Szkoła Główna Gospodarstwa Wiejskiego w Warszawie

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Analiza związków bioaktywnych w wybranych żywnościowych produktach ubocznych i ich potencjału do ponownego wykorzystania

Analysis of the bioactive compounds in selected food by-products and their re-utilisation potential

Rozprawa doktorska

Doctoral thesis

Rozprawa doktorska wykonana pod kierunkiem

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Spis treści

Streszczenie

Szacuje się, że około jednej trzeciej produkowanej żywności jest marnowana globalnie. Znaczna część tych strat wynika z wytwarzania produktów ubocznych w przemyśle spożywczym, które często nie są odpowiednio zagospodarowane mimo zawartości cennych składników bioaktywnych. Takie produkty uboczne wykorzystuje się obecnie głównie jako paszę dla zwierząt lub utylizuje, co oznacza niewykorzystany potencjał tych surowców. W obliczu rosnącego problemu przeludnienia i zmian klimatycznych istnieje istotna potrzeba wtórnego zagospodarowania surowców ubocznych w technologii żywności, biotechnologii i żywieniu człowieka. Celem pracy doktorskiej była szczegółowa analiza składu chemicznego, właściwości prozdrowotnych i funkcjonalnych oraz ocena możliwości wtórnego wykorzystania czterech produktów ubocznych: wytłoków jabłkowych, okryw nasiennych orzechów laskowych, młóta browarnianego i browarniczej gęstwy drożdżowej.

Analizy wykazały, że wytłoki jabłkowe, zastosowane w modelowych przekąskach roślinnych, sprzyjają wzrostowi bakterii probiotycznych i nadają produktom potencjalne właściwości prebiotyczne. Okrywy nasienne orzechów laskowych zawierają wysokie stężenia polifenoli o silnej aktywności przeciwutleniającej i działaniu bakteriostatycznym wobec patogenów, przy jednoczesnym braku efektu hamującego wzrost względem bakterii probiotycznych. Składniki młóta browarnianego wykazały zdolność do wspierania wzrostu bakterii probiotycznych oraz wpływały na korzystne zmiany składu bakteryjnej mikrobioty jelitowej w modelach *in vitro*. Odpadowa gęstwa drożdżowa była efektywnym podłożem wzrostowym dla *Propionibacterium freudenreichii*, umożliwiającym biosyntezę witaminy B_{12}, krótkołańcuchowych kwasów tłuszczowych i niezbędnych aminokwasów egzogennych.

Uzyskane wyniki potwierdzają możliwość ponownego zagospodarowania badanych produktów ubocznych. Ponowne włączenie ich do łańcucha żywnościowego lub wykorzystanie w biotechnologii pozwoli ograniczyć marnotrawstwo cennych surowców. Takie działania wpisują się w nurt gospodarki w obiegu zamkniętym oraz sprzyjają tworzeniu żywności o podwyższonej wartości zdrowotnej.

Słowa kluczowe: produktu uboczne, przemysł spożywczy, recykling, prebiotyk, związki biologicznie czynne, wzbogacanie

Abstract

Approximately one-third of all food produced globally is wasted. A significant share of these losses arises from the generation of by-products in the food industry, which are often underutilized despite being rich in valuable bioactive compounds. These materials leave much of their potential untapped because they are typically used as animal feed or wasted. In the face of increasing population growth and climate change, there is an urgent need to revalorize food industry by-products through their application in food technology, biotechnology, and nutrition.

This thesis aimed to conduct an in-depth analysis of the chemical composition, functional properties and health-promoting as well as to evaluate the potential for re-utilizing four selected by-products: apple pomace, hazelnut skin seeds, brewer's spent grains and spent brewery yeast.

The results revealed that when incorporated into model plant-based snacks, apple pomace promoted the growth of probiotic bacteria and imparted potential prebiotic properties to the final products. Hazelnut skin seeds contained high levels of polyphenols with antioxidant and bacteriostatic activity against pathogens while having no inhibitory effects on probiotic strains. Brewer's spent grain supported the growth of probiotic bacteria and induced beneficial modulations in the gut microbiota composition in the in vitro models. Spent brewery yeast served as an effective growth substrate for Propionibacterium freudenreichii, enabling the biosynthesis of vitamin B_{12}, short-chain fatty acids, and essential amino acids.

The findings confirm the relevance of reintroducing these by-products into the food chain or employing them in biotechnological applications. Such an approach can reduce the waste of valuable raw materials and support the principles of the circular economy while contributing to the development of functional foods with enhanced health benefits.

Keywords: by-products, food industry, recycling, prebiotic, bioactive compound, valorisation

Spis publikacji wchodzących w skład rozprawy doktorskiej

P1. *Kruk, M., Lalowski, P., Hoffmann, M., Trząskowska, M., & Jaworska, D. (2024). Probiotic Bacteria Survival and Shelf Life of High Fibre Plant Snack—Model Study. Plant Foods for Human Nutrition, 79(3), 586–593.*

Impact factor: 3,1; Punkty ministerialne: 100

P2. *Kruk, M., Ponder, A., Horoszewicz, J., Popławski, D., Król, K., Leszczyńska, J., Jaworska, D., & Trząskowska, M. (2024). By-product hazelnut seed skin characteristics and properties in terms of use in food processing and human nutrition. Scientific Reports, 14(1), 18835.*

Impact factor: 3,8; Punkty ministerialne: 140

P3. *Kruk, M., Lalowski, P., Płecha, M., Ponder, A., Rudzka, A., Zielińska, D., & Trząskowska, M. (2025). Prebiotic potential of spent brewery grain – In vitro study. Food Chemistry, 463, 141254.*

Impact factor: 8,5; Punkty ministerialne: 200

P4. *Kruk, M., Varmanen, P., Edelmann, M., Chamlagain, B., & Trząskowska, M. (2024). Food by-product valorisation in nutrients through spent brewer's yeast bioprocessing with Propionibacterium freudenreichii. Journal of Cleaner Production, 434, 140102.*

