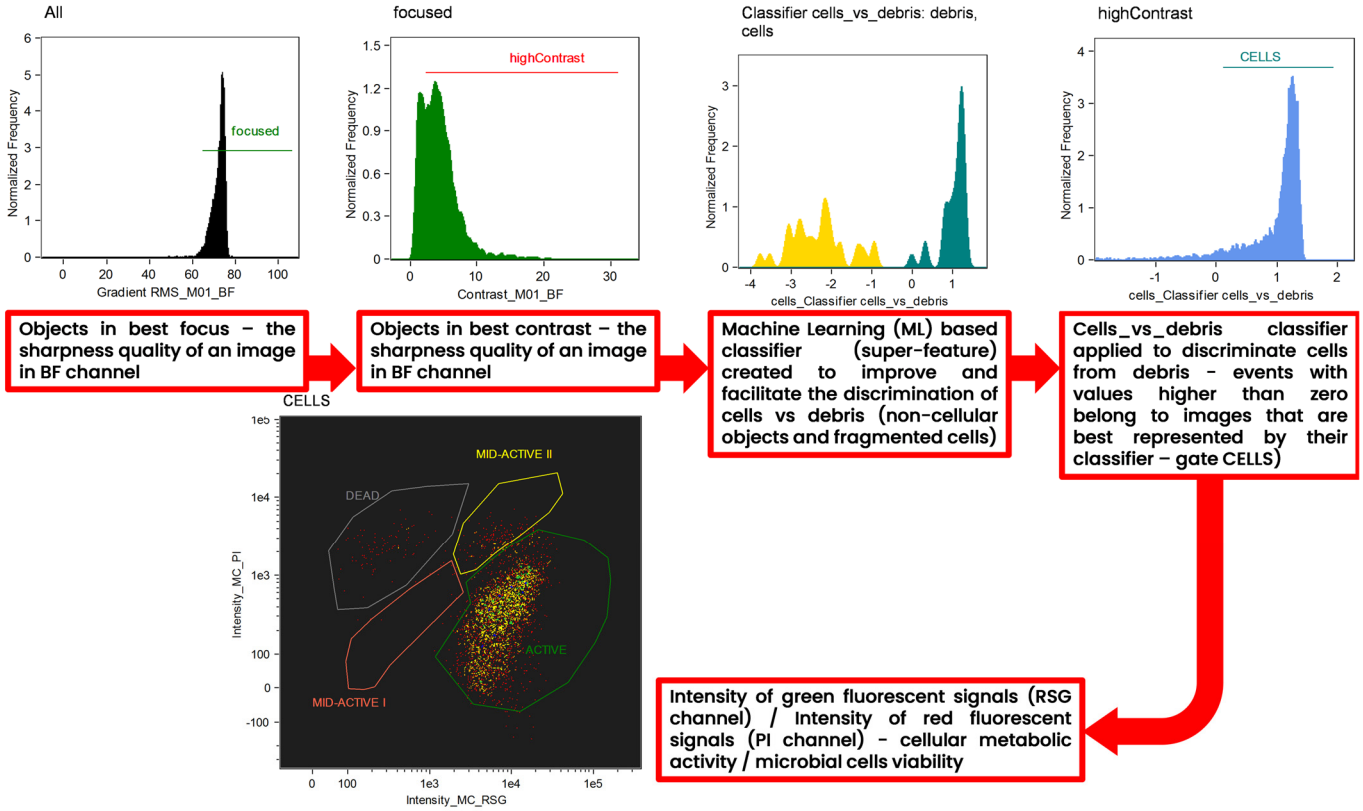


Figure 12. Gating strategy applied for the interpretation of the results of imaging flow cytometry (IFC) analysis. Analysis was assisted by an advanced tool to improve the interpretation of the cytometric results—the machine learning (ML) module of IDEAS software. "Batch" populations being representative images of microbial cells and debris (non-cellular objects and fragmented cells) were specified by the user to initiate a generation of the super feature (classifier) for the precise discrimination of single microbial cells vs. debris. Both classifiers were plotted into histograms, and events with values higher than zero belong to images that are best represented by their classifier.

Table 3. Components of the cells_vs_debris classifier—parameters and weights indicating the discrimination efficiency.

Parameters	Weights
H Entropy Std_M09_Ch09_9	−18.39
H Entropy Std_M01_BF_9	−17.57
H Homogeneity Std_M01_BF_11	−12.9
Area_MC	−12.89
H Entropy Std_M01_BF_11	−12.88
H Entropy Mean_M09_Ch09_9	−12.69
Major Axis Intensity_M09_Ch09	−12.68

4. Conclusions

In this study, the effects of stress conditions on three different fluid bed dried probiotic strains were measured, combining and comparing both plate cell counts and flow cytometry. Samples analyzed by flow cytometry were then further sorted into different identified groups: active, dead, mid-active I and mid active II (with mid-active I and mid active II possibly being VBNC). These selected groups were further analyzed to compare their viability, activity, and probiotic potential, and to compare their occurrence with the plate count method. Such an approach can help to identify the importance of VBNC cells in

probiotic preparations and can also prove the importance of supporting plate counts with additional, more accurate techniques such as flow cytometry for more viable and detailed probiotic cells assessment.

Author Contributions: Conceptualization, J.K., W.J., R.D.; Methodology, J.K., R.D., W.J.; Software, J.K., W.J.; Validation, J.K.; Formal Analysis, J.K.; Investigation, J.K.; Resources, J.K.; Data Curation, J.K.; Writing—Original Draft Preparation, J.K.; Writing—Review & Editing, J.K.; Visualization, J.K.; Supervision, R.D.; Project Administration, J.K.; Funding Acquisition, J.K. All authors have read and agreed to the published version of the manuscript.

Funding: This publication was co-financed within the framework of the Polish Ministry of Science and Higher Education's program: "Regional Initiative Excellence" in the years 2019–2022 (No. 005/RID/2018/19), financing amount 12,000,000 PLN.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

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

References

1. Gill, H.; Prasad, J. Probiotics, Immunomodulation, and Health Benefits. *Bioact. Compon. Milk* **2008**, *606*, 423–454. [CrossRef]
2. Santivarangkna, C.; Kulozik, U.; Foerst, P. Alternative Drying Processes for the Industrial Preservation of Lactic Acid Starter Cultures. *Biotechnol. Prog.* **2007**, *23*, 302–315. [CrossRef]
3. Broeckx, G.; Vandenheuvel, D.; Claes, I.J.J.; Lebeer, S.; Kiekens, F. Drying Techniques of Probiotic Bacteria as an Important Step towards the Development of Novel Pharmabiotics. *Int. J. Pharm.* **2016**, *505*, 303–318. [CrossRef] [PubMed]
4. Schell, D.; Beermann, C. Fluidized Bed Microencapsulation of Lactobacillus Reuteri with Sweet Whey and Shellac for Improved Acid Resistance and In-Vitro Gastro-Intestinal Survival. *Food Res. Int.* **2014**, *62*, 308–314. [CrossRef]
5. Jacobsen, N.M.Y.; Caglayan, I.; Caglayan, A.; Bar-Shalom, D.; Müllertz, A. Achieving Delayed Release of Freeze-Dried Probiotic Strains by Extrusion, Spheronization and Fluid Bed Coating—Evaluated Using a Three-Step in Vitro Model. *Int. J. Pharm.* **2020**, *591*, 120022. [CrossRef]
6. Fritzen-Freire, C.B.; Prudêncio, E.S.; Amboni, R.D.M.C.; Pinto, S.S.; Negrão-Murakami, A.N.; Murakami, F.S. Microencapsulation of Bifidobacteria by Spray Drying in the Presence of Prebiotics. *Food Res. Int.* **2012**, *45*, 306–312. [CrossRef]
7. Perdana, J.; Fox, M.B.; Siwei, C.; Boom, R.M.; Schutyser, M.A.I. Interactions between Formulation and Spray Drying Conditions Related to Survival of Lactobacillus Plantarum WCFS1. *Food Res. Int.* **2014**, *56*, 9–17. [CrossRef]
8. Nag, A.; Das, S. Improving Ambient Temperature Stability of Probiotics with Stress Adaptation and Fluidized Bed Drying. *J. Funct. Foods* **2013**, *5*, 170–177. [CrossRef]
9. Champagne, C.P.; Ross, R.P.; Saarela, M.; Hansen, K.F.; Charalampopoulos, D. Recommendations for the Viability Assessment of Probiotics as Concentrated Cultures and in Food Matrices. *Int. J. Food Microbiol.* **2011**, *149*, 185–193. [CrossRef] [PubMed]
10. Ouwehand, A.C.; Salminen, S.J. The Health Effects of Cultured Milk Products with Viable and Non-Viable Bacteria. *Int. Dairy J.* **1998**, *8*, 749–758. [CrossRef]
11. Foglia, C.; Allesina, S.; Amoruso, A.; De Prisco, A.; Pane, M. New Insights in Enumeration Methodologies of Probiotic Cells in Finished Products. *J. Microbiol. Methods* **2020**, *175*, 105993. [CrossRef] [PubMed]
12. Bensch, G.; Rüger, M.; Wassermann, M.; Weinholz, S.; Reichl, U.; Cordes, C. Flow Cytometric Viability Assessment of Lactic Acid Bacteria Starter Cultures Produced by Fluidized Bed Drying. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 4897–4909. [CrossRef]
13. Ayrapetyan, M.; Williams, T.; Oliver, J.D. Relationship between the Viable but Nonculturable State and Antibiotic Persister Cells. *J. Bacteriol.* **2018**, *200*, e00249-18. [CrossRef] [PubMed]
14. Veljović, K.; Popović, N.; Miljković, M.; Tolinački, M.; Terzić-Vidojević, A.; Kojić, M. Novel Aggregation Promoting Factor AggE Contributes to the Probiotic Properties of Enterococcus Faecium BGGO9-28. *Front. Microbiol.* **2017**, *8*, 1843. [CrossRef] [PubMed]
15. Yan, M.; Han, J.; Xu, X.; Liu, L.; Gao, C.; Zheng, H.; Chen, Y.; Tao, Y.; Zhou, H.; Li, Y.; et al. Gsy, a Novel Glucansucrase from Leuconostoc Mesenteroides, Mediates the Formation of Cell Aggregates in Response to Oxidative Stress. *Sci. Rep.* **2016**, *6*, 38122. [CrossRef]
16. Davis, C. Enumeration of Probiotic Strains: Review of Culture-Dependent and Alternative Techniques to Quantify Viable Bacteria. *J. Microbiol. Methods* **2014**, *103*, 9–17. [CrossRef]
17. Juzwa, W.; Myszka, K.; Białas, W. Investigation of the Population Dynamics within a *Pseudomonas Aeruginosa* Biofilm Using a Flow Based Biofilm Model System and Flow Cytometric Evaluation of Cellular Physiology. *Biofouling* **2018**, *34*, 835–850. [CrossRef]
18. Duber, A.; Zagrodnik, R.; Chwialkowska, J.; Juzwa, W.; Oleskowicz-Popiel, P. Evaluation of the Feed Composition for an Effective Medium Chain Carboxylic Acid Production in an Open Culture Fermentation. *Sci. Total Environ.* **2020**, *728*, 138814. [CrossRef]

19. Majeed, M.; Majeed, S.; Nagabhushanam, K.; Punnapuzha, A.; Philip, S.; Mundkur, L. Rapid Assessment of Viable but Non-Culturable Bacillus Coagulans MTCC 5856 in Commercial Formulations Using Flow Cytometry. *PLoS ONE* **2018**, *13*, e0192836. [CrossRef]
20. Michelutti, L.; Bulfoni, M.; Nencioni, E. A Novel Pharmaceutical Approach for the Analytical Validation of Probiotic Bacterial Count by Flow Cytometry. *J. Microbiol. Methods* **2020**, *170*, 105834. [CrossRef]
21. Chiron, C.; Tompkins, T.A.; Burguière, P. Flow Cytometry: A Versatile Technology for Specific Quantification and Viability Assessment of Micro-Organisms in Multistrain Probiotic Products. *J. Appl. Microbiol.* **2018**, *124*, 572–584. [CrossRef] [PubMed]
22. Konieczny, M.; Rhein, P.; Czaczyk, K.; Białas, W.; Juzwa, W. Imaging Flow Cytometry to Study Biofilm-Associated Microbial Aggregates. *Molecules* **2021**, *26*, 7096. [CrossRef] [PubMed]

nutrients

MDPI

Article

The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line

Jakub Kiepś [1,*], Wojciech Juzwa [1], Anna Olejnik [1], Anna Sip [1], Jolanta Tomaszewska-Gras [2] and Radosław Dembczyński [1,*]

[1] Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, 60-627 Poznan, Poland; wojciech.juzwa@up.poznan.pl (W.J.); anna.olejnik@up.poznan.pl (A.O.); anna.sip@up.poznan.pl (A.S.)

[2] Department of Food Safety and Quality Management, Poznan University of Life Sciences, 60-624 Poznan, Poland; jolanta.tomaszewska-gras@up.poznan.pl

* Correspondence: jakub.kieps@up.poznan.pl (J.K.); radoslaw.dembczynski@up.poznan.pl (R.D.)

Abstract: Adhesion is one of the main factors responsible for the probiotic properties of bacteria in the human gut. Membrane proteins affected by cellular damage are one of the key aspects determining adhesion. Fluid-bed-dried preparations containing probiotic bacteria were analyzed in terms of their stability (temperature of glass transition) and shelf life in different conditions (modified atmosphere, refrigeration). Imaging flow cytometry was utilized to determine four subpopulations of cells based on their physiological and morphological properties. Lastly, adhesion was measured in bacteria cultured in optimal conditions and treated with heat shock. The results show that the subpopulations with no or low levels of cell membrane damage exhibit the ability to adhere to Caco-2 cells. The temperature of protein denaturation in bacteria was recorded as being between 65 °C and 70 °C. The highest glass transition temperature (Tg) value for hydroxypropyl methylcellulose (used as a coating substance) was measured at 152.6 °C. Drying and coating can be utilized as a sufficient treatment, allowing a long shelf-life (up to 12 months). It is, however, worth noting that technological processing, especially with high temperatures, may decrease the probiotic value of the preparation by damaging the bacterial cells.

Keywords: viability; fluid bed drying; lactic acid bacteria; stress; quality control; imaging flow cytometry

Citation: Kiepś, J.; Juzwa, W.; Olejnik, A.; Sip, A.; Tomaszewska-Gras, J.; Dembczyński, R. The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line. *Nutrients* **2023**, *15*, 3484. https://doi.org/10.3390/nu15153484

Academic Editor: Francisco J. Pérez-Cano

Received: 13 July 2023
Revised: 31 July 2023
Accepted: 5 August 2023
Published: 7 August 2023

1. Introduction

Methods of microorganism preservation such as spray drying, freeze drying, vacuum drying, and fluidized bed drying allow probiotics to be obtained that retain their properties during storage and are easy to dose and apply [1]. The particular interest in probiotics stems from their beneficial effects on the health of humans and farm animals. Clinical trials confirm the beneficial effects of probiotics in the treatment of gastrointestinal conditions (GI) such as diarrhea, irritable bowel syndrome and inflammatory bowel disease [2]. In addition, their application in non-GI medical conditions is currently being researched, for example, in patients with atopic dermatitis and type-2 diabetes [3,4].

To obtain dried preparations of high quality and viability, various protection strategies can be incorporated into processing steps. In general, three strategies can be distinguished: adding protective agents, optimizing drying parameters, and prestressing the probiotic cells before drying [5]. The intentional use of stresses may increase the resistance of cells to unfavorable conditions during drying (dehydration, osmotic stress, shear stress, elevated temperature). All these stress conditions adversely affect the cells. As a result, damaged and dead cells appear in the preparation, reducing its quality (long-term stability and shelf-life) and affecting some of the probiotic properties (e.g., the ability to adhere to the intestinal epithelium).

Adhesion can be analyzed using various in vitro and in vivo methods. In vitro models include cell cultures (most notably Caco-2 and HT-29 cell lines), intestinal mucus cell models, organ culture models, and whole tissue models. Due to their ease of application and their relatively low cost, these models are used as a foundation for adhesion tests. They can be analyzed using simple imaging techniques like SEM. Additionally, the development of new tissue and organ models makes it possible to better represent the structural architecture of the intestinal tissues using 3d designs and allows for obtaining a multilayered system incorporating all types of cells found in the intestines. Their main disadvantage is that they do not represent the specific physiology of the host and do not take into consideration the intestinal microbiota [6]. To overcome these issues, ex vivo and in vivo models have been utilized. The intestinal tissue can also be analyzed afterward using microbiological and molecular analysis. Genomics is a non-invasive method that has also been utilized to identify the proteins associated with adhesion [7].

To evaluate the viability of probiotic cells, the most commonly used method is pour plate counts. This makes it possible to enumerate the viable probiotic cells; however, it is limited only to the subpopulation of cells with the ability to grow and divide. Inactivated, killed, or dead cells, which are unrecognizable using classical microbiological methods, also possess functional properties; however, live cells are more efficacious [8]. Treatment with sublethal temperatures can induce cross-protection mechanisms in probiotic bacteria. The exposure to sublethal temperatures can trigger the synthesis of heat shock proteins and other stress response elements, which not only protect probiotic cells from temperature stress, but also confer enhanced resistance to other stresses, such as those encountered in the gut environment, such as low pH [9], as well as those encountered during the treatment process, such as high temperature and dehydration [10]. As stated by Wang et al. [11], the live/dead state does not influence the adhesion ability of certain probiotic strains. Adhesion is mediated mainly by the components on the cell surface; it is, however, correlated with the integrity of the cell membrane. The health benefits conferred by live cells are more complex than those of dead cells [8].

This study aims to select fluid bed drying parameters (such as the application of the coating step and the selection of the coating material) for selected probiotic bacteria strains, as well as to assess the impact of culture conditions and drying processes on the survival, storage stability, and physiological characteristics of probiotic bacteria cells. Thermal analyses (DSC and TG/DTA) are utilized to assess the glass transition temperature of different coating substances applied in the process. The effect of technological parameters (temperature) on cell mortality was also determined using DSC analysis. An assessment of the impact of the drying method and drying conditions used on cell survival was performed using a cell line to model bacterial adhesion to the intestinal epithelium in vitro. Imaging flow cytometry (IFC) was applied to analyze cell adhesion and assess the physiological and morphological properties of the cell. The main impact and the significant novelty of the employed methodology lie in the prospect of facilitating the detection of dormant and injured bacterial cells as separate subpopulations. This makes it possible to determine more subpopulations than the limited routine discrimination of live and dead cells only; in addition to these two subpopulations, we also determined two subpopulations with varying levels of membrane damage and ongoing metabolic activity. Our research is focused on the assessment of cell cellular damage and metabolic activity in the samples after adhesion in cell lines and after long-term storage, and showcases a novel way of assessing the viability of probiotic bacteria. The adhesion results were also determined using the conventional pour plate method.

2. Materials and Methods

2.1. Inoculum Preparation

Leuconostoc mesenteroides 51 KBiMŻ, *Enterococcus faecium* 73 KBiMŻ, and *Carnobacterium divergens* 3 KbiMŻ were chosen as strains for assessment and were stored in the form of beads with cells in a freezer at −80 °C. MRS broth was used for propagation as a medium

ensuring optimal growth conditions for lactic acid bacteria. Inoculum was prepared in a volume of 10% of the bioreactor vessel to obtain the highest biomass yield. Two-step propagation also allowed for better adaptation of the microorganisms and shortened the resting phase in culture. The first step was carried out on ice to limit cell damage, which can result from thawing at room temperature. Afterward, the microorganisms were transferred to a 15 mL Falcon conical tube containing 9 mL MRS broth and sealed with parafilm, and incubated for 24 h at 30 °C. After incubation, 10 mL of the inoculum was transferred to a flask containing 90 mL of MRS broth, followed by another 24 h incubation at 30 °C. After these steps, the inoculum was ready to be used to start a culture in the bioreactor. All actions on microorganisms were carried out in a laminar chamber to reduce the risk of infection.

2.2. Bacterial Cultures

Biostat A plus bioreactors (Sartorius, Göttingen, Germany) were used for the cell culturing step. Firstly, the bioreactors and equipment were prepared: the pH electrode was calibrated against buffers at pH 4 and pH 9; next, 1 L of MRS broth medium was added to the vessel and then the bioreactor was autoclaved at 121 °C with a 20 min exposure time to provide sterile conditions. Inoculum was added, in a volume of 10% of the volume of the medium (100 mL), to a bioreactor cooled to 30 °C after sterilization. During this step, the bioreactor was pressurized with nitrogen to minimize the risk of infection with airborne microorganisms. After inoculation, the growth parameters were set at 30 °C, with a stirrer speed of 150 RPM and pH set at 6.5 (the optimal value for the selected strains). A 30% NaOH solution was used for pH regulation. NaOH consumption and pH changes over time were constantly monitored to determine the end of the exponential growth. The stabilization of pH at the set level with the simultaneous absence of base consumption indicated the end of microbial growth and inhibition of the production of acidifying metabolites. Heat shock stress was induced in certain samples by heating the culture in its stationary phase to 60 °C for 30 min. Samples were collected into sterile test tubes using a peristaltic pump connected to the sampling port.

2.3. Fluid Bed Drying

For the drying process, a GEA Strea-1 laboratory fluid-bed dryer was used. In the first step, the drum of the fluid-bed dryer was filled with the matrix (crystalline microcellulose). Next, the stream of filtered and heated air was introduced through the bottom perforated plate to keep the matrix in the fluid phase and to ensure even drying. The temperature range was set at up to 50 °C. Probiotic cells were suspended in the solution of a protective substance (5% trehalose) and fed to the dryer. They were then sprayed using an atomizing nozzle under a pressure of 2 bar. Drying and coating took approximately 30 min for each step.

2.4. Plate Count Method

The plate counting method was used to determine the number of live microorganisms present in the analyzed samples after drying and during the analysis of adhesion. Tubes containing 9 mL of solvent (0.9% NaCl) were prepared, and then 1 mL of the sample was added into the first tube so that a dilution of 10^{-1} was obtained. After thorough vortexing, 1 mL of the sample with a dilution of 10^{-1} was transferred to the next tube, and the whole process was repeated until the required order of dilution was obtained. For the culture, the fluid and suspension for drying the samples were taken directly from the suspension; however, in the case of the dried formulation, it was necessary to add a rehydration step. For that purpose, 1 g of the sample was weighed into a 99 mL flask with 0.9% NaCl, which was then placed in a 37 °C water bath. After 30 min of shaking in a water bath, further dilutions were prepared using the suspension. Finally, 1 mL of the diluted sample was applied to Petri dishes, and MRS agar medium (previously sterilized and stored at 55 °C to prevent solidification) was poured over the samples, mixed, and left to solidify. The

Petri dishes were incubated under aerobic conditions in an incubation chamber at 30 °C for 48 h, after which time visible colonies were counted and evaluated. The results obtained by counting visible colonies were considered statistically significant only for plates with dilution samples for which the number of colonies ranged from 30 to 300.

2.5. Intestinal Epithelial Cell Culture

The human intestinal epithelial Caco-2 cell line (HTB-37™) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 1% non-essential amino acids (100X NEAA, Sigma-Aldrich) and 20% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For intestinal barrier formation, Caco-2 cells were seeded on the PET membranes (Millicell® Cell Culture Inserts, 24 mm diameter, 0.4 μm pore size) (Millipore, Burlington, MA, USA, Merck Group) at an initial density of 4×10^5 cells/cm^2 and cultured for 21 days with a medium change three times a week. The integrity of the Caco-2 cell monolayers was monitored on the basis of transepithelial electrical resistance (TEER) measurements using the Millicell Electrical Resistance System (ERS-2, Millipore). Caco-2 cell cultures with TEER values $\geq 600\ \Omega \times$ cm^2 were used in the bacteria adhesion experiments.

Caco-2 is an epithelial cell line isolated from colon adenocarcinoma. One of the unique properties of the Caco-2 cell line is its ability to form a brush border with microvilli. Cells were cultivated for 21 days in 6-well plates with 0.4 μm cell culture inserts to reach the best divergence and full confluence. As a medium, D-MEM (Dulbecco's modified Eagle medium) was chosen, with the addition of an antibiotic (gentamicin). Cell lines were cultivated under the following conditions: temperature of 37 °C in a controlled atmosphere with 5% CO_2 concentration, and humidity exceeding 95%.

2.6. Adhesion Assay

Before the adhesion assay, Caco-2 cell monolayers were washed twice with PBS. Then, DMEM (without phenol red) with bacterial cells was added. The Caco-2 cell cultures combined with bacteria were incubated for 2 h at 37 °C. After incubation, the medium was removed from the epithelial cell cultures, and the cell monolayers were washed gently with PBS. Then, a cold 1% Triton X-100 solution was used to lyse the Caco-2 cells and release the adhered bacterial cells. Lysis was carried out for 3–5 min on ice. The cell lysates were centrifuged (10 min, 3.500 rpm), and the pellets were suspended in PBS. The number of bacterial cells was determined using IFC (Imaging Flow Cytometry) and pour plate counts in three replicates. Pour plate counts were performed in MRS agar and incubated under anaerobic conditions at 37 °C for 48 h.

2.7. Imaging Flow Cytometry

Imaging flow cytometry is a type of flow cytometry that combines the high-throughput analysis capabilities of flow cytometry with the imaging capabilities of microscopy. This method allows for the simultaneous analysis of multiple parameters, such as metabolic activity and cellular membrane integrity, in a single cell. The physiological and morphological properties of bacterial cells, such as metabolic activity and the integrity of the cellular membrane, were assessed using the imaging flow cytometer Amnis FlowSight™ (Luminex Corp., Austin, TX, USA). This imaging flow cytometer is equipped with 3 lasers (405 nm, 488 nm, and 642 nm), 5 fluorescence channels (acquisition by a multi-channel CCD camera), and a side scatter detector (SSC). Post-acquisition data analysis was performed using the IDEAS software ver. 6.2 (Luminex Corp., Austin, TX, USA). Morphological characteristics of the analyzed cells were determined using the Gradient RMS parameter from brightfield signals (Ch01) for the discrimination of high-resolution cell images. Additionally, brightfield digital image processing parameters: Aspect Ratio and Area were used to characterize the shape and size of the analyzed bacterial cells in combination with the discrimination

of bacterial cells from non-cellular debris (particles from prebiotic component) and single cells from aggregates. The viability and activity of probiotic bacteria cells in the samples were determined by performing fluorescent staining with RedoxSensorTM Green and PI (propidium iodide). The samples for analysis were prepared by centrifugation and then suspended in a 1% PBS buffer in 1:200 dilution. Then, the following dyes were added to the samples with a volume of 500 µL by being pipetted into Eppendorf tubes: 1.6 µL of RedoxSensorTM Green and 1.2 µL of PI. The cells in the samples were counted and assessed for morphology (microscopic image), activity (signal for RedoxSensorTM Green), and integrity of the cell membrane (signal for PI). Additional dying with DRAQ 5 and wheat germ agglutinin (WGA) was used to show the pattern of adhesion of the bacterial cells onto the Caco-2 cells. DRAQ 5 was added to dye the epithelial cell nuclei red and WGA to dye the cell membranes of the bacteria green. For all samples, a previously developed machine learning (ML) protocol [12] was utilized to help in the discrimination of cells from cellular and non-cellular debris. The ML module is a part of the IDEAS® 6.3. software, which was designed to process data acquired by Amnis Flow Sight imaging flow cytometer (Luminex Corp., Austin, TX, USA).

2.8. Scanning Electron Microscopy (SEM)

To visualize the morphology of the dried powder microparticles, selected samples were analyzed using scanning electron microscopy. Firstly, samples of the powder were coated with a thin layer of gold using the Q15OT ES coater. Images were obtained using a Quanta 250 microscope.

2.9. Thermogravimetry–Differential Thermal Analysis (TG/DTA)

The TG/DTA analysis was conducted using an STA 449 F5 Jupiter (Netzsch, Selb, Germany). First, 30 mg of sample was placed in the heating chamber and heated to 200 °C at the rate of 5 °C/min. Then, the chamber was filled with an inert gas (helium), and its flow was set at 20 mL/min.

2.10. Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter DSC 8500 (PerkinElmer Inc., Waltham, MA, USA) was used to determine the glass transition phenomena. The device, which was equipped with an Intracooler II and was running under Pyris 10.1 instrument management software, was calibrated using the standards of indium (T_m = 156.60 °C, ΔH = 28.45 J/g, PerkinElmer Inc.) and n-dodecane (99.8 purity, T_m =−9.65 °C, Merck). The samples (approximately 5–6 mg) were weighed into 20 µL aluminum pans (PerkinElmer, No. 0219–0062, Waltham, MA, USA) and hermetically sealed. The analysis of glass transition involved the following steps: (1) holding for 1.0 min at 30 °C; and (2) heating from 30 °C to 300 °C at 5 °C/min. The reference was an empty, hermetically sealed aluminum pan. Glass transition, as a second-order phase transition, was identified by a step in the baseline of the measurement curve and registered as a heat capacity change (ΔC_p, J/g °C) as a function of temperature. The glass transition temperature (T_g, °C) parameter was calculated as the inflection point. All samples were analyzed in two replicates.

2.11. Storage and Shelf-Life Tests

Samples after drying and coating were transferred to glass vials. A 1 g sample was placed in each vial and sealed using airtight caps in one of three variants: with atmospheric air, with nitrogen, and under vacuum. After sealing, the vials were stored at 3 different temperatures—−20 °C, 4 °C, and 20 °C—for 12 months. Samples for the pour plate tests were taken after 1, 2, 3, 6, 9, and 12 months of storage.

3. Results and Discussion

3.1. Glass Transition Temperature

The glass transition temperature was analyzed using both DSC and TG/DTA. This parameter is a property of amorphous materials that are formed, e.g., by removing the dispersing medium. This phenomenon occurs in the process of fluidized bed drying, where water (the dispersing medium) is removed and the duration of the process is insufficient for crystallization to occur; therefore, the dried material stays in an amorphous state. Above a critical temperature, described as the glass transition temperature, the dried material will start to change its structure from a glassy solid state to a rubbery form. This structural change can impact the physiochemical properties of a product and impact the viability of dried probiotics. Differential scanning calorimetry is a method that is well suited for measuring such changes in biological systems. It works by heating the sample with reference to an inert material (i.e., a material that is not undergoing a phase transition in a selected temperature range). Phase transitions such as glass transition are then registered as a difference in heat as a function of temperature between the sample and the reference material. The glass transition temperatures were measured for three dried samples coated with different coating materials (gum Arabic, hydroxypropyl methylcellulose, and shellac), as well as for dried, uncoated samples (Figure 1). All temperatures were high enough to provide stability and long shelf-life, and they are compared with the temperatures obtained using TG/DTA in Table 1. TG/DTA analysis was used to compare the T_g values. Figure 2 shows the recorded thermal properties of the samples, where it can be seen that the glass transition was recorded in a similar range as when using DSC. Samples after coating were also visualized using SEM (Figure 3) to show the surface of the pellets after using different coating materials. Additionally, bacterial cells immobilized on the surface of a single microcellulose pellet were observed (Figure 4).

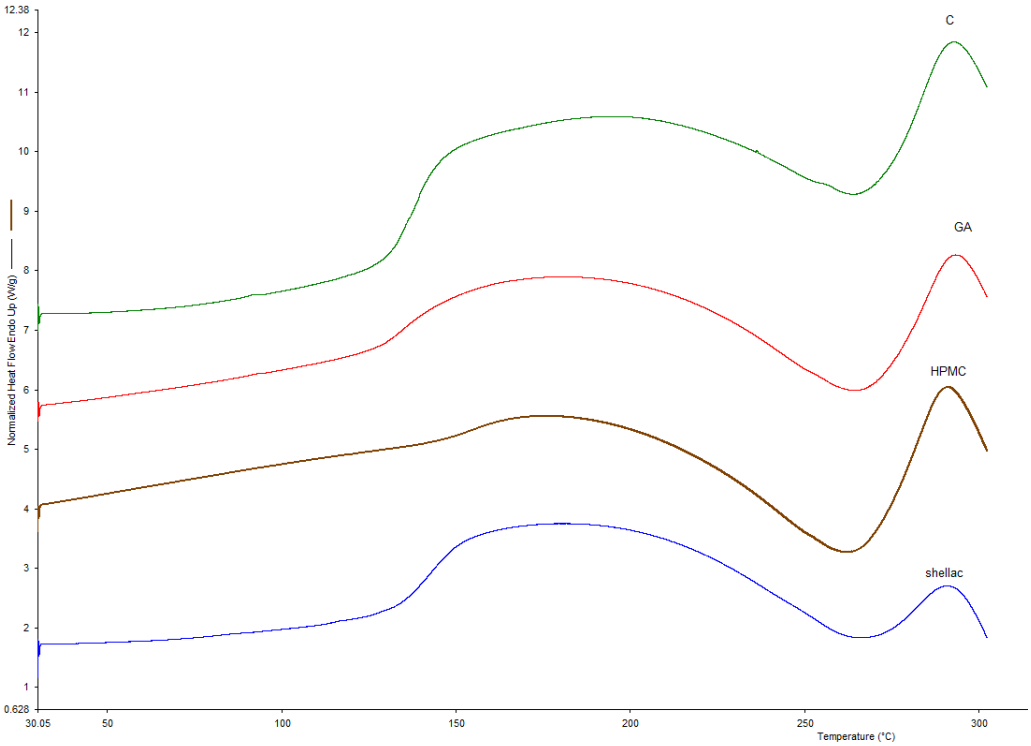


Figure 1. DSC graph of probiotic preparations with different coatings. C—dried, uncoated sample; GA—sample coated with gum arabic; HPMC—sample coated with hydroxypropyl methylcellulose; shellac—sample coated with shellac.

Table 1. Comparison of the temperature of glass transition (T_g) in samples coated with different materials measured using DSC and TG/DTA.

Sample	T_g Measured by DSC	T_g Measured by TG/DTA
uncoated	145.3 °C	141.3 °C
Gum arabic	144.8 °C	156.2 °C
HPMC	152.6 °C	156.1 °C
shellac	147.6 °C	140.3 °C

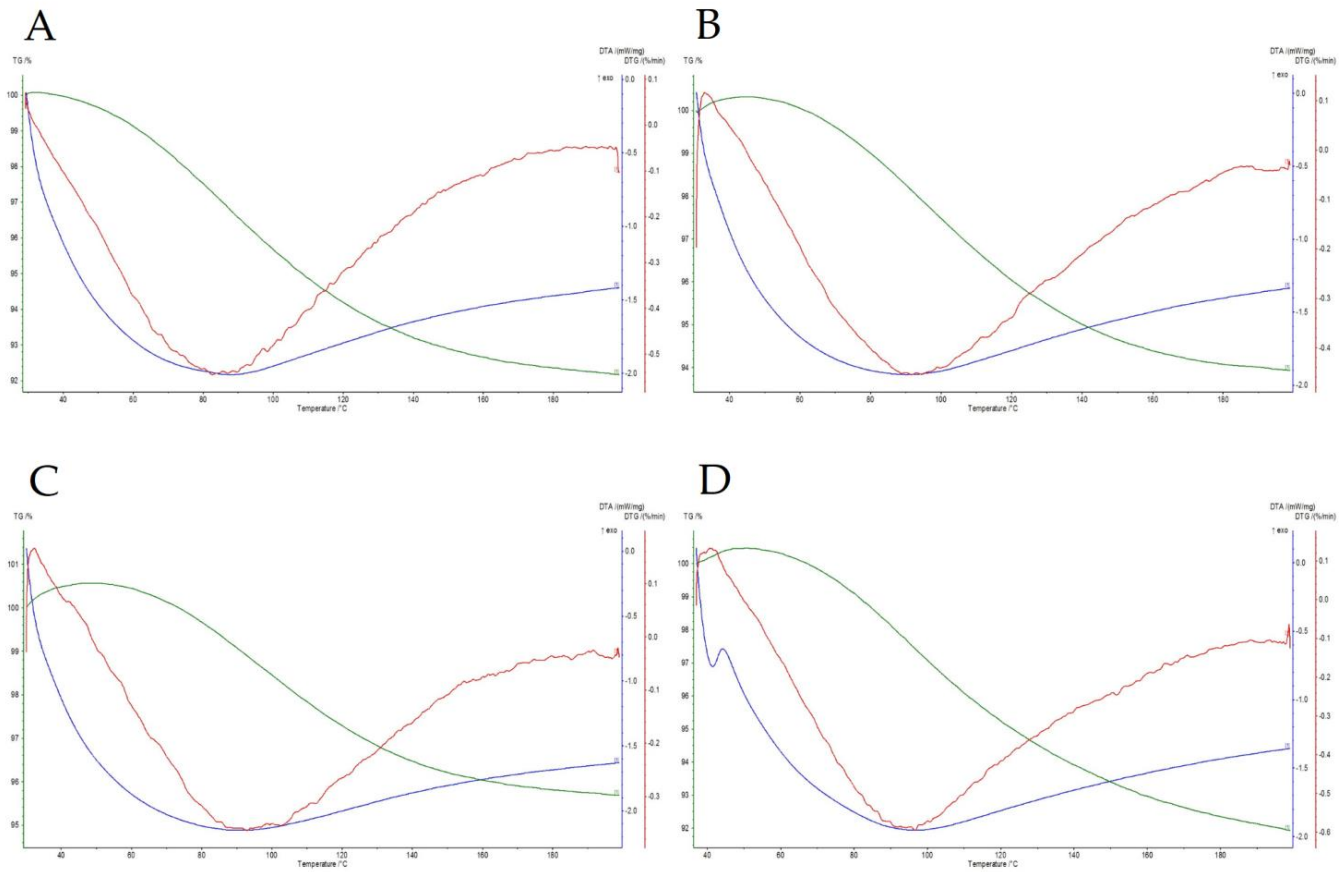


Figure 2. TG/DTA for (**A**) control (dried, uncoated); (**B**) samples coated with gum arabic; (**C**) samples coated with hydroxypropyl-methylcellulose; (**D**) samples coated with shellac.