Impact factor: 11,1; Punkty ministerialne: 140

Sumaryczny Impact factor: 26,5

Suma punktów ministerialnych: 580

Indeks skrótów

ABTS – kwas 2,2'-azino-bis(3-etylobenzotiazolino-6-sulfonowy)

ANOVA – analiza wariancji (analysis of variance)

a_w – aktywność wody

AX - arabinoksylan

AXOS –oligosacharydy arabinoksylanowe

BCFA – rozgałęzione krótkołańcuchowe kwasy tłuszczowe (branched-chain fatty acids)

DPPH – 2,2-difenylo-1-pikrylohydrazyl

ELISA – test immunoenzymatyczny (enzyme-linked immunosorbent assay)

GAE – ekwiwalent kwasu galusowego (gallic acid equivalent)

GC – chromatografia gazowa (gas chromatography)

his – histydyna

ser – seryna

arg – arginina

gly – glicyna

kw. – kwas

kw. asp – kwas asparaginowy

kw. glu – kwas glutaminowy

thr – treonina

ala – alanine

pro – prolina

cys – cysteine

lys – lizyna

tyr – tyrozyna

met – metionina

val – walina

ile – izoleucyna

leu – leucyna

phe – fenyloalanina

h-d pro – d-prolina

asn – asparagina

gln – glutamine

orn – ornityna

trp – tryptofan

HPLC – wysokosprawna chromatografia cieczowa (high performance liquid chromatography)

JTK – jednostki tworzące kolonie

MIC – minimalne stężenie hamujące (minimum inhibitory concentration)

OD – gęstość optyczna (optical density)

PCA – analiza głównych składowych (principal component analysis)

PCoA – analiza głównych współrzędnych (principal coordinates analysis)

SCFA – krótkołańcuchowe kwasy tłuszczowe (short-chain fatty acids)

SHIME – Simulator of the Human Intestinal Microbial Ecosystem

VCEAC – ekwiwalent pojemności przeciwutleniającej witaminy C (vitamin C equivalent antioxidant capacity

1. Przegląd literatury

Systemy żywnościowe stanęły w obliczu bezprecedensowych wyzwań w ciągu ostatnich kilku lat z powodu zaistnienia i trwania kilku zjawisk takich jak zmiany klimatyczne, wzrost wielkości populacji ludzkiej, zwiększonej urbanizacji, wybuchu pandemii COVID-19, a także konfliktów w wielu częściach świata. Globalne bezpieczeństwo żywnościowe stało się pilną kwestią i przyciągnęło uwagę społeczności naukowej (Tamasiga i in., 2023; Aït-Kaddour i in., 2024). Jednym ze znaczących dylematów, przed którymi stoi wdrażanie strategii bezpieczeństwa żywnościowego i zasad zrównoważonego rozwoju żywności, jest jej marnotrawstwo (Socas-Rodríguez i in., 2021). Znaczna część przetwarzanych surowców spożywczych nie trafia do konsumpcji, a straty te pociągają za sobą konsekwencje ekonomiczne i ekologiczne. W Unii Europejskiej całkowita ilość marnowanej żywności to około 60 milionów ton rocznie, z czego jej większość jest generowana w gospodarstwach domowych (EUROSTAT, 2021). Podczas produkcji pierwotnej oraz przetwarzania surowców spożywczych ilość otrzymywanych odpadów i produktów ubocznych sięga 15 milionów ton (EUROSTAT, 2021). Spośród tych strat żywotności duża cześć może zostać ponownie wykorzystana we wtórnych procesach przetwórczych jako żywność lub surowiec pośredni do produkcji związków bioaktywnych (Ryc. 1) (Rakesh i Mahendran, 2024).

Rycina 1. Schemat powstawania oraz składu produktów ubocznych przemysłu spożywczego

Istotną kategorię stanowią produkty uboczne procesów przetwórczych, które są bogate w składniki odżywcze i związki bioaktywne (Baglary i in., 2024). Mogą to być produkty uboczne pochodzenia roślinnego lub zwierzęcego (Trigo i in., 2020; Cao i in., 2022). Również produkty uboczne procesów fermentacyjnych dotyczących żywności, takie jak biomasa mikrobiologiczna uzyskiwana na wysoką skalę w przemyśle alkoholowym jest istotnym źródłem związków bioaktywnych i składników odżywczych

(Olivares-Galván i in., 2022). W dobie wyzwań ekologicznych oraz ekonomicznych spowodowanych stratami w produkcji żywności rośnie potrzeba zagospodarowania takich produktów ubocznych poprzez ich ponowne wykorzystanie w łańcuchu przetwórczym w biotechnologii lub przemyśle spożywczym. Wiele z nich ma obiecujące właściwości odżywcze i funkcjonalne, a odpowiednia obróbka może wyeliminować potencjalne zagrożenia dla zdrowia, umożliwiając bezpieczne zastosowanie ich jako składników żywności (Olivares-Galván i in., 2022). W odniesieniu do niniejszej pracy doktorskiej szczególną uwagę poświęcono wytłokom jabłkowym, okrywom nasiennym orzechów laskowych, młótu browarnianemu i odpadowej gęstwie drożdżowej z browarnictwa.

Odpady i produkty uboczne pochodzenia roślinnego, takie jak skórki, liście, nasiona, otręby, łuski zbóż, pestki oraz wytłoki, powstają zarówno na etapie produkcji pierwotnej, jak i podczas przetwarzania surowców roślinnych w przemyśle spożywczym. Skala ich generacji zależy od rodzaju surowca i technologii przetwórczej, jednak w wielu przypadkach odpady te stanowią znaczny odsetek masy całkowitej produktu roślinnego (Aït-Kaddour i in., 2024). Jedną z najpowszechniejszych grup takich produktów ubocznych są wytłoki owocowe, powstające w dużych ilościach w przemyśle sokowniczym. Szczególnym przykładem są wytłoki jabłkowe, które stanowią główny produkt uboczny w produkcji soków (Antonic i in., 2020). Szacuje się, że 20 - 30% masy przerabianych jabłek pozostaje w postaci wytłoków, co przy globalnej rocznej produkcji jabłek wynoszącej około 84,7 miliona ton, oznacza znaczącą produkcję materiału odpadowego o wysokim potencjale do zagospodarowania (Antonic i in., 2020). Tradycyjnie wytłoki są wykorzystywane jako nawóz organiczny, pasza dla zwierząt lub substrat do fermentacyjnej produkcji etanolu (Lyu i in., 2020). Wytłoki jabłkowe zawierają liczne związki biologicznie czynne, takie jak błonnik pokarmowy, w tym istotne ilości pektyn, polifenoli, tokoferoli, kwasu jabłkowego oraz cukrów prostych (Antonic i in., 2020). Ze względu na obecność tych składników, wytłoki jabłkowe postrzegane są obecnie jako wartościowy surowiec wtórny o potencjale aplikacyjnym w produkcji żywności funkcjonalnej, suplementów diety oraz jako źródło substancji bioaktywnych (Asif i in., 2024). Ze względu na wysoką zawartość wody i aktywność enzymatyczną, wytłoki jabłkowe są bardzo podatne na niekorzystne zmiany jakościowe, takie jak rozwój mikroorganizmów, utlenianie polifenoli, depolimeryzacja i deestryfikacja pektyn (Antonic i in., 2020; Bonnin i Pelloux, 2020). Procesy te nie tylko

ograniczają przydatność technologiczną tego surowca, ale także zmniejszają zawartość i biodostępność cennych składników. W związku z tym, dla zachowania jakości i wartości biologicznej wytłoków niezbędne jest ich szybkie przetwarzanie po oddzieleniu od soku (Sobczak i in., 2022). Pomimo ograniczeń technologicznych, wytłoki jabłkowe charakteryzują się wysoką zawartością związków bioaktywnych, co czyni je obiecującym surowcem do ekstrakcji substancji czynnych, składnikiem żywności o potencjalnym działaniu prebiotycznym oraz substratem do fermentacyjnej syntezy metabolitów bioaktywnych (Calvete-Torre i in., 2022; Kalinowska i in., 2023).

Okrywy nasienne orzechów laskowych powstają jako produkt uboczny podczas blanszowania lub prażenia orzechów i stanowią jedynie około 1% masy całego orzecha (Ceylan i in., 2023). Ze względu na skalę światowej produkcji orzechów laskowych, ilość generowanych w ten sposób odpadów jest znacząca (Food and Agriculture Organization of the United Nations, 2023). Dotychczas okrywy te nie znalazły szerokiego zastosowania wtórnego, mimo że charakteryzują się szeregiem właściwości technologicznych i prozdrowotnych, które predysponują je do ponownego wykorzystania w przemyśle spożywczym i biotechnologicznym. Jedną z kluczowych zalet okryw nasiennych jest niewielka zawartość wody, co przekłada się na dobrą trwałość, łatwość magazynowania oraz ograniczone ryzyko rozwoju niepożądanej mikroflory (Ceylan i in., 2023). Co istotne, to właśnie w okrywie znajduje się najwyższe stężenie związków bioaktywnych obecnych w całym orzechu, a zwłaszcza kwasu galusowego, katechiny i elagotaniny (Taş i Gökmen, 2015; Horoszewicz i in., 2022). Polifenole te nie tylko wykazują silne właściwości przeciwutleniające, ale również działanie bakteriostatyczne, co może zostać wykorzystane w celu ochrony żywności przed niekorzystnymi przemianami oksydacyjnymi oraz przed rozwojem mikroorganizmów chorobotwórczych lub saprofitycznych (Kępa i in., 2018). W ostatnich latach obserwuje się rosnące zainteresowanie aplikacyjnym wykorzystaniem okryw nasiennych orzechów laskowych jako dodatków funkcjonalnych do żywności. Podejmowane są próby ich zastosowania w produkcji jogurtów, słodkich przekąsek, makaronów oraz wyrobów mięsnych, w celu poprawy wartości odżywczej i stabilności składu chemicznego w czasie przechowywania (Longato i in., 2019; Dinkçi i in., 2021; Horoszewicz i in., 2022). Równolegle prowadzone są także badania nad wykorzystaniem tego surowca w żywieniu zwierząt, gdzie okrywy mogą wpływać korzystnie na jakość mięsa (Ceylan, 2024; Musati i in., 2024). Z punktu widzenia bezpieczeństwa żywności kluczowe znaczenie ma fakt, że

orzechy laskowe należą do najsilniejszych alergenów pokarmowych. Z tego względu konieczna jest ocena zawartości i stabilności alergenów w okrywach nasiennych oraz wpływu procesów technologicznych na ich alergenność (López i in., 2012; Camus-Ela i in., 2025). Doniesienia naukowe sugerują, że odpowiednia obróbka może znacząco obniżyć zawartość białek o działaniu alergennym, jednak ten aspekt wymaga dalszych badań. Warto również zaznaczyć, że profil sensoryczny okryw nasiennych oraz związki chemiczne odpowiedzialne za ich smaki aromat nie zostały dotąd szczegółowo scharakteryzowane (Ceylan i in., 2023). Zróżnicowany skład chemiczny tego materiału wskazuje na jego szerokie właściwości funkcjonalne i potencjał aplikacyjny, zarówno w przemyśle spożywczym, jak i poza nim.