3.2. Cells Pre Adhesion

Samples were taken pre adhesion from the suspension of probiotic cells used for incubation with epithelial cells. The following subpopulations were distinguished in the pre-adhesion samples using flow cytometry with RSG and PI staining: active (with confirmed metabolic activity and no cellular membrane damage); mid-active I (with lower-than-average levels of both metabolic activity and cellular membrane damage); mid-active II (with higher-than-average levels of both metabolic activity and cellular membrane damage) and dead (with no metabolic activity and high levels of cellular membrane damage). Detailed information on subpopulation determination was provided in our previous work [12]. The distribution of these groups for samples containing three strains of probiotic bacteria cultured under optimal conditions and after heat shock is presented in Figure 5, and the numbers of cells counted using the pour plate method is shown in Table 2. It can be observed that the majority of almost all samples corresponded to active cells, with the exception of *L. mesenteroides* after heat shock, which was more susceptible to

high temperature and consisted mainly of dead cells, with almost equal amounts of active and mid-active II subpopulations.

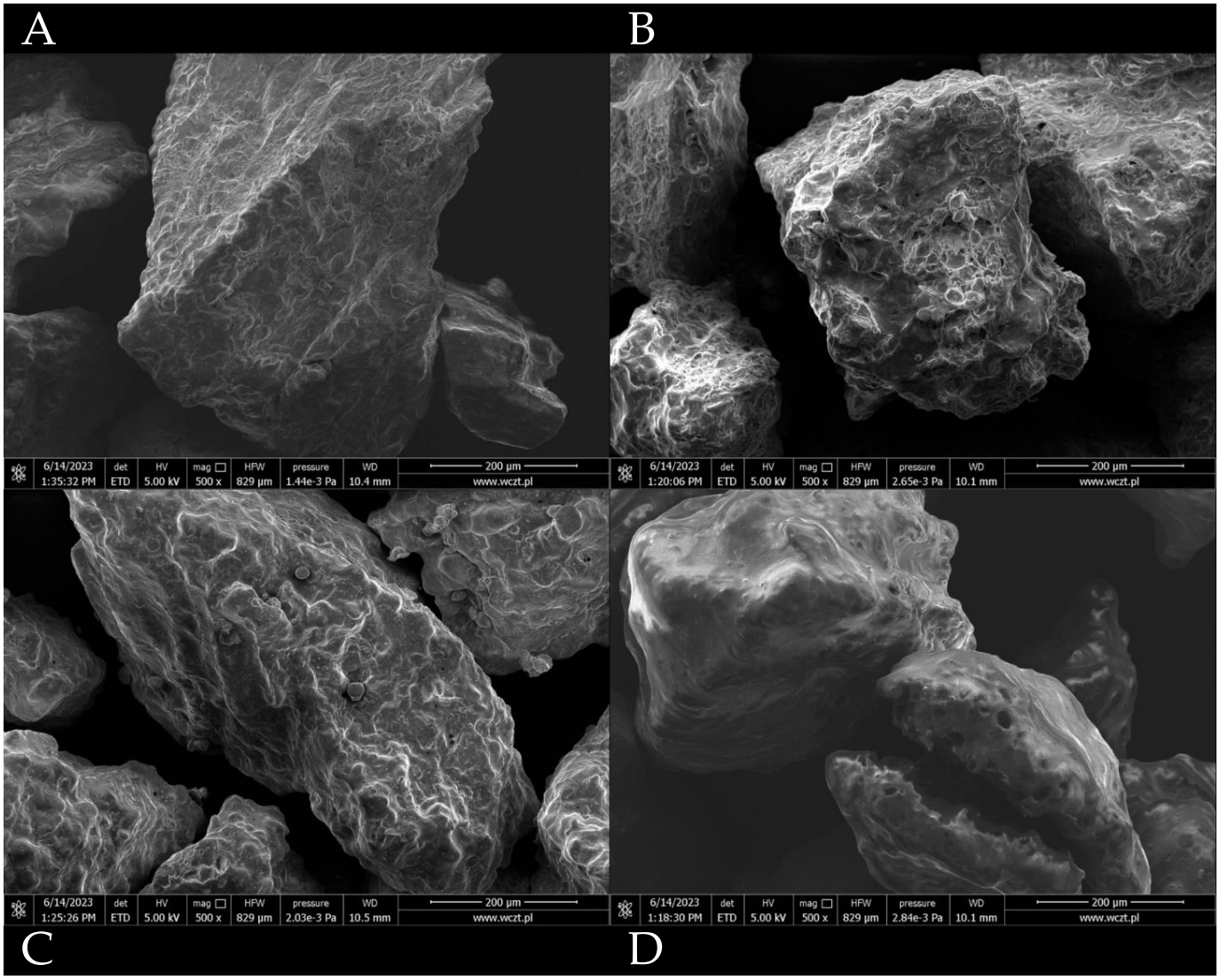


Figure 3. SEM pictures of dried probiotics coated with different materials. (**A**) Uncoated, (**B**) gum Arabic, (**C**) hydroxypropyl methylcellulose, (**D**) shellac.

Table 2. Enumeration of bacteria adhering to Caco-2 cells using the pour plate method.

Sample	Number of Cells Pre-adhesion [cfu/mL]	Adhered Cells [cfu/mL]	Adhered Cells [%]
L. mesenteroides dried	5.75×10^8 + SD	4.68×10^7	8.14
L. mesenteroides coated	5.20×10^8	2.88×10^7	5.54
E. faecium dried	9.25×10^8	3.16×10^8	34.12
E. faecium coated	6.55×10^8	1.50×10^8	22.96
C. divergens dried	4.00×10^8	3.64×10^7	9.10
C. divergens coated	1.5×10^8	1.96×10^7	13.07

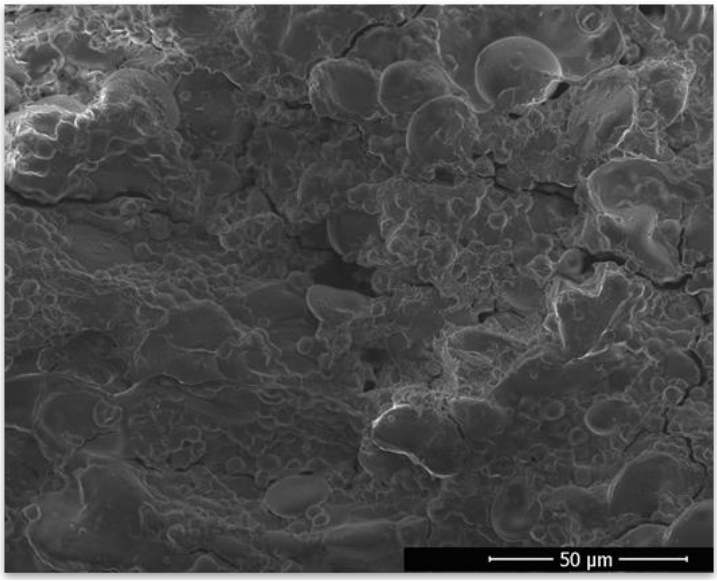


Figure 4. SEM picture of the surface of microcellulose matrix after drying with visible bacterial cells on its surface.

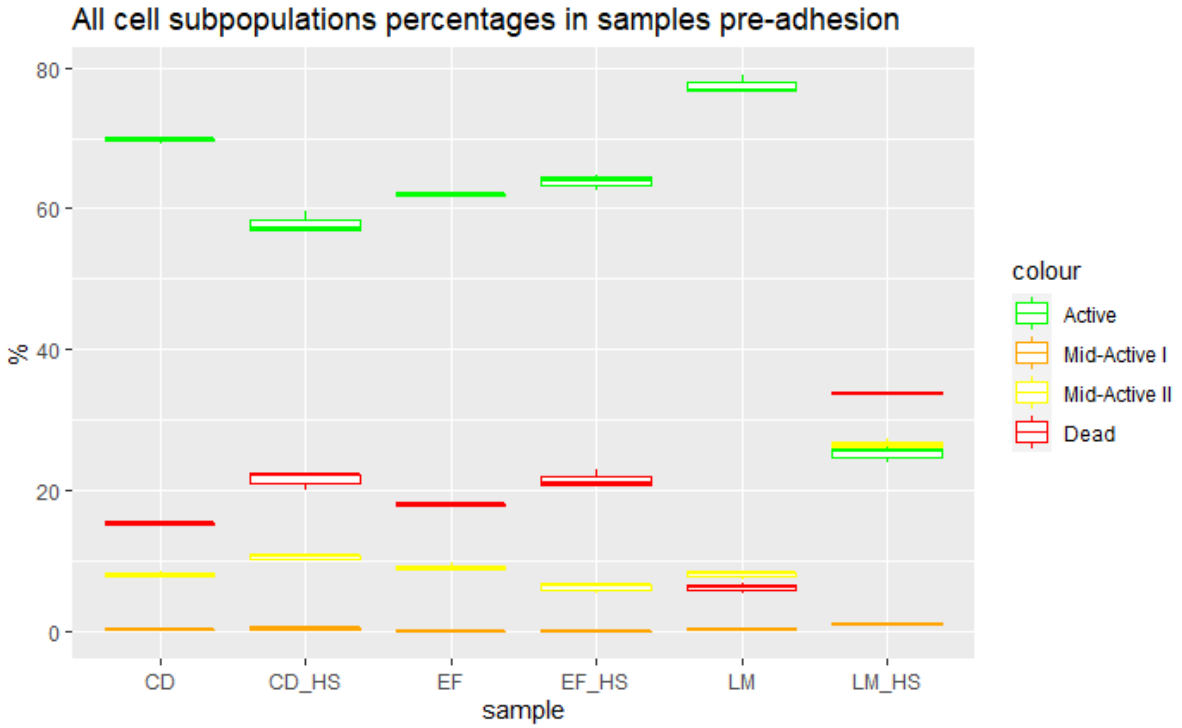


Figure 5. Percentages of cell subpopulations in samples pre adhesion. CD—*C. divergens*; CD_HS—*C. divergens* after heat shock; EF—*E. faecium*; EF_HS—*E. faecium* after heat shock; LM—*L. mesenteroides*; LM_HS—*L. mesenteroides* after heat shock.

3.3. Cells Post Adhesion

To measure the distribution of subpopulations in the post-adhesion samples, the supernatant left after incubation and washing of the cells was analyzed. This was necessary since the samples after trypsinization contained a combination of bacterial and epithelial cells, and as such were not suitable for flow cytometry. They were still enumerated using the pour plate method. The post-adhesion samples showed very low numbers of active cells. This led to the conclusion that the active subpopulation was adhering to the Caco-2 cells during incubation. The composition of the samples (Figure 6), which exhibited higher

contents of dead, mid-active I, and mid-active II cells than pre adhesion, showed that these subpopulations were unable to adhere, and were left suspended in the medium, which was analyzed for its contents. Caco-2 cells are a well-differentiated adherent cell line that is used for testing the adhesion of intestinal bacteria, studies of transmembrane transport, and studies of bacterial pathogen invasion. The results clearly show that the damage sustained by bacterial cells influences their ability to adhere to Caco-2 cells. All three groups—mid-active I and II and dead cells—showed low levels of adhesion compared to active cells. Cellular damage may alter the surface properties of probiotic cells, such as through changes in the expression or accessibility of adhesion-related molecules. For example, damage-induced modifications in the expression of lectins or surface proteins on probiotic cells may impact their ability to recognize and adhere to specific receptors on Caco-2 cells [11,13].

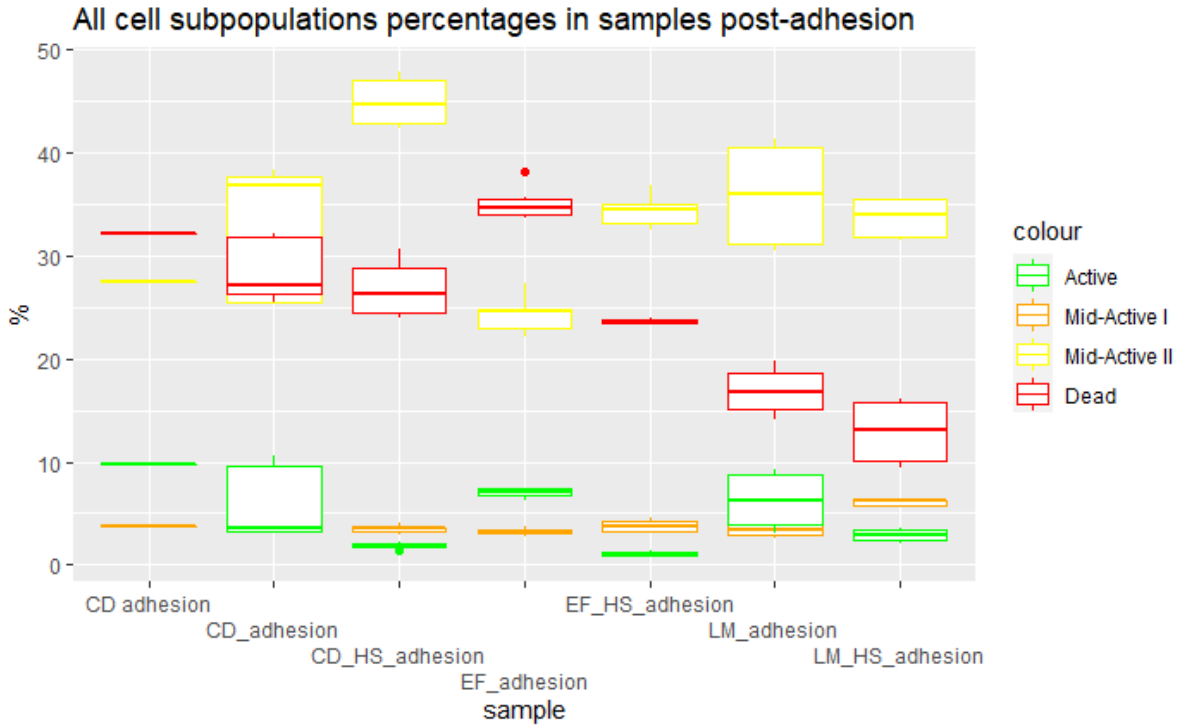


Figure 6. Percentages of cell subpopulations in samples post adhesion. CD—*C. divergens*; CD_HS—*C. divergens* after heat shock; EF—*E. faecium*; EF_HS—*E. faecium* after heat shock; LM—*L.mesenteroides*; LM_HS—*L. mesenteroides* after heat shock.

Cellular damage could disrupt the structures involved in probiotic cell adhesion, such as pili or fimbriae. These structures play a crucial role in facilitating adhesion to host cells. Damage-induced alterations in the structure or functionality of adhesion factors can reduce the ability of probiotic cells to adhere [14].

Damage may also play a role in the activation of various signaling pathways within probiotic cells, leading to changes in gene expression and cellular behavior. These changes can affect the expression of adhesion-related genes and proteins, influencing the adhesion capacity of probiotic cells to Caco-2 cells.

Cellular damage may cause physical damage to the outer membrane or cell wall of probiotic cells. This damage can expose inner components or disrupt the integrity of the cell surface, affecting the interaction between probiotic cells and Caco-2 cells.

Cellular damage could induce stress responses in probiotic cells, leading to the production of stress-related proteins and changes in cellular physiology. These stress responses can impact the adhesion properties of probiotic cells, potentially affecting their ability to adhere to Caco-2 cells.

Adhesion plays a crucial role in influencing the probiotic properties of bacterial cells. This is crucial for the colonization and persistence of probiotic bacteria in the gastrointestinal tract. Effective adhesion allows probiotic cells to establish a foothold in the gastrointestinal tract, which is essential for exerting their beneficial effects [15]. An important aspect of adhesion is the competitive exclusion of pathogens. Through adhesion, probiotic bacteria can competitively exclude or inhibit the attachment of pathogenic microorganisms to the intestinal epithelium. By occupying the adhesion sites on host cells, probiotic bacteria prevent the binding of pathogens, thus reducing their colonization, as well as potentially harmful effects [16,17]. Furthermore, adhesion to host cells allows probiotic bacteria to interact with other members of the gut microbiota. Through adhesion, probiotic bacteria can influence the composition and balance of the gut microbial community, promoting a beneficial microbial ecosystem [18]. The adhesion of probiotic bacteria to intestinal epithelial cells can also strengthen the epithelial barrier function. Probiotic bacteria can promote the production of tight junction proteins, strengthen cell–cell junctions, and enhance the integrity of the gut barrier. This can help prevent the translocation of pathogens or toxins across the intestinal epithelium [19]. Additionally, the adhesion of probiotic bacteria to gut epithelial cells can trigger immune responses and modulate the immune system. Probiotics can interact with immune cells present in the gut-associated lymphoid tissue, promoting beneficial immune responses and potentially regulating excessive inflammation [20]. Lastly, adherent probiotic bacteria can produce bioactive compounds near host cells. These compounds may include short-chain fatty acids [21], antimicrobial peptides [22], or metabolites, which can exert beneficial effects on the intestinal epithelium, immune system, or overall gut health.

It can be seen from these results that the bacterial cells exhibited the ability to adhere to intestinal epithelial Caco-2 cells by means of various mechanisms of cell adhesion. One such mechanism involves lectin–carbohydrate interactions, in which lectins, which are carbohydrate-binding proteins found on the surface of probiotic cells, recognize specific carbohydrates on the epithelial cell surface, thereby facilitating adhesion. For example, *Lactobacillus rhamnosus* LGG and *Lactobacillus mucosae* LM1 express lectins that interact with carbohydrates on the intestinal epithelial cells, promoting adhesion [13]. Another mechanism involves the role of surface proteins, such as adhesins, in promoting attachment. Adhesins and other surface proteins play a significant role in adhesion. These proteins on probiotic cells interact with specific receptors on Caco-2 cells. For instance, the adherence of *Lactobacillus acidophilus* to Caco-2 cells is mediated by the surface-associated protein MUB [23]. Probiotic cells can also adhere to the extracellular matrix (ECM) proteins secreted by Caco-2 cells, such as laminin or collagen. These interactions between probiotics and ECM components provide additional anchorage and stability. For example, *Lactobacillus plantarum* strains have been shown to adhere to fibronectin and mucin in the ECM [24]. Moreover, the mucus layer covering the intestinal epithelium can act as a substrate for probiotic adhesion. Mucus-binding proteins expressed on probiotic cells mediate this interaction. For instance, a comparison of patients with IBD and healthy individuals showed that some strains of *Lactobacillus* ssp. isolates exhibit weaker attachment to epithelial cells and adhere in lower numbers in the IBD patient group [25].

Cell adhesion is a complex process influenced by multiple factors. It plays a crucial role in the interaction between the probiotic cells and the gut of the host. Understating the various mechanisms involved can provide insights into the dynamics of probiotic–host interactions. One factor that impacts cell adhesion is the presence of prebiotics, such as fructooligosaccharides (FOS) or inulin. These compounds can enhance probiotic adhesion to Caco-2 cells by modifying the cell surface characteristics of both the probiotics and the Caco-2 cells. Prebiotics can promote the expression of adhesion-related molecules on probiotic cells and increase the accessibility of adhesion receptors on Caco-2 cells [26]. Gastrointestinal conditions also play a significant role in cell adhesion. Simulated gastrointestinal conditions have been shown to increase the adhesion of *Lactobacillus paracasei* to Caco-2 cells. This increase can be assigned to the increased production of extracellular

polymers [27]. Temperature is another factor that influences cell adhesion. It has been reported that growth temperature affects the production of exopolysaccharides (EPS) by *Lactobacillus paracasei*, with different levels of polymerization being observed at different temperatures [28]. EPS production can impact the ability of probiotic cells to adhere to Caco-2 cells and potentially modulate their colonization and functionality. Physiological levels of shear stress in the intestinal lumen can also modulate the adhesion of probiotic cells. Under conditions of flow, the adhesion forces between probiotic cells and Caco-2 cells may differ from those under static conditions. Shear stress can influence the expression of adhesion-related genes and alter the surface characteristics of both probiotic and Caco-2 cells, ultimately affecting cell adhesion [29,30]. Host factors, including mucins, antimicrobial peptides and cytokines, can influence probiotic cell adhesion to Caco-2 cells. For instance, mucins, which are major components of the mucus layer, can affect the accessibility of adhesion receptors on Caco-2 cells and modulate the adhesion of probiotic cells. Additionally, cytokines like tumor necrosis factor-alpha (TNF-α) or interleukin-8 (IL-8) released during inflammation influence the adhesion of probiotic cells to Caco-2 cells [31]. These host factors highlight the impact of the dynamic gut environment on probiotic adhesion.