Młóto browarniane jest najpowszechniejszym produktem ubocznym powstającym podczas produkcji piwa. Szacuje się, że na każde 100 litrów tego napoju generowane jest średnio około 20 kg młóta (Lynch i in., 2016; Nyhan i in., 2023). Według danych Eurostat, w 2022 roku w samej Unii Europejskiej wyprodukowano 34,3 miliarda litrów piwa, co odpowiada w przybliżeniu 6,8 milionom ton młóta browarnianego rocznie (EUROSTAT, 2023). Pomimo że jego najczęstszym zastosowaniem pozostaje wykorzystanie jako pasza dla zwierząt, skala produkcji sprawia, że znaczna część młóta nie znajduje wtórnego zastosowania i ulega marnowaniu (Nyhan i in., 2023). Świeże młóto browarniane zawiera wysoki udział wody oraz łatwo fermentujących składników, takich jak cukry i białka, co sprzyja szybkiemu rozwojowi mikroflory saprofitycznej i potencjalnie patogennej (Jackowski i in., 2020). Z tego względu surowiec ten wymaga szybkiego przetworzenia. Należy podkreślić, że jakość mikrobiologiczna młóta bezpośrednio po jego generacji jest zadowalająca dzięki działaniu wysokiej temperatury w procesach zacierania i filtracji brzeczki (Lao i in., 2020; Zeko-Pivač i in., 2022). Odpowiednie i szybkie działania przetwórcze mogą zatem zapewnić jego trwałość oraz bezpieczeństwo. Pod względem składu chemicznego młóto browarniane zawiera znaczną ilość błonnika pokarmowego (40 - 65% suchej masy), w tym przede wszystkim frakcje hemiceluloz, celulozy i ligniny. Szczególnie istotna jest hemiceluloza, która może stanowić nawet 40% suchej masy i składa się głównie z AX. Są to polimery zbudowane z cząsteczek D-ksylozy połączonych wiązaniami β-(1-4) glikozydowymi, podstawianych resztami L-arabinozy (Ikram i in., 2017). Oprócz błonnika, młóto zawiera również około 20% białka oraz niewielki udział tłuszczu (5–8%) (Lynch i in., 2016). Dodatkowo, surowiec ten jest źródłem związków fenolowych (Stefanello i in., 2018). Z uwagi na swój

skład, młóto browarniane powoduje coraz większe zainteresowanie jako składnik żywności funkcjonalnej, w szczególności jako czynnik prebiotyczny (Lao i in., 2020). Obecność frakcji błonnika predysponuje je do wpływania na skład mikrobioty jelitowej poprzez stymulację rozwoju korzystnych mikroorganizmów i produkcję SCFA. Co więcej, fermentacyjna obróbka młóta z udziałem wybranych szczepów mikroorganizmów może przyczynić się do zwiększenia jego wartości odżywczej poprzez konwersję związków fenolowych do form o większej aktywności biologicznej, poprawę strawności i struktur błonnika pokarmowego oraz wydłużenie trwałości mikrobiologicznej (Terefe, 2022; Yitayew i in., 2022). Cechy surowcowe młóta browarnianego jednoznacznie klasyfikują je jako surowiec o wysokim potencjale przetwórczym oraz funkcjonalnym, który może zostać wykorzystany w dziedzinach biotechnologii oraz żywieniu człowieka i technologii żywności.

Odpadowa gęstwa drożdżowa powstaje jako produkt uboczny przemysłu piwowarskiego i stanowi biomasę komórek drożdżowych i stałych produktów fermentacji osadzonych na dnie zbiorników fermentacyjnych po zakończeniu produkcji piwa. Szacuje się, że jej ilość wynosi od 2 do 4 kg na każde 100 litrów wyprodukowanego piwa (Puligundla i in., 2020). Choć drożdże piwowarskie są zazwyczaj wielokrotnie wykorzystywane w kolejnych cyklach fermentacyjnych, to po przekroczeniu pewnej liczby fermentacji ich aktywność i jakość końcowa piwa może ulec pogorszeniu. Efekt ten występuje w wyniku kumulacji martwych komórek i metabolitów wtórnych z procesów autolitycznych drożdży (Marson i in., 2020; Schlabitz i in., 2022). Powstająca w ten sposób biomasa, często jest traktowana jako odpad, a w rzeczywistości stanowi surowiec o wysokiej wartości odżywczej i użytkowej. Gęstwa drożdżowa zawiera znaczną ilość białka (40–50% w suchej masie), w tym szereg aminokwasów egzogennych, a także liczne witaminy z grupy B (z wyjątkiem witaminy B_{12}), składniki mineralne (fosfor, potas, magnez, cynk, selen, kobalt) oraz substancje o udowodnionym działaniu prebiotycznym obecne w ścianie komórkowej drożdży, głównie β-glukany i mannanooligosacharydy (Jaeger i in., 2020; Puligundla i in., 2020). Istotnym aspektem ograniczającym wykorzystanie gęstwy drożdżowej w żywieniu ludzi jest stosunkowo wysoka zawartość kwasów nukleinowych w jej składzie zwłaszcza RNA (Marson i in., 2020). Po spożyciu, RNA ulega rozkładowi do zasad purynowych, które metabolizowane są do kwasu moczowego. Nadmierne spożycie produktów bogatych w puryny może prowadzić do hiperurykemii, a w skrajnych przypadkach do rozwoju dny moczanowej

lub tworzenia kamieni nerkowych (Coelho i in., 2022). Z tego względu zastosowanie gęstwy drożdżowej w żywieniu człowieka wymaga wcześniejszego obniżenia zawartości RNA. W tym celu stosuje się różne strategie technologiczne, takie jak autoliza enzymatyczna, ultrafiltracja i obróbka cieplna (Jaeger i in., 2020; Marson i in., 2022). Dodatkowo ze względu na wysoką zawartość wody i aktywność biologiczną, gęstwa drożdżowa jest surowcem nietrwałym i wymaga szybkiego przetwarzania (Marson i in., 2020). Tradycyjnie wykorzystywana jest jako dodatek paszowy, surowiec do produkcji ekstraktu drożdżowego, wzmacniaczy smaku lub jako składnik w piekarnictwie wpływający na właściwości technologiczne ciasta i na wartość odżywczą gotowych wyrobów (Jaeger i in., 2020; Schlabitz i in., 2022). Obecnie zauważa się coraz większy potencjał gęstwy drożdżowej w przemyśle spożywczym i biotechnologicznym jako alternatywnego źródła białka, witamin z grupy B i nośnika mikroelementów oraz jako substratu do syntezy związków bioaktywnych. W rezultacie dzięki coraz bardziej intensywnym badaniom nad tym produktem ubocznym, biomasa drożdżowa może znaleźć zastosowanie jako funkcjonalny składnik o określonych właściwościach technologicznych (Puligundla i in., 2020).