3.4. Adherence Patterns and Membrane Staining with DRAQ-5

IFC was also utilized to visualize the adherence of the bacterial cells after incubation with Caco-2 epithelial cells (Figure 7). DRAQ 5 was added to stain the Caco-2 cell (Ch11), and WGA was used to stain the bacterial cells (Ch02). The combined image (Ch02/Ch11) and brightfield view (Ch01) show that the bacteria adhered to the entirety of the accessible surface of the Caco-2 cell. The fluorescence channels for the detection of emitted light (Ch02 and Ch11) were selected to match the emission wavelength of selected dyes. Ch01 contains brightfield images recorded for individual events as they pass through the flow cell.

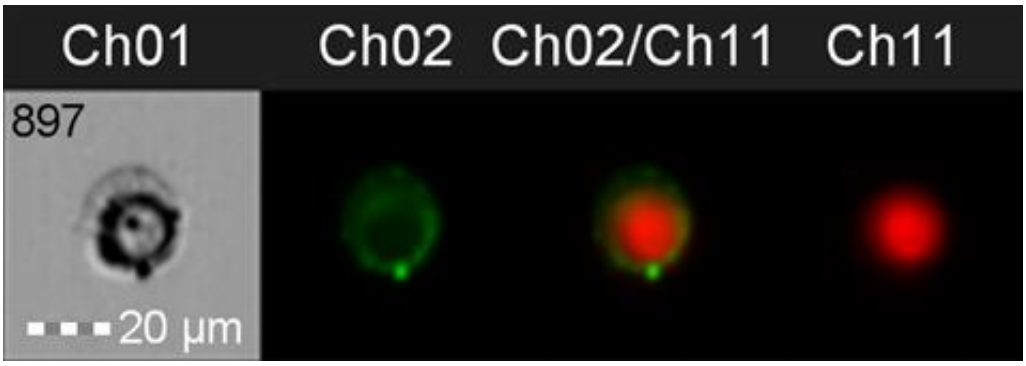


Figure 7. Example of adherence pattern in *E. faecium* visualized using DRAQ5 and WGA membrane dyes.

3.5. The Thermal Resistance of Bacteria

Thermal stress is a primary factor, occurring during drying, influencing cell viability and probiotic properties. DSC is sensitive to protein denaturation and can be used to determine the thermal resistance of cells. Lepock [32] reported that the onset temperature of protein denaturation is correlated with growth inhibition and the onset of cell lethality. It is, however, highly strain specific, and dependent on growth conditions. For example, the onset of denaturation for *Bacillus psychrophilus* was measured at only 30 °C, while it reached 55 °C and 65 °C for *Bacillus stearothermophilus* WAT and *Bacillus stearothermophilus* ATCC12016, respectively. Figure 8 summarizes the DSC measurements of thermal stability for *E.facium* and *C. divergens* grown under optimal conditions and after heat shock. The start of the denaturation process can be observed between 65 °C and 70 °C. The exact temperatures were 68.05 °C and 66.94 °C for *E. faecium* with and without heat shock, respectively, and 68.02 °C and 64.39 °C for *C. divergens*.

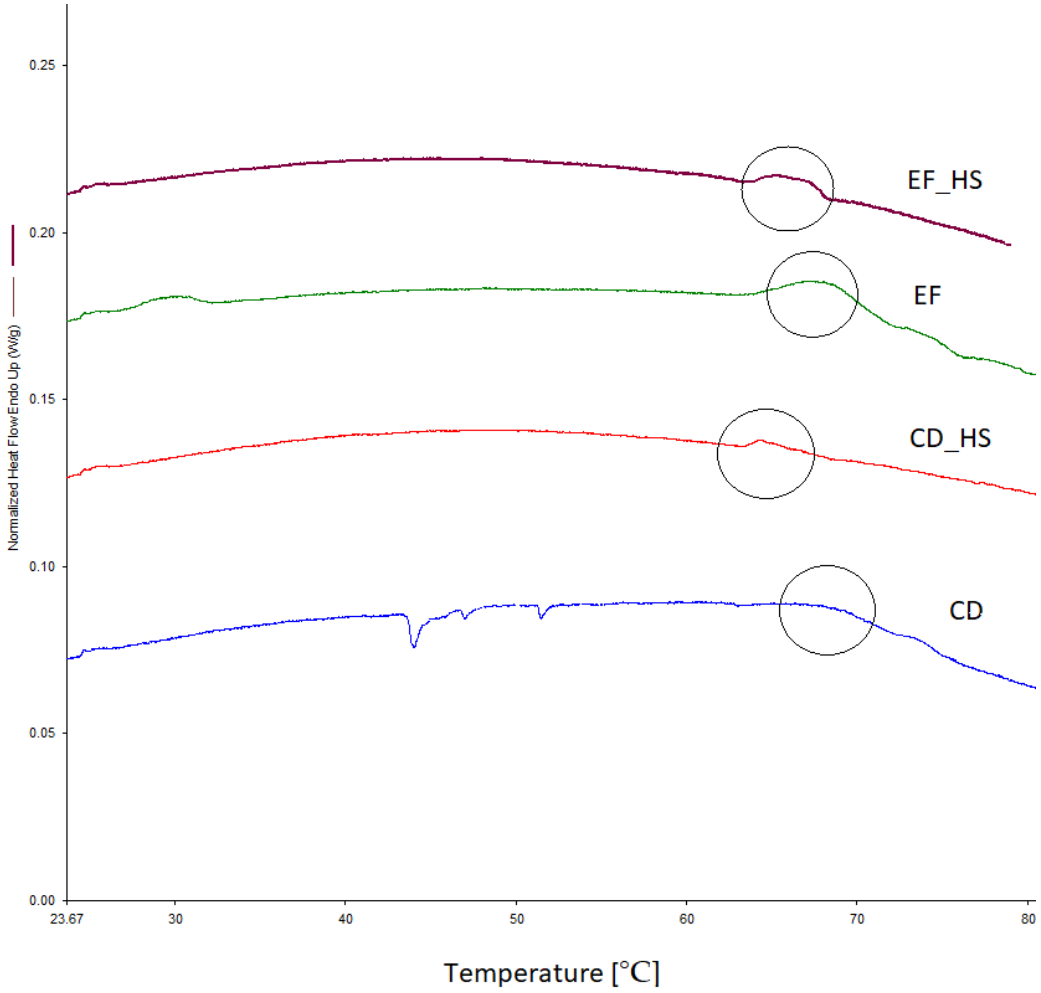


Figure 8. DSC graph of thermal stability for *E. faecium* and *C. divergens* cultured under optimal conditions and after heat shock. The curves for denaturation are circled.

3.6. Analysis of Samples after Storage

The dried preparations were stored for 12 months in different atmospheres and at different temperatures. Samples were measured after 1, 2, 3, 6, 9 and 12 months of storage or until the point at which their viability decreased below the 10^6 cfu/g threshold. The results (shown in Figure 9) show that the viability of stored preparations (measured in cfu/g) was highest for the samples stored at −20 °C, as those were the only samples to have a recorded viability of over 10^6 cfu/g for the whole 12 months. Samples stored at 4 °C also showed an acceptable shelf-life of 6–9 months. Samples stored at 20 °C lost their viability the fastest (after only 3 months). Modified atmospheres (N_2 and vacuum) also improved the shelf-life of samples compared to those kept in contact with air. For uncoated samples in particular, both variants had a prolonged shelf-life of 9 months compared with the 6 months for samples in contact with air. The beneficial effect of a modified atmosphere can be attributed to limiting the oxidation processes, effectively achieving the same level of stability as obtained by coating. The subpopulations in the samples were also determined using IFC, both fresh after drying and after 12 months. Most notable was the reduction in the number of active cells and the increase in both dead and mid-active subpopulations (Figure 10).

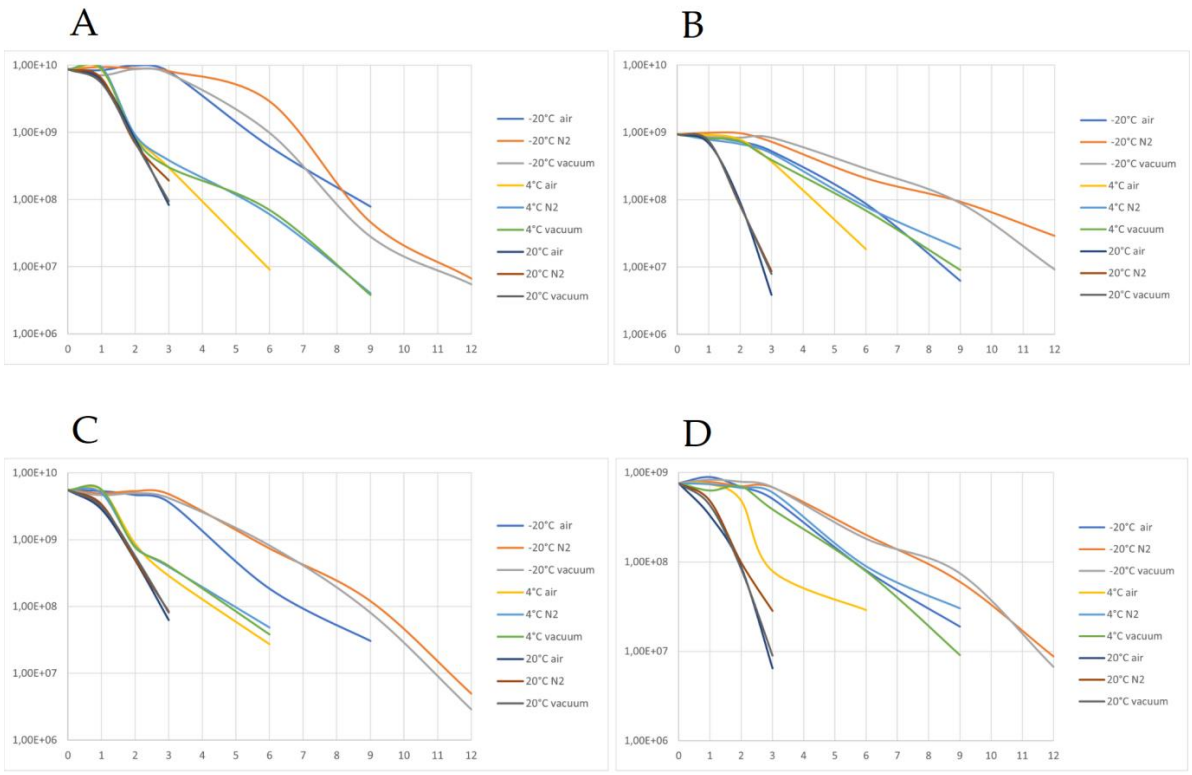


Figure 9. (**A**) *E. faecium* viability after storage under different conditions (−20 °C, 4 °C, 20° and packaging atmosphere with air, N2, and vacuum) for dried samples; (**B**) *E. faecium* viability after storage under different conditions (−20 °C, 4 °C, 20° and packaging atmosphere with air, N2, and vacuum) for coated samples; (**C**) *L. mesenteroides* viability after storage under different conditions (−20 °C, 4 °C, 20° and packaging atmosphere with air, N2, and vacuum) for dried samples; (**D**) *E. faecium* viability after storage under different conditions (−20 °C, 4 °C, 20° and packaging atmosphere with air, N2, and vacuum) for coated samples.

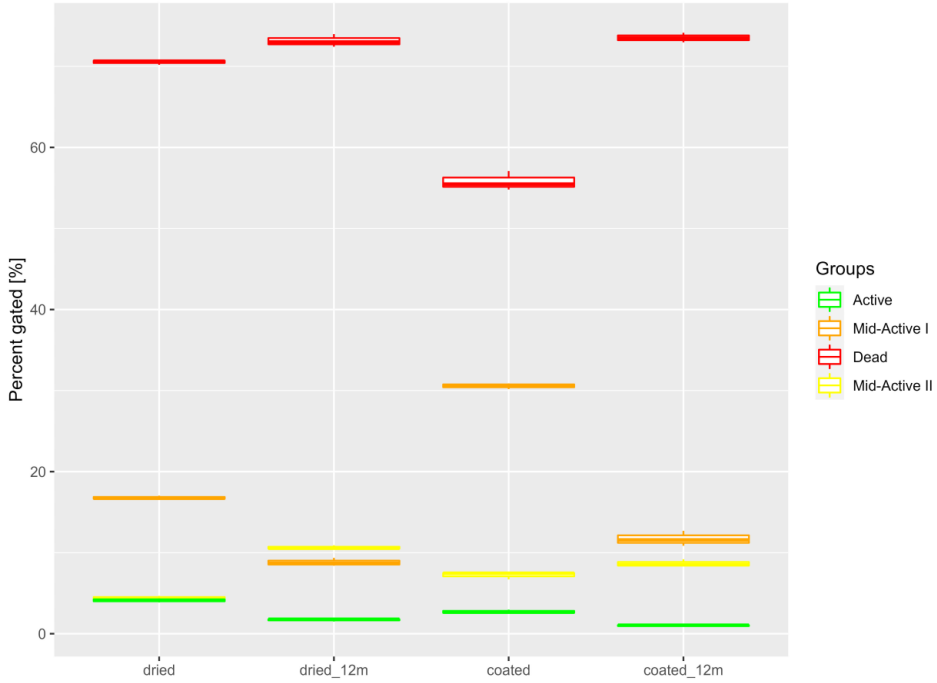


Figure 10. Comparison of percentages of cell subpopulations in dried and coated samples directly after processing and after 12 months of storage.

4. Conclusions

In conclusion, the cells that sustained membrane damage were divided into three groups (mid-active I, mid-active II, and dead) using IFC on the basis of the severity of the damage. A fourth group, containing cells with no recognizable damage, was determined and labeled as active. Only active cells exhibited the ability to adhere to the Caco-2 epithelial cell line, as confirmed by IFC. These results show that any, even minimal, damage to the cell membrane negatively affects the ability of the cell to adhere to the epithelium. This occurs even when damage to the cell membrane does not lead to cell death and the cell retains high enzymatic activity and is able to regenerate enough to maintain the ability to divide cells. DSC analysis of cell denaturation was used to determine the temperature at which denaturation started in our samples, which was between 65 and 70 °C. For dried preparations, the thermophysical analyses showed that the coating material with the highest T_g was HPMC, and that the samples stored at −20 °C and under a modified atmosphere had the longest shelf-life, at 12 months. Samples stored at −4 °C had a shelf-life of 6–9 months, while also being easier to store, potentially making this a better choice for commercial purposes. A comparison of the results of the IFC and pour plate counts also showed that the mid-active I and II subpopulations could not be recognized using the pour plate method. Cells with intermediate activity and low levels of cellular damage can still be viable in terms of their probiotic properties and ability to confer beneficial health effects. Commonly used classical microbiological methods are, however, unable to detect this important subpopulation, which could be included in viable cell counts for the assessment of probiotics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15153484/s1, Table S1: Dried samples. Table S2. Coated samples.

Author Contributions: Conceptualization, J.K.; methodology, J.K., W.J., A.O., A.S., J.T.-G. and R.D.; software, J.K.; validation, J.K.; formal analysis, J.K.; investigation, J.K.; resources, J.K., W.J., A.O., A.S., J.T.-G. and R.D.; data curation, J.K.; writing—original draft preparation, J.K.; writing—review and editing, J.K.; visualization, J.K.; supervision, R.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Materials.

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

References

1. Kiepś, J.; Dembczyński, R. Current Trends in the Production of Probiotic Formulations. *Foods* **2022**, *11*, 2330. [CrossRef]
2. Islam, S.U. Clinical Uses of Probiotics. *Medicine* **2016**, *95*, e2658. [CrossRef]
3. Prakoeswa, C.R.S.; Bonita, L.; Karim, A.; Herwanto, N.; Umborowati, M.A.; Setyaningrum, T.; Hidayati, A.N.; Surono, I.S. Beneficial Effect of Lactobacillus Plantarum IS-10506 Supplementation in Adults with Atopic Dermatitis: A Randomized Controlled Trial. *J. Dermatol. Treat.* **2022**, *33*, 1491–1498. [CrossRef]
4. Sáez-Lara, M.J.; Robles-Sanchez, C.; Ruiz-Ojeda, F.J.; Plaza-Diaz, J.; Gil, A. Effects of Probiotics and Synbiotics on Obesity, Insulin Resistance Syndrome, Type 2 Diabetes and Non-Alcoholic Fatty Liver Disease: A Review of Human Clinical Trials. *Int. J. Mol. Sci.* **2016**, *17*, 928. [CrossRef]
5. Broeckx, G.; Vandenheuvel, D.; Claes, I.J.J.; Lebeer, S.; Kiekens, F. Drying Techniques of Probiotic Bacteria as an Important Step towards the Development of Novel Pharmabiotics. *Int. J. Pharm.* **2016**, *505*, 303–318. [CrossRef]
6. Alp, D.; Kuleaşan, H. Adhesion Mechanisms of Lactic Acid Bacteria: Conventional and Novel Approaches for Testing. *World J. Microbiol. Biotechnol.* **2019**, *35*, 156. [CrossRef]
7. Byakika, S.; Mukisa, I.M.; Byaruhanga, Y.B.; Muyanja, C. A Review of Criteria and Methods for Evaluating the Probiotic Potential of Microorganisms. *Food Rev. Int.* **2019**, *35*, 427–466. [CrossRef]
8. Sarkar, S. Whether Viable and Dead Probiotic Are Equally Efficacious? *NFS* **2018**, *48*, 285–300. [CrossRef]

9. Kulkarni, S.; Haq, S.F.; Samant, S.; Sukumaran, S. Adaptation of Lactobacillus Acidophilus to Thermal Stress Yields a Thermotolerant Variant Which Also Exhibits Improved Survival at PH 2. *Probiotics Antimicro. Prot.* **2018**, *10*, 717–727. [CrossRef]
10. Bommasamudram, J.; Muthu, A.; Devappa, S. Effect of Sub-Lethal Heat Stress on Viability of Lacticaseibacillus Casei N in Spray-Dried Powders. *LWT* **2022**, *155*, 112904. [CrossRef]
11. Wang, L.-Q.; Zhao, F.; Liu, F.; Meng, X.-C. Live/Dead State Is Not the Factor Influencing Adhesion Ability of Bifidobacterium Animalis KLDS2.0603. *J. Microbiol.* **2013**, *51*, 584–589. [CrossRef]
12. Kiepś, J.; Juzwa, W.; Dembczyński, R. Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups. *Int. J. Mol. Sci.* **2023**, *24*, 6841. [CrossRef]
13. Valeriano, V.D.; Bagon, B.B.; Balolong, M.P.; Kang, D.-K. Carbohydrate-Binding Specificities of Potential Probiotic Lactobacillus Strains in Porcine Jejunal (IPEC-J2) Cells and Porcine Mucin. *J. Microbiol.* **2016**, *54*, 510–519. [CrossRef]
14. Nishiyama, K.; Sugiyama, M.; Mukai, T. Adhesion Properties of Lactic Acid Bacteria on Intestinal Mucin. *Microorganisms* **2016**, *4*, 34. [CrossRef]
15. Gorreja, F.; Walker, W.A. The Potential Role of Adherence Factors in Probiotic Function in the Gastrointestinal Tract of Adults and Pediatrics: A Narrative Review of Experimental and Human Studies. *Gut Microbes* **2022**, *14*, 2149214. [CrossRef]
16. Jessie Lau, L.Y.; Chye, F.Y. Antagonistic Effects of Lactobacillus Plantarum 0612 on the Adhesion of Selected Foodborne Enteropathogens in Various Colonic Environments. *Food Control* **2018**, *91*, 237–247. [CrossRef]
17. Walsham, A.D.S.; MacKenzie, D.A.; Cook, V.; Wemyss-Holden, S.; Hews, C.L.; Juge, N.; Schüller, S. Lactobacillus Reuteri Inhibition of Enteropathogenic Escherichia Coli Adherence to Human Intestinal Epithelium. *Front. Microbiol.* **2016**, *7*, 244. [CrossRef]
18. Wang, T.; Teng, K.; Liu, G.; Liu, Y.; Zhang, J.; Zhang, X.; Zhang, M.; Tao, Y.; Zhong, J. Lactobacillus Reuteri HCM2 Protects Mice against Enterotoxigenic Escherichia Coli through Modulation of Gut Microbiota. *Sci. Rep.* **2018**, *8*, 17485. [CrossRef]
19. Bron, P.A.; Kleerebezem, M.; Brummer, R.-J.; Cani, P.D.; Mercenier, A.; MacDonald, T.T.; Garcia-Ródenas, C.L.; Wells, J.M. Can Probiotics Modulate Human Disease by Impacting Intestinal Barrier Function? *Br. J. Nutr.* **2017**, *117*, 93–107. [CrossRef]
20. Monteagudo-Mera, A.; Rastall, R.A.; Gibson, G.R.; Charalampopoulos, D.; Chatzifragkou, A. Adhesion Mechanisms Mediated by Probiotics and Prebiotics and Their Potential Impact on Human Health. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 6463–6472. [CrossRef]
21. Chang, Y.H.; Jeong, C.H.; Cheng, W.N.; Choi, Y.; Shin, D.M.; Lee, S.; Han, S.G. Quality Characteristics of Yogurts Fermented with Short-Chain Fatty Acid-Producing Probiotics and Their Effects on Mucin Production and Probiotic Adhesion onto Human Colon Epithelial Cells. *J. Dairy Sci.* **2021**, *104*, 7415–7425. [CrossRef]
22. Mohanty, D.; Panda, S.; Kumar, S.; Ray, P. In Vitro Evaluation of Adherence and Anti-Infective Property of Probiotic Lactobacillus Plantarum DM 69 against Salmonella Enterica. *Microb. Pathog.* **2019**, *126*, 212–217. [CrossRef]
23. Xiong, R.; Pan, D.; Wu, Z.; Guo, Y.; Zeng, X.; Lian, L. Structure and Immunomodulatory Activity of a Recombinant Mucus-Binding Protein of Lactobacillus Acidophilus. *Future Microbiol.* **2018**, *13*, 1731–1743. [CrossRef]
24. Yadav, A.K.; Tyagi, A.; Kumar, A.; Saklani, A.C.; Grover, S.; Batish, V.K. Adhesion of Indigenous Lactobacillusplantarum to Gut Extracellular Matrix and Its Physicochemical Characterization. *Arch. Microbiol.* **2015**, *197*, 155–164. [CrossRef]
25. Najafi, S.; Sotoodehnejadnematalahi, F.; Amiri, M.M.; Pourshafie, M.R.; Rohani, M. Decreased Mucosal Adhesion of Lactobacillus Species in Patients with Inflammatory Bowel Disease. *Casp. J. Intern. Med.* **2022**, *13*, 713–720. [CrossRef]
26. Krausova, G.; Hynstova, I.; Svejstil, R.; Mrvikova, I.; Kadlec, R. Identification of Synbiotics Conducive to Probiotics Adherence to Intestinal Mucosa Using an In Vitro Caco-2 and HT29-MTX Cell Model. *Processes* **2021**, *9*, 569. [CrossRef]
27. Bengoa, A.A.; Zavala, L.; Carasi, P.; Trejo, S.A.; Bronsoms, S.; de los Ángeles Serradell, M.; Garrote, G.L.; Abraham, A.G. Simulated Gastrointestinal Conditions Increase Adhesion Ability of Lactobacillus Paracasei Strains Isolated from Kefir to Caco-2 Cells and Mucin. *Food Res. Int.* **2018**, *103*, 462–467. [CrossRef]
28. Bengoa, A.A.; Llamas, M.G.; Iraporda, C.; Dueñas, M.T.; Abraham, A.G.; Garrote, G.L. Impact of Growth Temperature on Exopolysaccharide Production and Probiotic Properties of Lactobacillus Paracasei Strains Isolated from Kefir Grains. *Food Microbiol.* **2018**, *69*, 212–218. [CrossRef]
29. Delon, L.C.; Guo, Z.; Oszmiana, A.; Chien, C.-C.; Gibson, R.; Prestidge, C.; Thierry, B. A Systematic Investigation of the Effect of the Fluid Shear Stress on Caco-2 cells towards the Optimization of Epithelial Organ-on-Chip Models. *Biomaterials* **2019**, *225*, 119521. [CrossRef] [PubMed]
30. Mays, Z.J.S.; Chappell, T.C.; Nair, N.U. Quantifying and Engineering Mucus Adhesion of Probiotics. *ACS Synth. Biol.* **2020**, *9*, 356–367. [CrossRef]
31. Saeed, A.; Yasmin, A.; Baig, M.; Ahmed, M.A.; Farooqi, Z.-R. Streptococcus Lactarius MB622 and Streptococcus Salivarius MB620 Isolated from Human Milk Reduce Chemokine IL-8 Production in Response to TNF-α in Caco-2 Cell Line, an Exploratory Study. *Cytokine* **2023**, *168*, 156232. [CrossRef]
32. Lepock, J.R. Measurement of Protein Stability and Protein Denaturation in Cells Using Differential Scanning Calorimetry. *Methods* **2005**, *35*, 117–125. [CrossRef]

applied sciences

MDPI

Article

Economic analysis of the production process of probiotics based on the biological and physiological parameters of the cells

Jakub Kiepś [1,*], Anna Olejnik [1], Wojciech Juzwa [1], and Radosław Dembczyński [1,*]

[1] Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-627 Poznań, Poland

* Correspondence: jakub.kieps@up.poznan.pl; radoslaw.dembczynski@up.poznan.pl

Featured Application: The presented analysis provides a concise depiction of the fluid bed drying process of probiotics. It also introduces a new method of evaluation for probiotics, based on the actual number of biologically useful cells. This factor is used to evaluate and economically justify the introduction of technological procedures, such as sublethal stresses and coating.

Abstract: Probiotic bacteria confer a range of health benefits and are a focus of a growing number of studies. One of the main issues is their stability during drying and storage, which is why techniques, such as fluid bed drying and coating or treatment with stress factors during culturing are used. The methods of the evaluation of probiotic viability and quality are however lacking and need a way of distinguishing between different subpopulations of probiotic bacteria. To address this issue imaging flow cytometry (IFC) has been used to assess cells after simulated *in vitro* digestion of dried and coated preparations treated with pH stress and heat shock. Samples were analyzed fresh and after 12 months of storage using RedoxSensor Green and propidium iodide dyes to assess metabolic activity and cell membrane integrity of the cells. The results were then used to design a drying process on an industrial scale and evaluate the economic factors in the Superpro Designer v13 software. Based on the number of biologically active and beneficial cells obtained using tested methods the coating process and treatment with heat shock and pH stress have been the most effective and up to 10 times cheaper to produce than only by drying. Additionally, samples after 12 months of storage have shown an increase in the proportion of cells with intermediate metabolic activity and low amounts of cell membrane damage, which are still viable in probiotic products. This subpopulation of bacteria can still be considered live in probiotic products but is not necessarily effectively detected by pour plate counts.

Keywords: SuperPro Designer, drying, shelf-life, quality control, simulated digestion, adhesion

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

1. Introduction

In recent decades, there has been a growing recognition of the importance of gut microbiota for human health and well-being. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. Cells are often enumerated using plate pour counts directly after the production process or after storage. Such values do not directly represent the number of probiotic cells that reach the colon after the gastric transit. Additionally, pour plate enumeration does not take into consideration cell subpopulations with intermediate activity (i.e. viable but nonculturable - VBNC) [2]. Therefore the number of colony forming units determined using classic microbiology is not synonymous with the number of probiotic cells in the preparation. To address this issue we determined that for more precise enumeration the viable and beneficial (VB) cells enumerated using imaging flow cytometry should be

considered, namely those cells that remain useful and confer health benefits to the host after technological processes and digestion. We also look deeply into the process of production of probiotics and determine the cost and quality of the product based on those assumptions.

New reports on the numerous health benefits of probiotic bacteria, such as their role in the positive regulation of inflammatory bowel diseases (IBD), treatment of allergies, and urogenital infections have led to increased interest in their commercial production [3]. Existing studies on probiotic production often utilize drying techniques such as spray or freeze-drying. The quality parameters of dry cells (number of living cells, biological activity) usually depend on the type of drying method used. In the case of thermolabile materials, including bacterial cells, the smallest reduction in quality after drying is observed when freeze-drying is used. However, this method is expensive and time-consuming [4]. Therefore, freeze-drying is increasingly being replaced by spray drying. However, during spray drying, it is often difficult to obtain the quality of bacterial preparations similar to that obtained during freeze-drying. One of the reasons is the relatively high drying temperature, which will usually not be lower than 100^{0}C for inlet and 60^{0}C for outlet air. Meanwhile, in fluid bed dryers it is possible to carry out the process at much lower temperatures, close to 30-40^{0}C, thus minimizing heat inactivation [5,6]. Dryers operating in fluidized bed conditions have very good mass and energy transfer parameters, therefore the drying time is short [7]. Additionally, the investment and operating costs of fluid bed drying are lower than those of freeze or spray drying [8]. In a fluidized bed, the cell matrix can be easily coated with an additional protective layer, improving its shelf-life and stability during storage. Effective application of probiotics is, however, limited due to the lack of standardized requirements for efficacy and the number of viable cells (colony forming units, CFU/mL) in the final product. Additionally, the decrease in cell viability during the passage through the gastrointestinal (GI) tract is not taken into consideration [9]. Recent advances in gastrointestinal simulation techniques have made it possible to better understand the dynamic interactions that influence the survival and activity of probiotics along their journey through the digestive tract. The limited survival of probiotics during in vitro digestion is a complex process that is influenced by many factors, including the acidity of the stomach, the presence of bile salts and enzymes, the gut microbiota and its colonization resistance, and properties of the probiotic strain itself [10]. The acidic environment of the stomach is one of the most significant challenges to the survival of probiotics. To survive the acidic environment of the stomach, probiotics must have some protective mechanisms in place. These mechanisms include the production of acids and enzymes that can neutralize stomach acid [11], the formation of protective biofilms [12,13], and the ability to adhere to the stomach lining [14]. Bile salts and enzymes are also major challenges to the survival of probiotics. Bile salts are produced by the liver and are released into the small intestine. Bile salts have many antimicrobial properties that can kill probiotic bacteria [15]. Antimicrobial proteins are also present in the small intestine and act as a defense mechanism against external microorganisms [16]. To survive the bile salts and enzymes, bacteria must have the ability to adhere to the intestinal lining and to produce protective substances (bile salt hydrolase) that can neutralize the bile salts [17]. In addition to the acidity of the stomach and the presence of bile salts and enzymes, the physical and chemical properties of the probiotic strain also play a role in its survival during in vitro digestion. Probiotic strains that are more resistant to acidity, bile salts, and enzymes are more likely to survive the in vitro digestion process. Additionally, probiotic strains that can adhere to the intestinal lining and produce protective substances are also more likely to survive the in vitro digestion process [18]. Coating is also one of the strategies utilized to improve the survival of probiotics during digestion and to improve the shelf-life of the product [19]. Different functional materials can be used as coating substances to tackle various challenges expected during the oral delivery of probiotics [20]. Polysaccharides, such as alginate, as well as cellulose and lipid-based coating are commonly used to protect the probiotics from low pH of the gastrointestinal tract. Coatings

can also be utilized to supply the probiotic cells with prebiotics by using inulin or polydextrose as coating material. Additionally, coating can improve the intestinal retention by strengthening the interaction between probiotics and the intestinal mucus layer. Composite biomagnetic materials can be utilized in the coating layer to improve both the retention and cellular localization of the probiotics [21]. Also, natural ingredients such as red ginseng dietary fiber can improve the intestinal adhesion of probiotics [22].

The first section of the paper discusses the mechanisms underlying the survival of probiotic LAB during in vitro digestion, which is especially important for the evaluation of possible probiotics since only living bacteria can fully confer their health benefits after successfully passing through the GI tract [23]. The acidic environment of the stomach and the presence of bile salts and enzymes pose significant challenges to the viability of these microorganisms. For the *in vitro* digestion we have chosen samples that were cultured in stress conditions (of high temperature and pH shock) that contained freshly cultured bacteria, dried and coated preparations, as well as preparations after storage to check their influence on the survival of probiotic bacteria during the gastrointestinal passage.

The second section of the paper presents a comprehensive project focused on the production of probiotics. This project details each step of the process, from strain selection and fermentation to drying and formulation. The integration of scientific principles with technological innovations ensures the production of high-quality probiotics with optimal viability. Commonly, pour plate counts are used as a standard evaluation method for probiotic enumeration. The results are then calculated as colony forming units (cfu) per gram or milliliter of preparation. However, not all bacterial cells present in the final product are active and can confer health benefits to the host. Furthermore, not all of the live cells (VBNC) can grow on standard Agar media [24]. Flow cytometry allows to count and assess other subpopulations than fully active cells [25], for example, based on their metabolic activity and cellular membrane damage. Flow cytometry provides a more comprehensive characterization of probiotics, while being a more rapid technique, allowing for the analysis of thousands of cells per second [26]. To better describe the amount of biologically active and useful cells the results of digestion analyzed by flow cytometry were used for economic analysis as they provide a more reliable parameter for process design and planning of production steps.

2. Materials and Methods

2.1. Strains and cultures

The strain of lactic acid bacteria (LAB) used in this study was *Enterococcus faecium* 73 KBiMŻ. MRS broth was selected as the medium because it provides the required growth conditions for these bacteria. To achieve the highest amount of biomass, an inoculum was prepared in a volume that represented 10% of the medium volume for the bioreactor culture. To gradually achieve a culture volume of 1 liter, the inoculum was first seeded in two stages. This allowed for improved adaptation of the microorganisms and shortened the lag phase.

All steps of the inoculum preparation were conducted in a laminar flow hood for a minimized contamination risk. First, the strain was thawed on ice to minimize cell damage. Once the microorganisms had warmed to an ambient temperature and thawed, they were transferred to a larger, 15-milliliter Falcon conical tube filled with 9 milliliters of MRS broth (Oxoid Ltd., Basingstoke, Hampshire, UK). The tube was then sealed with parafilm and incubated for 24 hours at 30°C. Following incubation, 10 milliliters of the inoculum were transferred to a flask containing 90 milliliters of MRS broth. The flask was incubated for an additional 24 hours at 30°C. After these steps, the inoculum was used to initiate a culture in the bioreactor.

The cell biomass was cultured using Biostat A plus bioreactors (Sartorius AG, Göttingen, Germany), equipped with a 5L culture vessel. The bioreactor was heated using a heating blanket system. Agitation was provided by a Rushton impeller. Process control

and data acquisition were performed using the BioPAT MFCS Software. The pH electrode was calibrated against buffers at pH 4 and pH 9 before sterilization and culturing. Next, MRS broth medium in the volume of 1Lwas added to the bioreactor vessel. To create aseptic environment all parts of the bioreactor and the medium were then autoclaved at 121°C for 30 minutes. Afterwards, nitrogen was run through the aeration system to minimize chance of pathogens entering the bioreactor with air. After the medium in the bioreactor has cooled to 30°C the inoculum was added. Bacteria were cultured at 30°C, with the pH set at 6.5 and maintained by adding a 30% NaOH solution. The culture was grown for 24 hours, with constant stirring at 150 RPM. The end of the exponential growth phase was determined by the cessation of NaOH consumption and the stabilization of pH. These factors, in combination with the simultaneous end of base consumption were used as indicators for the beginning of the stationary growth phase. Lastly, after the biomass growth has stopped, the culture was transferred into sterile centrifuge vessels using a peristaltic pump.

2.2. Stresses

To investigate the impact of stress on bacterial cell survival during fluid bed drying, various cultures were subjected to distinct stress environments. Two types of stresses were introduced: heat shock cultures were exposed to short-term thermal stress by increasing the temperature to 50°C for 30 minutes. During pH stress cultures were exposed to short-term acid stress by lowering the pH to 2.0 for 30 minutes. These two types of stresses were also determined to be the easiest to apply in an industrial setting. Osmotic stress and culturing without pH control were also considered as possible stress factors for bacterial adaptation. They were, however, not used since osmotic stress proved to be troublesome to implement on a larger scale, after adding salts to the medium to expose the bacteria to stress conditions the whole biomass needed to be centrifuged and resuspended in a freshly made medium. Additionally culturing without pH control was not used in the research, since it did not provide the expected split into the subpopulations with intermediate metabolic activity, as observed with pH and heat shock. The main goal of introducing sublethal stress conditions during the culturing stage was to determine whether the stress adaptation of bacterial cells had a significant impact on their survival during fluid bed drying.

2.3. Fluid bed drying and coating

Both the drying and coating process were conducted in the Strea-1 (GEA, Oelde, Germany) laboratory fluid bed dryer. First, the matrix (crystalline microcellulose [Ingredientpharm, Pratteln, Switzerland]) was added to the product container in the dryer. To put the product in a fluid state a stream of hot (up to 50°C) air was introduced.

Bacteria for drying were suspended in a solution of a protective substance (5% trehalose [Sigma-Aldrich, St. Louis, MO, USA]) and pumped to the dryer. They entered an atomizing nozzle, which was operating under the pressure of 2 bar. Higher pressures could damage the cells due to the shear forces and reduce their viability. The drying and coating process took approximately 30 minutes each. 100 g of matrix and 100 ml of 2% hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich, St. Louis, MO, USA) were used for coating. Drying was carried out in the top-spraying system, where the nozzle was placed above the fluidized bed. For coating the Wurster system was utilized, where the nozzle sprays the coating agent from below and is kept shielded from the matrix to avoid clumping. Ready powder was packed for further analysis and shelf-life assessment.