Jednym z perspektywicznych kierunków nadawania wartości dodanej produktom ubocznym przemysłu spożywczego jest ich wykorzystanie w procesach biotechnologicznych z udziałem mikroorganizmów (Dadhaneeya i in., 2024). Produkty uboczne bogate w składniki odżywcze mogą stanowić atrakcyjne podłoże hodowlane dla wybranych szczepów mikroorganizmów, które w toku fermentacji prowadzą do biosyntezy związków bioaktywnych o pożądanym działaniu fizjologicznym (Martí-Quijal i in., 2021). Przykładem takiego mikroorganizmu jest *Propionibacterium freudenreichii*, zdolny do syntezy aktywnej formy witaminy B_{12} oraz SCFA (Thierry i in., 2011). Na podstawie danych literaturowych można stwierdzić, że odpadowa gęstwa drożdżowa spełnia wymagania wzrostowe tego mikroorganizmu, co czyni ją potencjalnym, tanim i zrównoważonym substratem fermentacyjnym (Puligundla i in., 2020). Z drugiej strony, skład chemiczny surowców spożywczych może wpływać na wzrost i aktywność mikroorganizmów poprzez ich selektywną stymulację lub inhibicję (Gyawali i Ibrahim, 2014; Guil-Guerrero i in., 2016). Zjawisko to może być wykorzystane do naturalnej konserwacji produktów żywnościowych, poprzez odpowiednio kontrolowaną fermentację i obecność związków bioaktywnych o działaniu przeciwdrobnoustrojowym lub antyoksydacyjnym. Przykładem mogą być związki

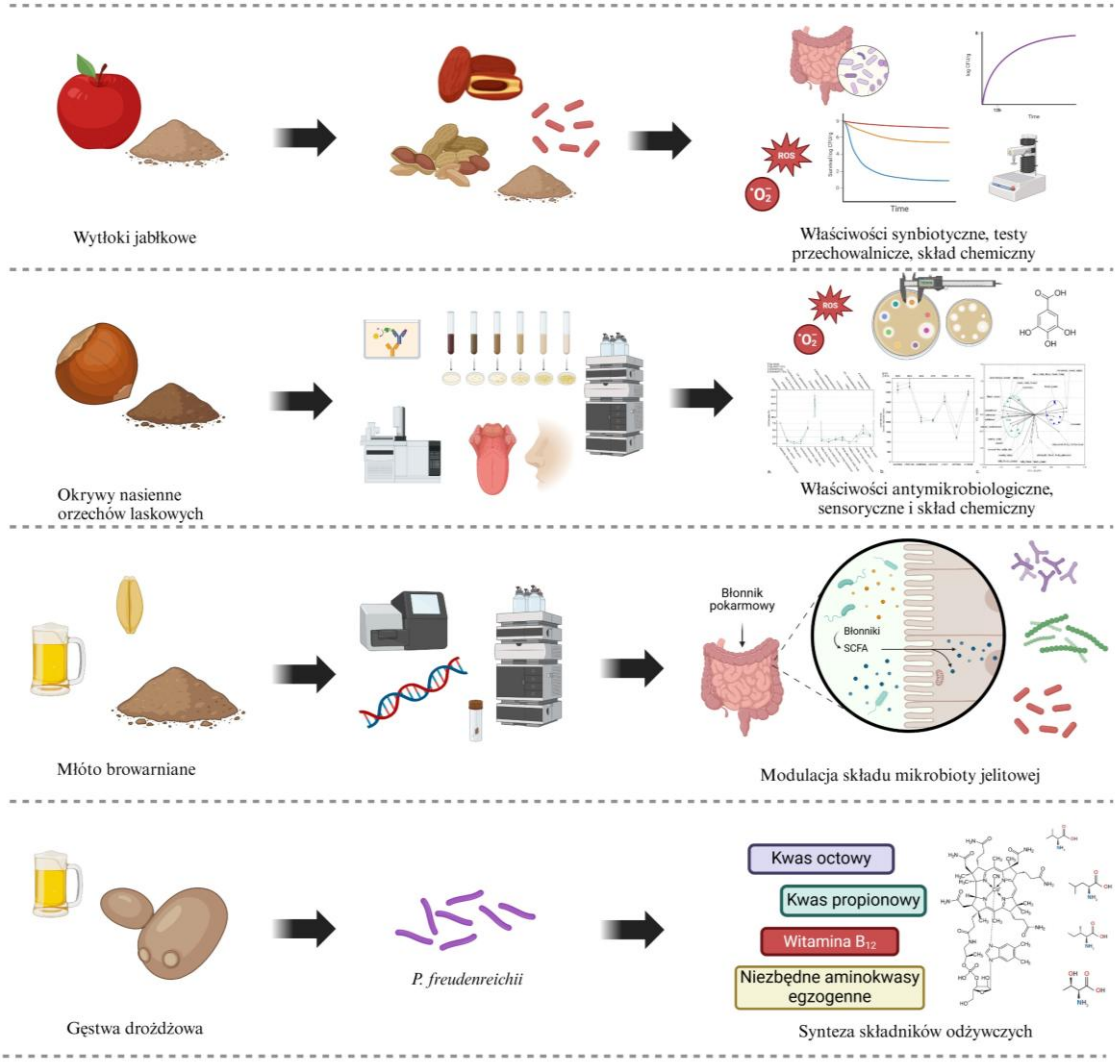
polifenolowe występujące w okrywach nasiennych orzechów laskowych, które wykazują zarówno silne właściwości antyoksydacyjne, jak i zdolność do ograniczania wzrostu niepożądanych mikroorganizmów (Horoszewicz i in., 2022; Ceylan, 2024). Co więcej drobnoustroje, odgrywają również istotną rolę w utrzymaniu homeostazy organizmu gospodarza, zwłaszcza poprzez interakcje na poziomie mikrobioty własnej człowieka (Hou i in., 2022). W tym kontekście kluczowym komponentem wielu produktów ubocznych i decydującym o ich potencjale prozdrowotnym, jest błonnik pokarmowy. Selektywne frakcje błonnika stanowią źródło energii dla szerokiego spektrum mikroorganizmów jelitowych, modulując ich skład i aktywność metaboliczną (Delzenne i in., 2025). Wśród badanych produktów ubocznych szczególną uwagę zwracają młóto browarniane oraz wytłoki jabłkowe, które charakteryzują się wysoką zawartością błonnika, odpowiednio AX oraz pektyn (Lynch i in., 2016; Antonic i in., 2020). Zarówno AX, jak i pektyny oraz ich pochodne oligosacharydy zostały scharakteryzowane jako związki o potencjalnych właściwościach prebiotycznych. Ich efektywność zależy przede wszystkim od struktury chemicznej i masy cząsteczkowej, jednak liczne badania wskazują, że ich regularne spożycie może prowadzić do korzystnych zmian w składzie mikrobioty jelitowej, zwiększonej produkcji SCFA oraz szeregu pozytywnych efektów metabolicznych i immunologicznych u gospodarza (Broekaert i in., 2011; Pascale i in., 2022; Rudjito i in., 2023; del Amo-Mateos i in., 2024).

Aktualna problematyka zagospodarowania produktów ubocznych przemysłu spożywczego koncentruje się na poszukiwaniu zrównoważonych metod ich przetwarzania oraz możliwości ich ponownego wykorzystania w przetwórstwie spożywczym i żywieniu człowieka. Wykorzystanie mikroorganizmów do biosyntezy związków bioaktywnych z produktów ubocznych, a także wprowadzanie komponentów tych surowców do żywności, stanowi obiecującą strategię, łączącą korzyści środowiskowe z potencjalnym działaniem prozdrowotnym. Dzięki odpowiedniej obróbce możliwe jest przekształcenie produktów ubocznych przemysłu spożywczego w składniki funkcjonalne lub wykorzystanie ich naturalnych właściwości. Tego rodzaju podejście sprzyja realizacji idei zrównoważonego rozwoju, ogranicza marnotrawstwo żywności i jednocześnie przyczynia się do poprawy zdrowia konsumentów oraz stanu środowiska naturalnego.

2. Cel pracy, hipoteza badawcza i zakres pracy

Celem pracy doktorskiej była szczegółowa analiza składu chemicznego, właściwości prozdrowotnych i funkcjonalnych oraz ocena możliwości wtórnego wykorzystania wybranych produktów ubocznych z przemysłu spożywczego. Główną hipotezę badawczą zdefiniowano jako:

„Produkty uboczne z przetwórstwa spożywczego posiadają właściwości klasyfikujące je do dalszego wykorzystania w przemyśle spożywczym i żywieniu człowieka a także mogą być wykorzystywane w celu podnoszenia wartości zdrowotnej żywności”.



Rycina 2. Schematyczny zakres pracy.

Zakres badań obejmował analizy poszczególnych produktów ubocznych w czterech modelach eksperymentów (Ryc. 2 i 3). Podczas badań z zakresu niniejszej pracy doktorskiej przeanalizowano wytłoki jabłkowe, okrywy nasienne orzechów

laskowych, młóto browarniane i browarniczą odpadową gęstwę drożdżową. Dla każdego z analizowanych produktów ubocznych zdefiniowano oddzielny cel badawczy, który umieszczono przy opisie poszczególnych artykułów naukowych wchodzących w zakres tej pracy doktorskiej. W przypadku wytłoków jabłkowych przeprowadzono badania aplikacyjne, gdzie dodano je do modelowych produktów spożywczych. W kwestii pozostałych produktów ubocznych, przeprowadzone badania miały charakter badań podstawowych.

3. Materiały i metody badawcze

Materiałami do badań były produkty uboczne pochodzące z przemysłu spożywczego. Wytłoki jabłkowe pozyskano z przedsiębiorstwa Doehler (Kozietuły Nowe, Polska), okrywy nasienne orzechów laskowych z przedsiębiorstwa Ferrero (Belsk Duży, Polska), młóto browarniane z Kampanii Piwowarskiej Dojlidy Brewery (Białystok, Polska, natomiast gęstwy drożdżowe z kilku browarów z terenu Polski (Browar Ciechan, Ciechanów; Browar Jabłonowo, Jabłonowo; Browar Palatum, Warszawa; Browar Perła, Lublin). Schemat badań przedstawiono na Rycinie 3.

Metody użyte w badaniu opisanym w publikacji P1

→ Metody mikrobiologiczne (hodowle komórkowe płynne i stałe z wykorzystaniem podłoży selektywnych).
→ Metody kolorymetryczne pomiaru siły antyoksydacyjnej (ABTS, DPPH).
→ Metoda kolorymetryczna Folina-Ciocialtu pomiaru całkowitej zawartości polifenoli.
→ Metoda sekwencjonowania genetycznego pierwszej generacji Sangera.
→ Metoda Soxleta pomiaru całkowitej zawartości tłuszczu.
→ Metoda enzymatyczno-wagowa pomiaru zawartości błonnika z rozbiciem na frakcję rozpuszczalną i nierozpuszczalną.
→ Metody teksturometryczne, deformacji i siły cięcia.
→ Metoda fizykochemiczna pomiaru aktywności wody.
→ Metody sensoryczne (Ilościowa Analiza Opisowa, analiza konsumencka).
→ Metody statystyczne (ANOVA z testem Tukeya, test t-Studenta, PCA).