2.4. Simulated gastrointestinal conditions

Based on the method reported by Minekus et al. [27] the simulated gastrointestinal conditions were divided into gastric and intestinal phases, without introducing the oral

phase as it was deemed optional by this method authors. Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were made up as recommended by Minekus et al. with the addition of enzymes and $CaCl_2$ (Table 1.). 1M NaOH and 1M HCl were used for pH regulation in both digestion fluids. For the simulated gastric digestion rehydrated sample and gastric fluid were combined in a 50:50 (v/v) ratio for a final volume of 40 ml. Samples under digestion were incubated at 37 °C for 2h. After this step, the sample (40 ml) was combined with 40 ml of intestinal fluid and incubated at 37 °C for 2h.

Table 1. Simulated digestion fluids composition.

Constituent	SGF	SIF
pepsin	2000 U/ml	-
pancreatin	-	100 U/ml (based on trypsin)
bile	-	10 mM
$CaCl_2$	0.075 mM	0.3 mM
pH	**3**	**7**

2.5. Intestinal Epithelial Cell Culture

The human intestinal epithelial Caco-2 cell line (HTB-37™) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). It is a well-established model for studying intestinal barrier function. The cells were isolated from colon adenocarcinoma and can form a tight monolayer in culture.

To culture Caco-2 cells, the cells are placed on PET membranes (Millicell® Cell Culture Inserts, 24 mm diameter, 0.4 μm pore size) (Millipore, Burlington, MA, USA, Merck Group) The initial density is 4×10^5 cells/cm^2. The cells are cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA) with the addition of 1% non-essential amino acids (100X NEAA, Sigma-Aldrich) and 20% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA). The medium is changed three times a week, and the cells are maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The integrity of the Caco-2 cell monolayer is monitored based on transepithelial electrical resistance (TEER) measurements. TEER is a measure of the electrical resistance across the cell monolayer. Caco-2 cell cultures with TEER values ≥ 600 Ω × cm^2 are considered to have formed a tight monolayer and are used in the bacteria adhesion experiments.

2.6. Adhesion assay

The adhesion assay was performed according to the methodology described in our previous work [28]. The Caco-2 cells were prepared by first washing the monolayers twice with PBS to remove any loosely attached cells. DMEM (without phenol red) with bacterial cells was then added to the cells, and the cultures were incubated for 2 hours at 37°C to allow the bacterial cells to adhere to the Caco-2 cells. After incubation, the medium was removed from the cultures, and the cell monolayers were washed with PBS to remove any unbound bacterial cells. Lysis was performed next to release the adhered bacterial cells and the lysates were centrifuged and analyzed. The distribution of subpopulations in the samples post-adhesion was measured by analyzing the supernatant left after incubation and washing of the cells.

2.7. Imaging flow cytometry

Flow cytometry was used to examine bacterial cells for their metabolic activity and viability. Amnis FlowSight™ (Luminex Corp., USA) is a flow cytometer with imaging and was used to examine bacterial cells. The flow cytometer allows for the use of 3 lasers (405 nm, 488 nm, and 642 nm), 5 fluorescence channels (acquisition by a multi-channel CCD

camera), and a side scatter detector (SSC). We utilized the protocol described in our previous work [24], using RedoxSensor™ Green and PI (propidium iodide) to assess the metabolic activity of the cell and the integrity of the cell membrane accordingly.

2.8. Process simulation in Superpro Designer

To simulate the large-scale production of fluid bed dried probiotics, SuperPro Designer v13 (Intelligen, USA) was employed. The key conditions for the process were determined based on experimental results obtained from laboratory-scale processes. The final product was prepared in the form of a dried powder containing the biomass of *Enterococcus faecium* 73 KBiMŻ, standardized to contain 1×10^8 of viable bacterial cells per gram of the product. The project simulation provides information on economic evaluation. In the evaluation the process flowsheets, operating costs, estimated capital, raw material and equipment costs, and profitability analysis are included. The cost of the equipment was provided from local supplier information and the sizing was calculated based on the process needs and throughput. Raw material prices were based on quotations from global suppliers (i.e. Sigma-Aldrich, St. Louis, MO, US). Other economic variables, such as water (2.67 \$/m^3), electricity (0.20\$/kW-h), income tax (19%), and labor cost (5.50 \$/h) were established based on local (Poland) values and may vary based on location. The project was assumed to operate for 330 days annually, with a 15-year lifespan. Additionally, one year was planned for construction and six months for start-up. The efficiency in the first year was estimated to be 50% due to the start-up period and lower occupancy. Described assumptions were constant and applied to all six projects.

2.9. Sensitivity analysis

Crystal Ball software (Oracle, USA) was used to perform a sensitivity analysis for the key parameters of the technological process to assess their impact on the unit production cost. Using the COM function of SuperPro designer simulations were performed for a range of values for the following parameters: cost (normal distribution) of trehalose, HPMC, and medium and process time (triangular distribution) of fermentation, drying, and coating. The analysis was conducted for a range of values for those parameters from -20% to +20% of the base value.

2.10. Statistical analysis

Statistical analysis was performed using R version 4.3.1 run in RStudio (Posit, Boston, MA, USA). Statistical differences between the analyzed groups were determined by a one-way ANOVA with Tukey's post hoc test. A p-value below 0.05 was considered statistically significant. The results are presented as mean values of three repetitions ± standard deviation.

3. Results and discussion

3.1. In vitro simulated digestion

In this part of the research, different samples were treated with two-step gastrointestinal digestion and subjected to adhesion assay afterward. The following samples were used: free bacteria cells, cultured in optimal conditions, and treated with heat shock and pH stress (Figure 1.); samples after fluid bed drying and coating were analyzed both fresh and after 12 months of storage after no treatment, heat shock, and pH shock (Figures 2. and 3.). Heat shock and pH shock were chosen as two types of the most common stress factors for lactic acid bacteria. These stresses are also present during drying (heat shock) and digestion (pH shock). Additionally, cross-protection systems can help the cells adapt to different types of stresses sharing similar resistance mechanisms *[29].* The obtained results were used in the project of the technological process to represent the subpopulation spread for different variants. Four

subpopulations of cells were determined using IFC, based on their metabolic activity (measured by RedoxSensor Green) and cellular membrane damage (measured by propidium iodide). The active subpopulation includes the cells with high levels of metabolic activity and no cellular membrane damage, the mid-active I cells show low levels of both metabolic activity and cellular membrane damage, while the mid-active II cells show high levels of both, the dead cell subpopulation contains cells with no metabolic activity and high degree of cellular membrane damage.

Free cells presented in Figure 1. show high levels of activity before treatment and after pH shock. The cells after heat shock show already much lower levels of active cells than two other variants. After *in vitro* digestion, all free cell samples show a significant decrease in active cell subpopulation, especially after the second stage representing the small intestine. For both dried and coated samples (also after storage) the results show that the first stage of digestion (S1), representing the gastric conditions led to a decrease in active cells in all samples. For free cells cultured in optimal conditions and after pH shock the mid-active II subpopulation has increased after S1, which shows that the damage may not be permanent, as these cells have shown the ability to regenerate in optimal conditions *[24]*. A similar increase in mid-active II cells was also observed in dried cells treated with pH and heat shock after 12 months of storage. The second stage of digestion (S2), representing the small intestine was more lethal for cells than S1, as also observed by Rodrigues et al.*[30]*. In all samples reduction of active cells was observed with a simultaneous decrease in overall observed cells, caused by cell breakdown. An increase in cellular debris, that could not be assigned to any of the four subpopulations was observed in IFC as a result of the cell decomposition. Cells after coating were overall less affected by the GI conditions than uncoated cells. Similar results were observed for *Lactobacillus salivarius* NRRL B-30514 coated with rice protein-shellac composite *[31]*. Cells after S2 were subjected to adhesion assay, which showed an increase in active subpopulation, further proving that the mid-active cells were able to resume their activity after being placed in optimal conditions during adhesion assay. Mid-active cells would not be enumerated using plate cell counts, and with such methods, a reduction in the number of microorganisms would be noticed. However, the preparations evaluated using our method show activity even after a year, thanks to the detection of VBNC cells. Samples after 12 months of storage show an increase in the proportion of cells with intermediate metabolic activity and low amounts of cellular damage. After such selection, the remaining cells are more resistant and tolerate the digestion conditions better than cells directly after drying.

Figure 1.

Subpopulation distribution in samples from bacterial cultures grown in optimal conditions, with heat shock and pH shock. Shown in four stages – pre-adhesion, after the first stage of *in vitro* digestion, after the second stage of *in vitro* digestion, and after adhesion.

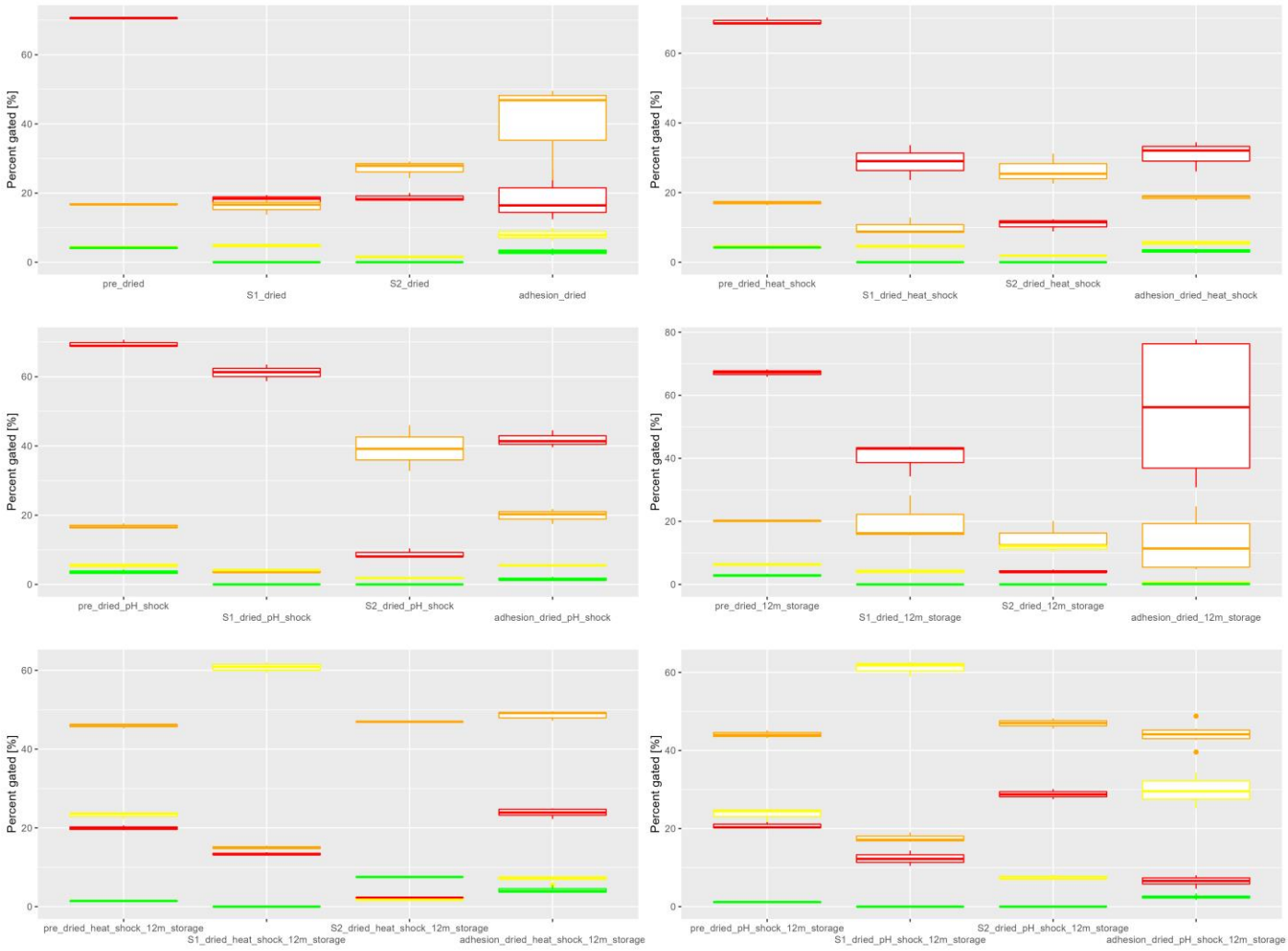


Figure 2.

Subpopulation distribution in fluid bed-dried samples treated with heat shock, pH shock, and untreated. Shown in four stages – pre-adhesion, after the first stage of *in vitro* digestion, after the second stage of *in vitro* digestion, and after adhesion.

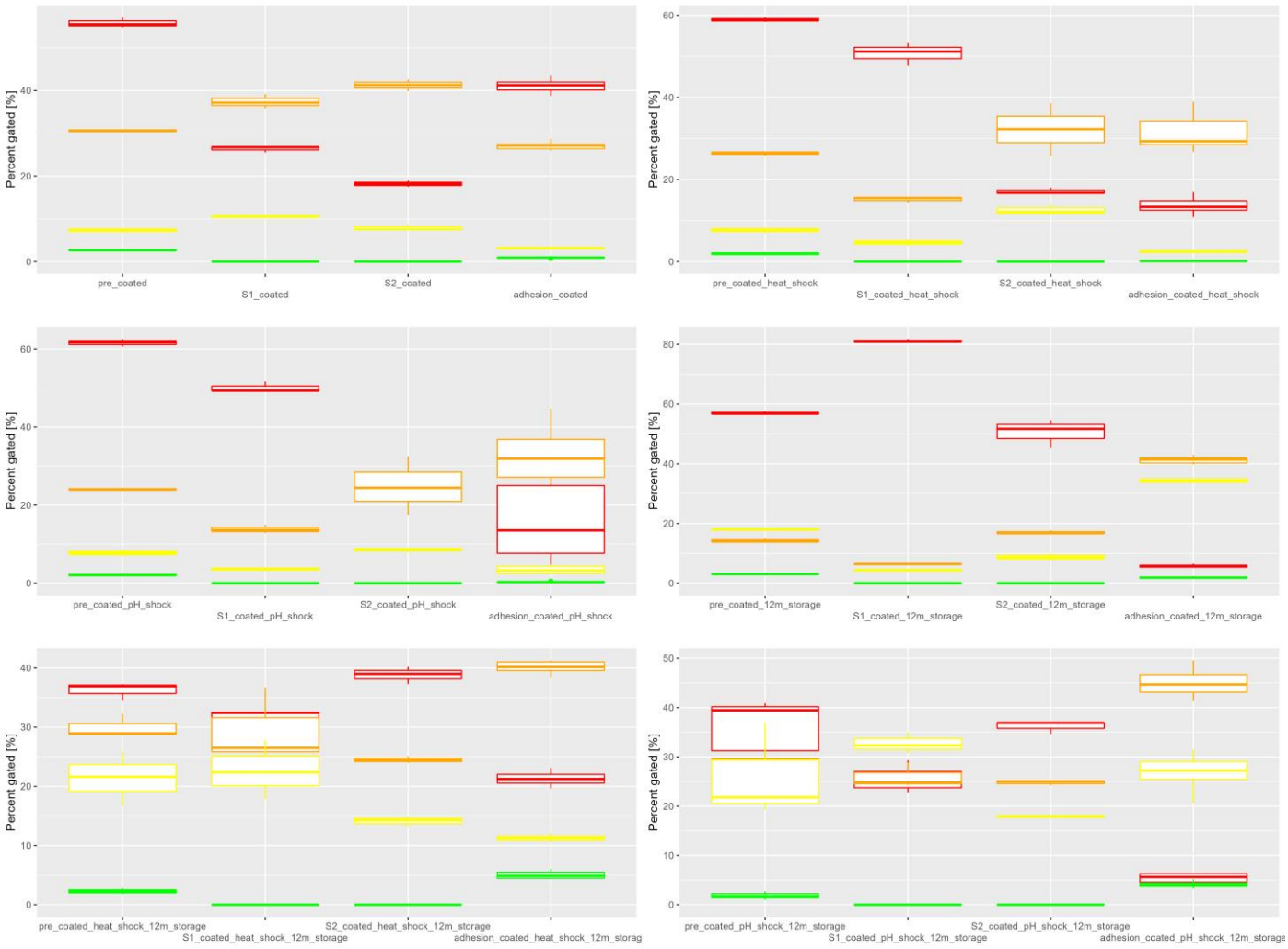


Figure 3.

Subpopulation distribution in fluid bed-dried and coated samples treated with heat shock, pH shock, and untreated. Shown in four stages – pre-adhesion, after the first stage of *in vitro* digestion, after the second stage of *in vitro* digestion, and after adhesion.

3.2. Process design and economic analysis

The preliminary project of the technological process for dried probiotic production was prepared based on laboratory-scale experiments. Its process flow diagram shows the necessary steps and procedures in the variant including fluid bed drying and coating (Figure 4.). Overall 6 project variants were prepared – for drying with no stress, with pH shock, and with heat shock and similarly for coating with no stress, with pH shock, and with heat shock. Triggering bacterial stress adaptation was introduced as a way of improving the viability of probiotics during drying and storage *[32]*. All projects were divided into 3 sections: Medium Preparation, Seed Culture and Fermentation, and Fluid bed Drying and Coating. Medium preparation is a step where the MRS medium for culturing is mixed from base ingredients, heat sterilized, and distributed to corresponding culturing vessels. The second section contains culturing containers with increasing volumes – from 2L shake flasks to 250L bioreactor. In the final stage, the biomass is mixed with a protective substance (5% trehalose) and dried in a fluid bed dryer with crystalline microcellulose as a matrix. The protective effects of using trehalose come from its ability to replace the water in the intracellular macromolecules during drying *[33]*. In an additional step, the powder can then be coated with 2%

HPMC. The final number of cells in the product was set at 10^8 cfu/g as recommended by industry standards *[34]*. Economic calculations have been performed for the process, taking into consideration the cost of raw materials, waste treatment, energy consumption and heat transfer agents, equipment cost, and the direct fixed capital (DFC). DFC consists of total plant direct cost (TPDC), total plant indirect cost (TPIC), and contractors fee and contingency (CFC). The economic evaluation containing all of the mentioned costs has been summarized in Table 2. The cost of purchasing equipment needed for the process is summarized in Table 3. Additionally, the cost of installment for the equipment was calculated as 1.5 x the purchase cost. To evaluate the economic profitability of certain technological treatments, such as coating and stresses during culturing the production costs of obtaining preparations for six variants (dried, dried with heat shock, dried with pH shock, coated, coated with heat shock, coated with pH shock) were compared. As proven by our previous research the cells that are beneficial for the patient, namely metabolically active and adherent to the Caco-2 cells, are present in two of the described subpopulations – active and mid-active II. Only those two groups were taken into consideration when assessing the cost of production of probiotic powder, based on the definition of probiotics, which describes them as live cells *[35]*. Using imaging flow cytometry the mid-active II cell subpopulation was determined to suit the description of VBNC *[36]* and therefore was also included in the project. The results of simulated *in vitro* digestion experiments were used, where the subpopulation composition of preparation after ingestion was assessed. Using those values we determined the viable and beneficial (VB) cells, which are the cells that remain useful and confer health benefits to the host after technological processes and digestion. The individual cost of production of 1kg of VB cells using different technological variants was calculated in Table 4. For the samples measured directly after drying the coated variant treated with heat shock provided the best value in terms of price per 1kg of VB cells after digestion. As for the samples after 12 months of storage, the coated samples after pH shock showed the best value. The results for samples after adhesion were not included in the economic assessment of the project, since only the non-adherent cells present in the suspension after incubation could be measured using flow cytometry. Based on the results it can be concluded that the number of viable cells after drying is not a good indicator of the product quality. For better assessment number of cells surviving the technological process, storage and digestion should be most important for the consumer. Economic evaluation based on the price of achieving preparation containing 1kg of cells that meet those requirements should also be considered by the producer while planning the process.