Metody użyte w badaniu opisanym w publikacji P2

→ Metoda wagowa pomiaru wydajności ekstrakcji.
→ Metoda kolorymetryczna Folina-Ciocialtu pomiaru całkowitej zawartości polifenoli.
→ Metody kolorymetryczne pomiaru siły antyoksydacyjnej (ABTS, DPPH).
→ Metoda HPLC pomiaru zawartości związków fenolowych.
→ Metoda ELISA pomiaru zawartości alerganów (Bet v 1 i profiliny).
→ Metoda mikrobiologiczna rozcieńczeń agarowych mająca na celu określenie minimalnej dawki hamującej wzrost mikroorganizmów.
→ Metoda GC - elektroniczny nos, służąca do pomiaru związków aromatycznych.
→ Metoda instrumentalna profilu smakowego, elektroniczny język.
→ Metody statystyczne (ANOVA z testem Tukeya, test t-Studenta, PCA).

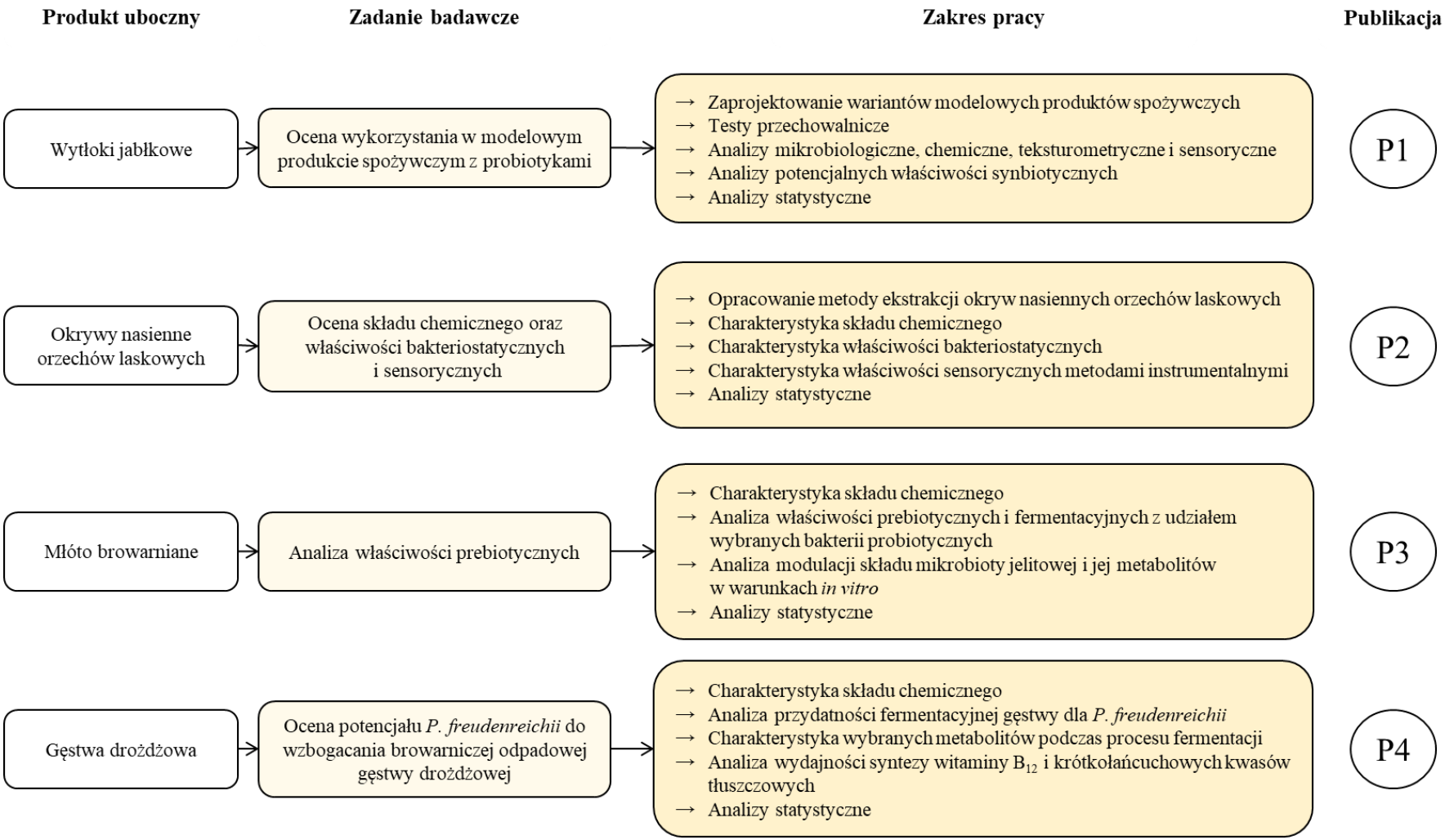
Metody użyte w badaniu opisanym w publikacji P3

→ Metoda Kjeldahla do oznaczania całkowitej zawartości białka.
→ Metoda GC z uprzednią hydrolizą polisacharydów w celu określenia zawartości polisacharydów nieskrobiowych rozpuszczalnych i nierozpuszczalnych.
→ Metoda enzymatyczna określania zawartości β-glukanów.
→ Metoda wagowa określania zawartości ligniny.
→ Metoda HPLC do analizy cukrów, kwasów organicznych, SCFA i BCFA.
→ Metody mikrobiologiczne (hodowle komórkowe płynne i stałe z wykorzystaniem podłoży selektywnych).
→ Metoda HPLC pomiaru zawartości związków fenolowych.
→ Metoda potencjometryczna pomiaru pH.
→ Metoda mikrobiologiczna określania aktywności prebiotycznej i indeksu prebiotycznego.
→ Metoda trawienia dynamicznego i fermentacji charakterystycznej dla jelita grubego *in vitro* (SHIME).
→ Metoda fermentacji statycznej charakterystycznej dla jelita grubego.
→ Metoda sekwencjonowania z wykorzystaniem platformy Illumina MiSeq oraz analiza bioinformatyczna.
→ Metody statystyczne (ANOVA, test t-Studenta, PCoA).