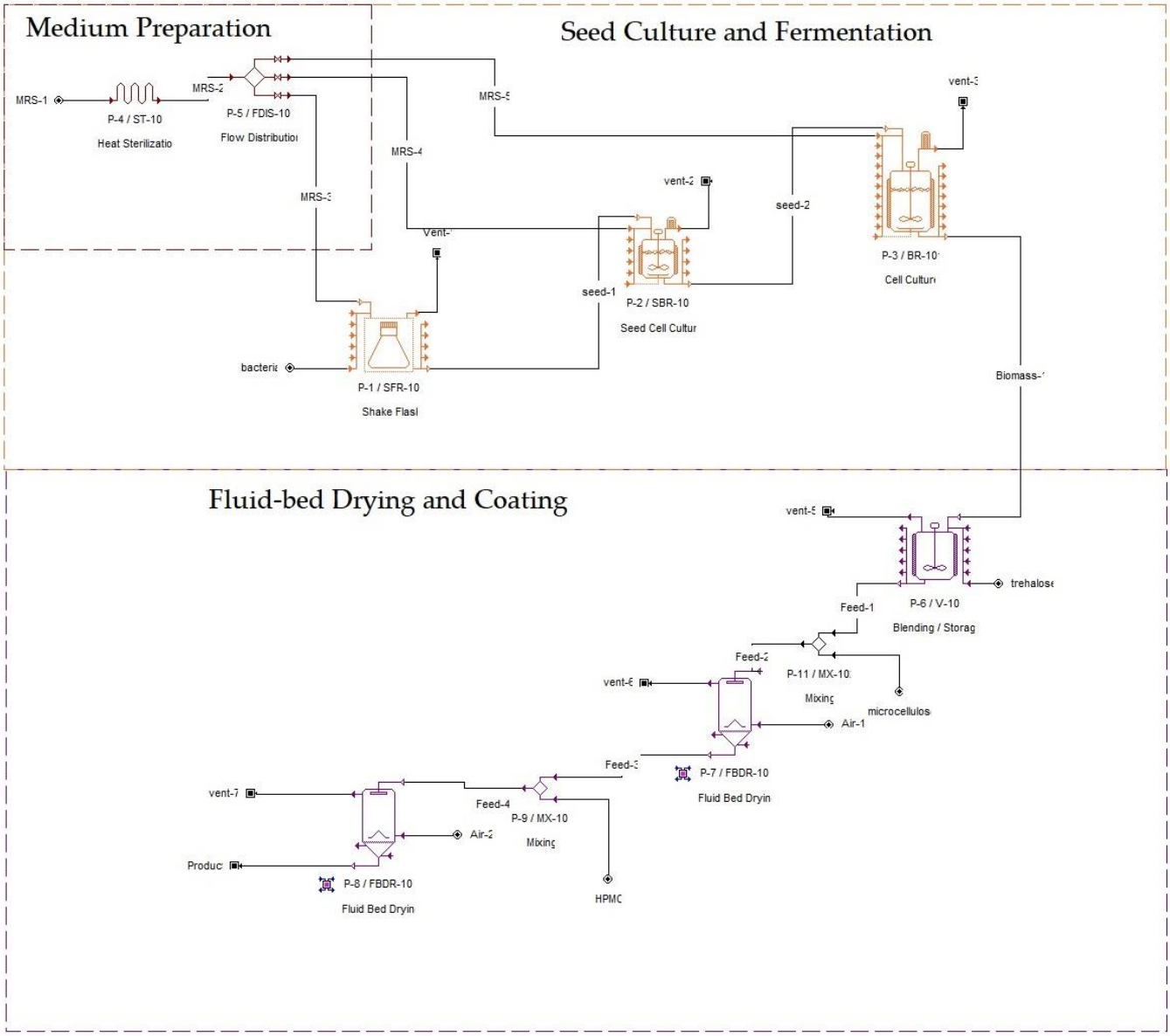


Figure 4.

Process flow diagram for fluid bed drying and coating of probiotics.

Table 2. Economic evaluation summary

Parameter	Unit	
DFC	$	1,584,000
TPDC	$	861,000
TPIC	$	516,000
CFC	$	207,000
Operating cost	$	100,000
Batch size	kg	326.91
Cost basis annual	kg/year	34,979
Gross margin	%	71.98
Return on investment (ROI)	%	11.51
Payback time (PBT)	year	4.33
Net present value (NPV at 7%)	$	909,000
Revenues [per year]	$	350,000

Table 3. Equipment and its purchase cost summary

Equipment	Size	Purchase cost (PC) [$]
Fluid Bed Dryer	415.97 L	107,000
Bioreactor	296.11 L	150,000
Seed Bioreactor	18.34 L	29,950
Heat sterilizer	66.17 L/h rated by throughput	30,000
Blending tank	308.29 L	11,400
Shake flask rack	2 L	1000

Table 4. Economic evaluation of different variants

variant	Unit production cost [$/kg]	Production cost [$/1kg VB cells pre-digestion]	Production cost [$/1kg VB cells after digestion]
Dried [A]	2.59	30.58 ^{cde} ± 1.05	173.83 ^{a} ± 32.59
Dried, 12 months [B]	2.59	28.09 ^{cde} ± 2.45	22.19 ^{cde} ± 2.11
Dried heat shock [A]	2.59	29.40 ^{cde} ± 0.13	134.20 ^{b} ± 12.05
Dried heat shock 12 months [B]	2.59	10.52 ^{e} ± 0.36	27.91 ^{cde} ± 0.98
Dried pH shock [A]	2.63	29.35 ^{cde} ± 1.99	144.51 ^{b} ± 10.82
Dried pH shock 12 months [B]	2.63	10.58 ^{e} ± 0.37	35.11 ^{cd} ± 2.87
Coated [A]	3.01	30.31 ^{cde} ± 1.73	38.18 ^{c} ± 3.36
Coated 12 months [B]	3.01	14.36 ^{cde} ± 0.27	34.13 ^{cde} ± 4.06
Coated heat shock [A]	3.01	31.45 ^{cde} ± 2.77	24.03 ^{cde} ± 2.48
Coated heat shock 12 months [B]	3.01	12.73^{de} ± 1.87	21.38 ^{cde} ± 1.41
Coated pH shock [A]	3.05	31.00 ^{cde} ± 1.77	36.22 ^{cd} ± 2.44
Coated pH shock 12 months [B]	3.05	10.95 ^{e} ± 1.24	17.02 ^{de} ± 0.35

[A] measured directly after drying/coating

[B] measured after 12 months of storage

Results are mean values of three repetitions ± standard deviation. Values sharing the same letter are not significantly different ($p \leq 0.05$).

Sensitivity analysis was provided for key assumptions in reference to the unit production cost and is presented in Figure 5. The baseline unit production price was 2.75 $/kg and the baseline number of batches was 10^7. Those values were then tested in the range of -20% to 20%. Results show that the main impact on product cost has the medium price, which confirms the need for seeking alternative carbon sources and media based on recycled waste materials. Additionally, the drying process has some impact, mainly because of the dryer purchase price, as well as the amount of trehalose and energy used. Trehalose cost, HPMC cost, and fermentation time both show the expected impact on the price. Little to no change was observed for coating, mainly since this process utilizes the same equipment as the drying step and therefore only the resource and energy demand is considered. Figures 6. and 7. both show the probability distribution based on 10,000 trials for the unit production cost and annual number of batches, accordingly. The probability of achieving unit production prices under 3.01 was calculated at 91.88 %. The probability of achieving the desired annual number of batches in the range of 102-112 was certain in 86.79%. The

variability of the main product cost and number of batches per year has been demonstrated in Figure 8.

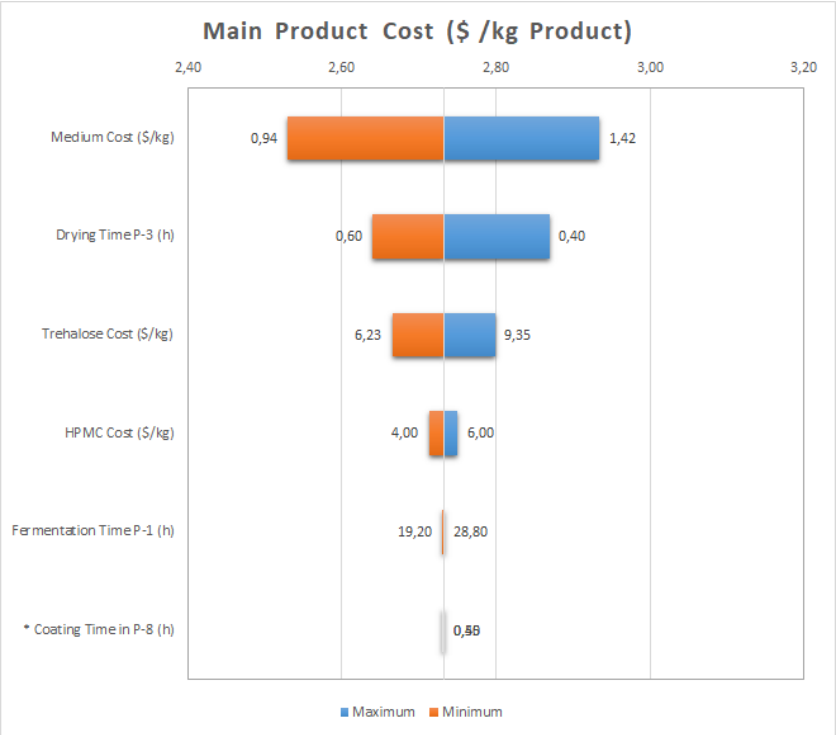


Figure 5.

Tornado plot depicting the impact of variables on unit production cost.

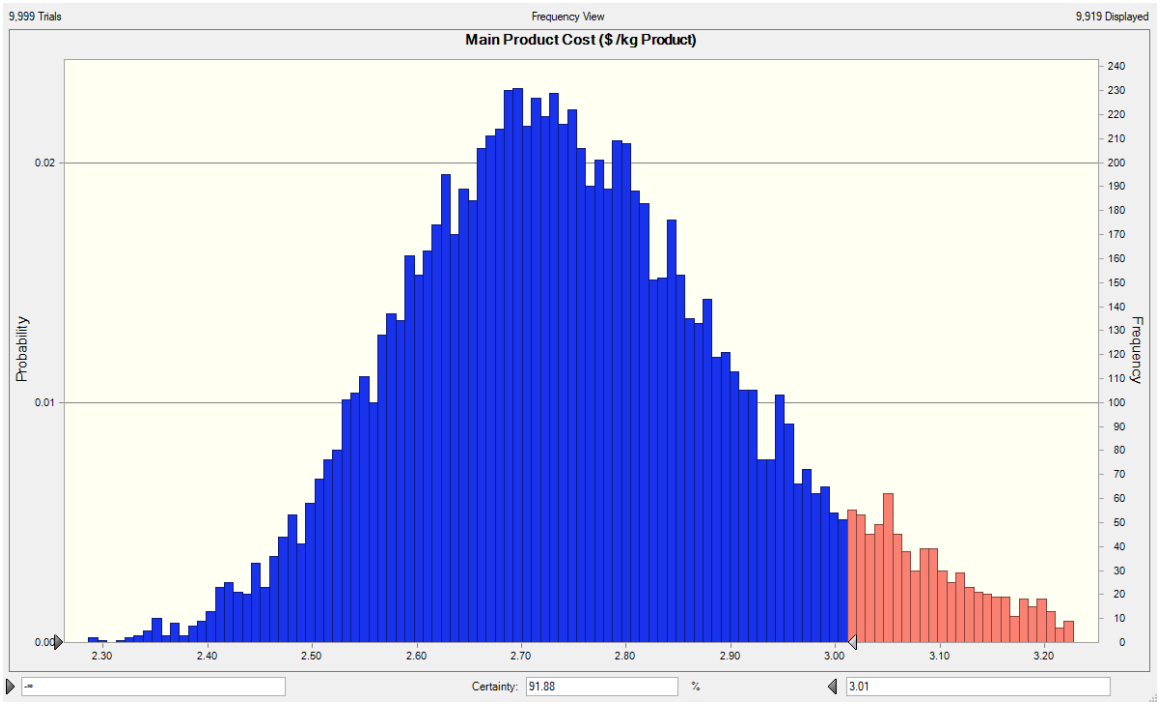


Figure 6.

Probability distribution of the unit production cost, based on 10,000 trials.

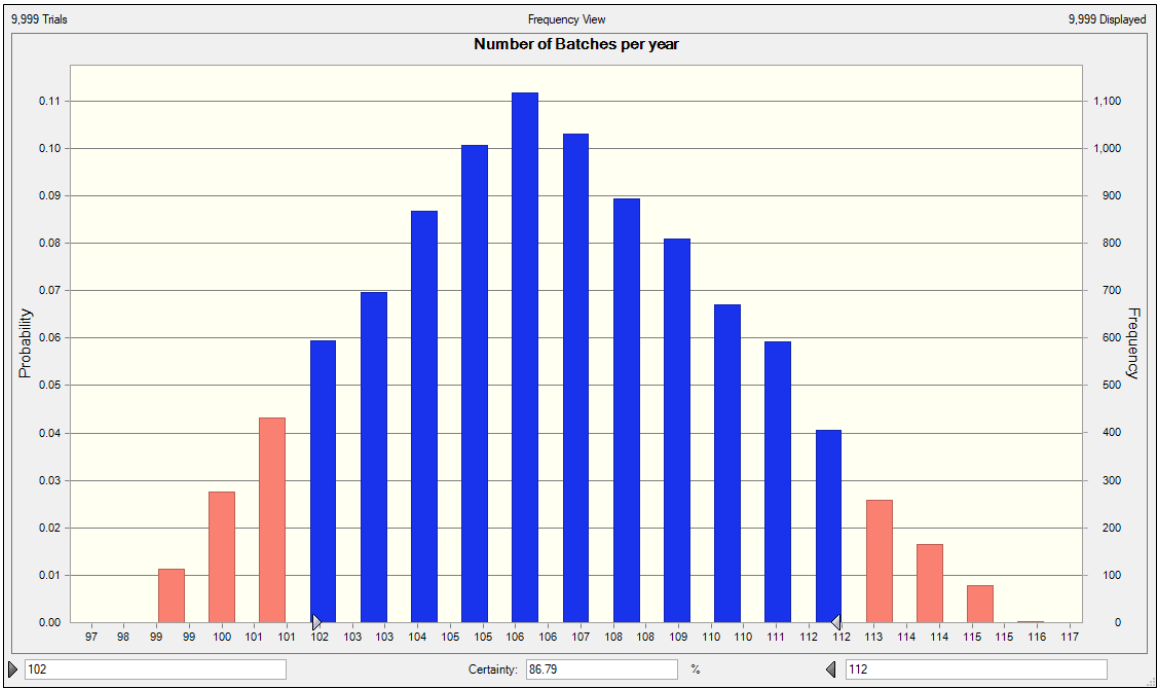


Figure 7.

Probability distribution of the annual number of batches, based on 10,000 trials.

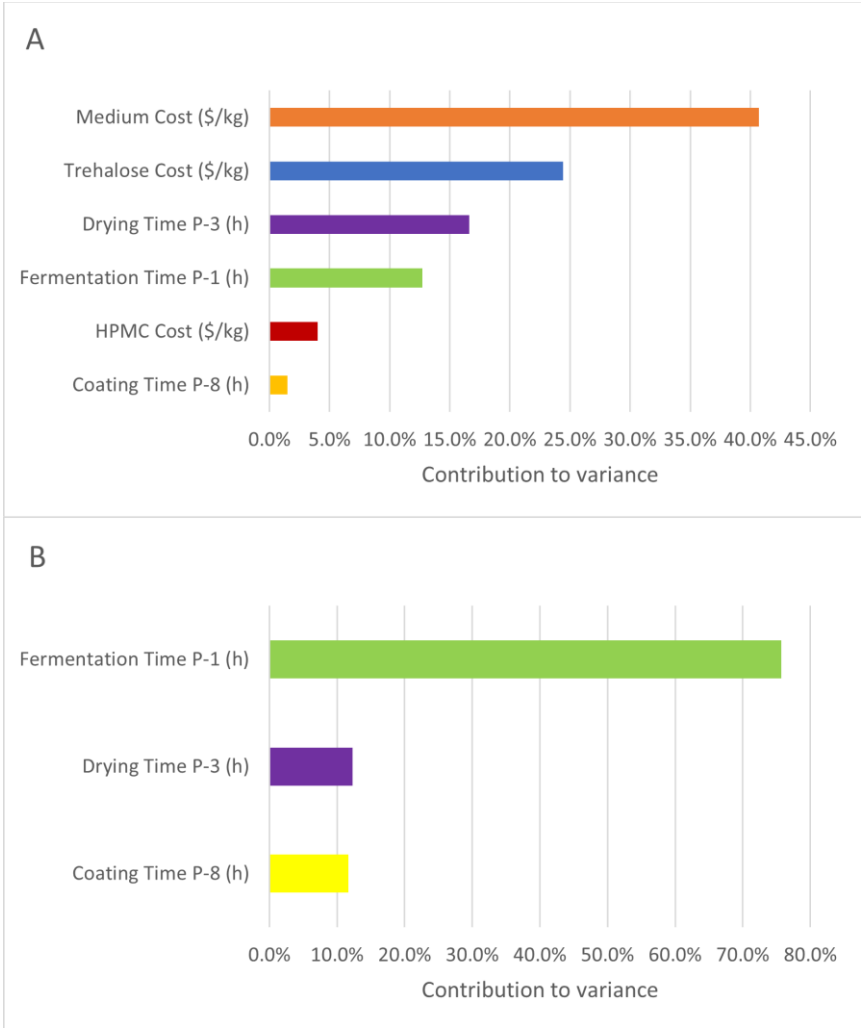


Figure 8.

Sensitivity of main product cost (A) and number of batches per year (B) to deviation in variables.

4. Conclusions

This paper explores the *in vitro* digestion and production of probiotic LAB, with a focus on the physiological and economic aspects of this process. Widely adopted enumeration methods (such as pour plate counts) should be supported by modern tools (IFC) for a better assessment of the quality of probiotics. The importance of cell subpopulations with intermediate metabolic activity (VBNC) cannot be understated, as they vastly contribute to the amount of beneficial cells in the preparation after technological processes, storage, and digestion. Economic analysis was conducted to identify the key factors that influence the cost of production. These factors include the cost of the medium, drying time, and costs of trehalose (protective substance) and HPMC (coating substance). This study contributes to the holistic understanding of probiotics as a bridge between scientific innovation and consumer well-being. By synthesizing scientific insights with practical applications and financial considerations, this study provides a multidimensional perspective on the probiotics field. A comprehensive approach that encompasses scientific, technological, and economic dimensions is essential for advancing the probiotics field and obtaining more beneficial products.

Supplementary materials:

Table S1: Mean values with standard deviation for all subpopulations after *in vitro* digestion and adhesion tests. Means and SD were calculated based on 3 repetitions.

Sample	Gated Active	Gated Dead	Gated Mid-Active I	Gated Mid-Active II
adhesion_coated	0.92 ± 0.12	41.1 ± 1.66	27.09 ± 1.02	3.15 ± 0.27
adhesion_coated_12m_storage	1.88 ± 0.32	5.77 ± 0.48	41.27 ± 1.2	34.35 ± 0.68
adhesion_coated_heat_shock	0.14 ± 0.07	13.68 ± 2.14	31.41 ± 4.8	2.44 ± 0.27
adhesion_coated_heat_shock_12m_storage	5.03 ± 0.69	21.31 ± 1.25	40.1 ± 1.13	11.25 ± 0.59
adhesion_coated_pH_shock	0.32 ± 0.11	15.51 ± 10.3	32.97 ± 7.46	3.41 ± 1.09
adhesion_coated_pH_shock_12m_storage	4.1 ± 0.66	5.46 ± 0.95	45.03 ± 2.99	26.85 ± 3.78
adhesion_culture	12.94 ± 2.13	7.23 ± 2.67	24.99 ± 2.6	20.91 ± 1.96
adhesion_culture_heat_shock	14.36 ± 0.74	34.25 ± 6.4	5.25 ± 0.67	15.99 ± 1.89
adhesion_culture_pH_shock	19.27 ± 0.48	12.58 ± 0.64	16.93 ± 1.63	11.37 ± 0.1
adhesion_dried	3 ± 0.96	18.48 ± 7.32	40.04 ± 14.23	7.98 ± 1.9
adhesion_dried_12m_storage	0.13 ± 0.07	55.72 ± 22.67	12.95 ± 8.77	0.43 ± 0.31
adhesion_dried_heat_shock	3.23 ± 0.68	30.85 ± 4.32	18.55 ± 0.77	5.53 ± 0.72
adhesion_dried_heat_shock_12m_storage	4.21 ± 0.74	23.82 ± 1.05	48.67 ± 1.02	7.05 ± 0.82
adhesion_dried_pH_shock	1.58 ± 0.6	41.84 ± 2.48	19.84 ± 2.17	5.51 ± 0.29
adhesion_dried_pH_shock_12m_storage	2.48 ± 0.59	6.49 ± 1.27	44.16 ± 3.05	29.77 ± 3.49
pre_coated	2.71 ± 0.25	55.79 ± 1.17	30.57 ± 0.36	7.22 ± 0.45
pre_coated_12m_storage	3.02 ± 0.09	57 ± 0.53	14.28 ± 0.67	17.94 ± 0.32
pre_coated_heat_shock	1.93 ± 0.41	58.91 ± 0.55	26.36 ± 0.52	7.64 ± 0.64
pre_coated_heat_shock_12m_storage	2.28 ± 0.5	36.23 ± 1.53	29.94 ± 2	21.37 ± 4.52
pre_coated_pH_shock	2.09 ± 0.3	61.62 ± 0.98	24.14 ± 0.28	7.74 ± 0.86
pre_coated_pH_shock_12m_storage	1.84 ± 0.88	34.45 ± 9.95	29.51 ± 0.29	26.01 ± 9.53
pre_culture	83.03 ± 2.55	3.01 ± 0.36	0.15 ± 0.05	3.13 ± 0.31
pre_culture_heat_shock	8.83 ± 0.78	67.05 ± 0.56	0.04 ± 0.05	14.77 ± 0.79
pre_culture_pH_shock	86.81 ± 0.15	3.42 ± 0.31	0.23 ± 0.15	2.85 ± 0.25

pre_dried	4.12 ± 0.24	70.54 ± 0.31	16.77 ± 0.24	4.35 ± 0.33
pre_dried_12m_storage	2.92 ± 0.39	67.11 ± 1.17	20.05 ± 0.24	6.31 ± 0.44
pre_dried_heat_shock	4.23 ± 0	69.07 ± 1.05	16.97 ± 0.55	4.58 ± 0.04
pre_dried_heat_shock_12m_storage	1.4 ± 0.12	19.96 ± 0.64	45.87 ± 0.56	23.2 ± 0.91
pre_dried_pH_shock	3.58 ± 0.73	69.44 ± 1.1	16.82 ± 0.73	5.39 ± 0.66
pre_dried_pH_shock_12m_storage	1.13 ± 0.21	20.8 ± 1	44.12 ± 1	23.72 ± 1.81
S1_coated	0.02 ± 0.03	26.39 ± 0.76	37.41 ± 1.7	10.45 ± 0.26
S1_coated_12m_storage	0	81.14 ± 0.58	6.36 ± 0.24	4.35 ± 0.45
S1_coated_heat_shock	0	50.71 ± 2.81	15.18 ± 0.79	4.64 ± 0.76
S1_coated_heat_shock_12m_storage	0	31.92 ± 1.03	29.48 ± 6.32	22.68 ± 5
S1_coated_pH_shock	0	50.12 ± 1.35	13.82 ± 1.08	3.59 ± 0.46
S1_coated_pH_shock_12m_storage	0	25.61 ± 3.36	25.98 ± 2.48	32.7 ± 2.2
S1_culture	2.53 ± 0.55	52.76 ± 1.5	1.76 ± 0.27	15.91 ± 0.61
S1_culture_heat_shock	0.19 ± 0.07	58.54 ± 0.29	2.45 ± 0.29	6.99 ± 0.28
S1_culture_pH_shock	5.94 ± 0.54	43.26 ± 1.52	3.25 ± 0.19	33.04 ± 0.97
S1_dried	0	17.8 ± 1.96	16.14 ± 2.18	4.82 ± 0.58
S1_dried_12m_storage	0	40.33 ± 5.26	20.01 ± 7.15	4.19 ± 0.68
S1_dried_heat_shock	0	28.75 ± 5.01	10.03 ± 2.42	4.66 ± 0.42
S1_dried_heat_shock_12m_storage	0	13.38 ± 0.4	15.03 ± 0.44	60.72 ± 1.47
S1_dried_pH_shock	0	61.19 ± 2.39	3.66 ± 0.3	4.11 ± 0.14
S1_dried_pH_shock_12m_storage	0	12.34 ± 1.96	17.59 ± 1.27	61.11 ± 2.02
S2_coated	0.01 ± 0.02	18.17 ± 0.74	41.24 ± 1.35	7.88 ± 0.71
S2_coated_12m_storage	0.01 ± 0.02	50.53 ± 4.81	17.07 ± 0.55	8.81 ± 1.2
S2_coated_heat_shock	0	17.16 ± 0.8	32.19 ± 6.44	12.53 ± 1.38
S2_coated_heat_shock_12m_storage	0	38.83 ± 1.45	24.49 ± 0.56	14.08 ± 0.87
S2_coated_pH_shock	0	22.67 ± 1.47	24.79 ± 7.51	8.42 ± 0.5
S2_coated_pH_shock_12m_storage	0	36.15 ± 1.29	24.82 ± 0.58	17.92 ± 0.36
S2_culture	0	17.02 ± 1.72	10.28 ± 1.09	4.66 ± 1.16
S2_culture_heat_shock	0	26.38 ± 4.8	5.57 ± 1.98	2.72 ± 1.48
S2_culture_pH_shock	0.01 ± 0.02	27.06 ± 1.8	11.26 ± 1.21	8.79 ± 3.21
S2_dried	0	18.65 ± 1.23	27.11 ± 2.49	1.49 ± 0.33
S2_dried_12m_storage	0	4.15 ± 0.48	14.58 ± 4.84	11.67 ± 1.19
S2_dried_heat_shock	0	10.87 ± 1.81	26.37 ± 4.4	1.93 ± 0.19
S2_dried_heat_shock_12m_storage	7.53 ± 0.51	2.28 ± 0.43	46.94 ± 3.99	1.75 ± 0.34
S2_dried_pH_shock	0	8.83 ± 1.36	39.31 ± 6.64	1.82 ± 0.12
S2_dried_pH_shock_12m_storage	0.01 ± 0.02	28.81 ± 1.3	46.94 ± 1.31	7.48 ± 0.67

Author Contributions: Conceptualization, J.K.; methodology, J.K., A.O., W.J., R.D. software, J.K.; validation, J.K.; formal analysis, J.K.; investigation, J.K.; resources, J.K., A.O., W.J.; data curation, J.K.; writing—original draft preparation, J.K.; writing—review and editing, J.K., R.D.; visualization, J.K.; supervision, R.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

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

5. References

1. Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation ; Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina, 1 - 4 October 2001 ; Report of a Joint FAO/WHO Working

Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, 30 April - 1 May 2002; Joint FAO WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, Ed.; FAO food and nutrition paper; Food and Agriculture Organization of the United Nations [u.a]: Rome, 2006; ISBN 978-92-5-105513-7.

2. Pereira, J.V.; Gamage, H.K.A.H.; Cain, A.K.; Hayes, E.; Paulsen, I.T.; Tetu, S.G. High-Throughput Viability Testing of Microbial Communities in a Probiotic Product Using Flow Cytometry. Applied Microbiology **2023**, 3, 1068–1082, doi:10.3390/applmicrobiol3030074.
3. George Kerry, R.; Patra, J.K.; Gouda, S.; Park, Y.; Shin, H.-S.; Das, G. Benefaction of Probiotics for Human Health: A Review. Journal of Food and Drug Analysis **2018**, 26, 927–939, doi:10.1016/j.jfda.2018.01.002.
4. Broeckx, G.; Vandenheuvel, D.; Claes, I.J.J.; Lebeer, S.; Kiekens, F. Drying Techniques of Probiotic Bacteria as an Important Step towards the Development of Novel Pharmabiotics. International Journal of Pharmaceutics **2016**, 505, 303–318, doi:10.1016/j.ijpharm.2016.04.002.
5. Fu, N.; Chen, X.D. Towards a Maximal Cell Survival in Convective Thermal Drying Processes. Food Research International **2011**, 44, 1127–1149, doi:10.1016/j.foodres.2011.03.053.
6. Vass, P.; Domokos, A.; Pantea, E.; Szilágyi, B.; Molnár, M.; Záhonyi, P.; Nagy, B.; Nagy, Z.K. Processing of Thermosensitive Biological API from Suspension Using an Integrated Continuous Granulation – Drying – Milling Line into Powder Ready for Tableting. Drying Technology **2023**, 41, 492–502, doi:10.1080/07373937.2022.2101474.
7. Assari, M.R.; Basirat Tabrizi, H.; Najafpour, E. Energy and Exergy Analysis of Fluidized Bed Dryer Based on Two-Fluid Modeling. International Journal of Thermal Sciences **2013**, 64, 213–219, doi:10.1016/j.ijthermalsci.2012.08.020.
8. Mohideen Batcha, M.F.; Amirnordin, S.H.; Md Yudin, A.S. 4 - Fluidized Bed Dryers. In Drying Technology in Food Processing; Jafari, S.M., Malekjani, N., Eds.; Woodhead Publishing, 2023; pp. 67–122 ISBN 978-0-12-819895-7.
9. Hathi, Z.; Mettu, S.; Priya, A.; Athukoralalage, S.; Lam, T.N.; Choudhury, N.R.; Dutta, N.K.; El-Omar, E.M.; Gong, L.; Mohan, G.; et al. Methodological Advances and Challenges in Probiotic Bacteria Production: Ongoing Strategies and Future Perspectives. Biochemical Engineering Journal **2021**, 176, 108199, doi:10.1016/j.bej.2021.108199.
10. Han, S.; Lu, Y.; Xie, J.; Fei, Y.; Zheng, G.; Wang, Z.; Liu, J.; Lv, L.; Ling, Z.; Berglund, B.; et al. Probiotic Gastrointestinal Transit and Colonization After Oral Administration: A Long Journey. Front. Cell. Infect. Microbiol. **2021**, 11, 609722, doi:10.3389/fcimb.2021.609722.
11. Qi, Y.; Huang, L.; Zeng, Y.; Li, W.; Zhou, D.; Xie, J.; Xie, J.; Tu, Q.; Deng, D.; Yin, J. Pediococcus Pentosaceus: Screening and Application as Probiotics in Food Processing. Frontiers in Microbiology **2021**, 12.
12. Song, A.-X.; Mao, Y.-H.; Siu, K.-C.; Tai, W.C.S.; Wu, J.-Y. Protective Effects of Exopolysaccharide of a Medicinal Fungus on Probiotic Bacteria during Cold Storage and Simulated Gastrointestinal Conditions. International Journal of Biological Macromolecules **2019**, 133, 957–963, doi:10.1016/j.ijbiomac.2019.04.108.
13. Wang, X.; Cao, Z.; Zhang, M.; Meng, L.; Ming, Z.; Liu, J. Bioinspired Oral Delivery of Gut Microbiota by Self-Coating with Biofilms. Science Advances **2020**, 6, eabb1952, doi:10.1126/sciadv.abb1952.
14. Lu, Y.; Han, S.; Zhang, S.; Wang, K.; Lv, L.; McClements, D.J.; Xiao, H.; Berglund, B.; Yao, M.; Li, L. The Role of Probiotic Exopolysaccharides in Adhesion to Mucin in Different Gastrointestinal Conditions. Current Research in Food Science **2022**, 5, 581–589, doi:10.1016/j.crfs.2022.02.015.
15. Chai, L.-N.; Wu, H.; Wang, X.-J.; He, L.-J.; Guo, C.-F. The Mechanism of Antimicrobial Activity of Conjugated Bile Acids against Lactic Acid Bacilli. Microorganisms **2023**, 11, 1823, doi:10.3390/microorganisms11071823.
16. Mukherjee, S.; Hooper, L.V. Antimicrobial Defense of the Intestine. Immunity **2015**, 42, 28–39, doi:10.1016/j.immuni.2014.12.028.

17. Long, S.L.; Gahan, C.G.M.; Joyce, S.A. Interactions between Gut Bacteria and Bile in Health and Disease. Molecular Aspects of Medicine **2017**, 56, 54–65, doi:10.1016/j.mam.2017.06.002.
18. Zielińska, D.; Rzepkowska, A.; Radawska, A.; Zieliński, K. In Vitro Screening of Selected Probiotic Properties of Lactobacillus Strains Isolated from Traditional Fermented Cabbage and Cucumber. Curr Microbiol **2015**, 70, 183–194, doi:10.1007/s00284-014-0699-0.
19. Safeer Abbas, M.; Afzaal, M.; Saeed, F.; Asghar, A.; Jianfeng, L.; Ahmad, A.; Ullah, Q.; Elahi, S.; Ateeq, H.; Shah, Y.A.; et al. Probiotic Viability as Affected by Encapsulation Materials: Recent Updates and Perspectives. International Journal of Food Properties **2023**, 26, 1324–1350, doi:10.1080/10942912.2023.2213408.
20. Yang, X.; Wang, C.; Wang, Q.; Zhang, Z.; Nie, W.; Shang, L. Armored Probiotics for Oral Delivery. Smart Medicine **2023**, doi:10.1002/SMMD.20230019.
21. Buss, M.T.; Ramesh, P.; English, M.A.; Lee-Gosselin, A.; Shapiro, M.G. Spatial Control of Probiotic Bacteria in the Gastrointestinal Tract Assisted by Magnetic Particles. Advanced Materials **2021**, 33, 2007473, doi:10.1002/adma.202007473.
22. Son, Y.S.; Kwon, M.; Son, N.; Kim, S.-K.; Son, M.-Y. Multilayer Coating with Red Ginseng Dietary Fiber Improves Intestinal Adhesion and Proliferation of Probiotics in Human Intestinal Epithelial Models. J Microbiol Biotechnol **2023**, 33, 1–10, doi:10.4014/jmb.2305.05013.
23. Rajam, R.; Subramanian, P. Encapsulation of Probiotics: Past, Present and Future. Beni-Suef Univ J Basic Appl Sci **2022**, 11, 46, doi:10.1186/s43088-022-00228-w.
24. Kiepś, J.; Juzwa, W.; Dembczyński, R. Imaging Flow Cytometry Demonstrates Physiological and Morphological Diversity within Treated Probiotic Bacteria Groups. International Journal of Molecular Sciences **2023**, 24, 6841, doi:10.3390/ijms24076841.
25. Anandharaj, M.; Rani, R.P.; Swain, M.R. Production of High-Quality Probiotics by Fermentation. In Microbial Functional Foods and Nutraceuticals; Gupta, V.K., Treichel, H., Shapaval, V. (Olga), Antonio De Oliveira, L., Tuohy, M.G., Eds.; Wiley, 2017; pp. 235–266 ISBN 978-1-119-04901-2.
26. Fiore, W.; Arioli, S.; Guglielmetti, S. The Neglected Microbial Components of Commercial Probiotic Formulations. Microorganisms **2020**, 8, 1177, doi:10.3390/microorganisms8081177.
27. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A Standardised Static in Vitro Digestion Method Suitable for Food – an International Consensus. Food Funct. **2014**, 5, 1113–1124, doi:10.1039/C3FO60702J.
28. Kiepś, J.; Juzwa, W.; Olejnik, A.; Sip, A.; Tomaszewska-Gras, J.; Dembczyński, R. The Effects of Cellular Membrane Damage on the Long-Term Storage and Adhesion of Probiotic Bacteria in Caco-2 Cell Line. Nutrients **2023**, 15, 3484, doi:10.3390/nu15153484.
29. Bucka-Kolendo, J.; Sokołowska, B. Lactic Acid Bacteria Stress Response to Preservation Processes in the Beverage and Juice Industry. Acta Biochim Pol **2017**, 64, 459–464, doi:10.18388/abp.2017_1496.
30. Rodrigues, V.C. da C.; Silva, L.G.S. da; Simabuco, F.M.; Venema, K.; Antunes, A.E.C. Survival, Metabolic Status and Cellular Morphology of Probiotics in Dairy Products and Dietary Supplement after Simulated Digestion. Journal of Functional Foods **2019**, 55, 126–134, doi:10.1016/j.jff.2019.01.046.
31. Wang, A.; Lin, J.; Zhong, Q. Enteric Rice Protein-Shellac Composite Coating to Enhance the Viability of Probiotic Lactobacillus Salivarius NRRL B-30514. Food Hydrocolloids **2021**, 113, 106469, doi:10.1016/j.foodhyd.2020.106469.
32. Huang, S.; Vignolles, M.-L.; Chen, X.D.; Le Loir, Y.; Jan, G.; Schuck, P.; Jeantet, R. Spray Drying of Probiotics and Other Food-Grade Bacteria: A Review. Trends in Food Science & Technology **2017**, 63, 1–17, doi:10.1016/j.tifs.2017.02.007.

33. Fu, N.; Huang, S.; Xiao, J.; Chen, X.D. Producing Powders Containing Active Dry Probiotics With the Aid of Spray Drying. Adv Food Nutr Res **2018**, 85, 211–262, doi:10.1016/bs.afnr.2018.02.003.
34. Haffner, F.B.; Diab, R.; Pasc, A.; Haffner, F.B.; Diab, R.; Pasc, A. Encapsulation of Probiotics: Insights into Academic and Industrial Approaches. AIMSMATES **2016**, 3, 114–136, doi:10.3934/matersci.2016.1.114.
35. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. The International Scientific Association for Probiotics and Prebiotics Consensus Statement on the Scope and Appropriate Use of the Term Probiotic. Nat Rev Gastroenterol Hepatol **2014**, 11, 506–514, doi:10.1038/nrgastro.2014.66.
36. Ayrapetyan, M.; Williams, T.; Oliver, J.D. Relationship between the Viable but Nonculturable State and Antibiotic Persister Cells. J Bacteriol **2018**, 200, doi:10.1128/JB.00249-18.