Metody użyte w badaniu opisanym w publikacji P4

→ Metoda Kjeldahla do oznaczania całkowitej zawartości białka.
→ Metoda HPLC do analizy cukrów, kwasów organicznych, SCFA.
→ Metoda HPLC do analizy wolnych aminokwasów.
→ Metoda HPLC do analizy zawartość ryboflawiny.
→ Metoda HPLC do analizy zawartości kobalaminy.
→ Metoda kolorymetryczna Folina-Ciocialtu pomiaru całkowitej zawartości polifenoli.
→ Metoda spektometrii mas z plazmą wzbudzaną indukcyjnie do analizy zawartości kobaltu.
→ Metody mikrobiologiczne (hodowle komórkowe płynne i stałe z wykorzystaniem podłoży selektywnych).
→ Metoda spektrofotometryczna do analizy OD hodowli bakteryjnych.
→ Metody statystyczne (ANOVA z testem Tukeya, test t Studenta, PCA)

Metody i materiały badawcze wykorzystane w tej pracy zostały szczegółowo scharakteryzowane w tekstach artykułów naukowych. Informacje o przygotowaniu próbek z zakresu każdej z publikacji znajdują się w tym opracowaniu przy opisie poszczególnych etapów badań.

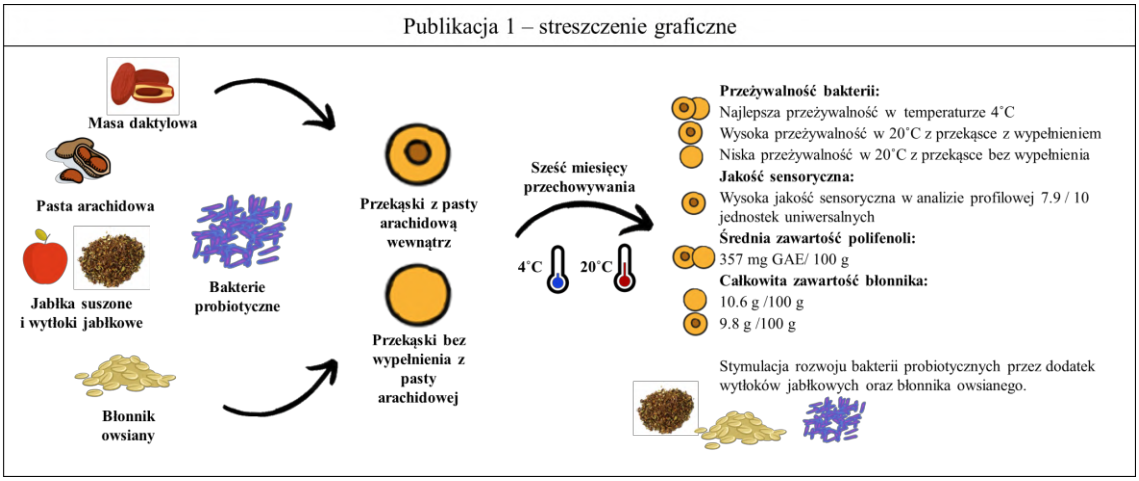


Rycina 3. Schemat badań.

4. Opis wyników i dyskusja

4.1. Wytłoki jabłkowe jako dodatek funkcjonalny do modelowych przekąsek

Abstrakt graficzny dotyczący badania na temat wytłoków jabłkowych przedstawiono na Rycinie 4.



Rycina 4. Abstrakt graficzny odnoszący się do Publikacji 1.

Szczególną uwagę w tym badaniu poświęcono zastosowaniu wytłoków jabłkowych jako dodatku funkcjonalnego do żywności, wpisującego się w strategię zrównoważonego zagospodarowania produktów ubocznych z przemysłu sokowniczego. Celem badań było wykorzystanie wytłoków jabłkowych w modelowych przekąskach o wysokiej zawartości błonnika pokarmowego, wzbogaconych w bakterie probiotyczne *Lacticaseibacillus rhamnosus* ATCC 53103, charakteryzujących się długim okresem przydatności do spożycia. Zakres pracy został przedstawiony na Rycinie 3.

Przekąski składały się z masy daktylowej, suszonych jabłek bez skórki, wytłoków jabłkowych, błonnika owsianego i pasty arachidowej. Do wariantów poddanych przechowywaniu przez sześć miesięcy (4°C i 20°C) wprowadzono bakterie probiotyczne w różnej formie (biomasa, liofilizat lub mikrokapsułki). Bakterie były umieszczone w masie bazowej przekąski lub nadzieniu z pasty arachidowej. W eksperymencie oceniono również podstawowy skład chemiczny przekąsek oraz zmiany sensoryczne, teksturalne, zawartość związków polifenolowych i aktywność antyoksydacyjną w trakcie przechowywania. Rezultaty badania przechowalniczego potwierdziły wysoką przeżywalność bakterii probiotycznych w przekąskach z nadzieniem z pasty arachidowej

charakteryzującej się niską aktywnością wodną (a_w = 0,27), zapewniającą utrzymanie liczebności bakterii powyżej 6 log JTK/g przez okres 5 miesięcy w 20°C. Stwierdzono również wysoką aktywność antyoksydacyjną zaprojektowanych produktów spożywczych (średnio 300 mg VCEAC/100 g) oraz wysoką zawartość polifenoli ogółem (średnio 357 mg GAE/100 g). Analizy sensoryczne wskazały na bardzo dobrą jakość ogólną przekąsek (średnio 7,1/10 punktów), przy czym próbki zawierające nadzienie z pasty arachidowej cechowały się wyższą akceptacją konsumencką i atrakcyjniejszym profilem sensorycznym.

Kluczowym eksperymentem w odniesieniu do wykorzystania wytłoków jabłkowych było przetestowanie kilku wariantów modelowych przekąsek z dodatkiem różnych źródeł błonnika, jako czynnika różnicującego aktywność bakterii probiotycznych. Skład recepturowy tych wariantów przedstawiono w Tabeli 1. Ten etap badania przeprowadzono niezależnie od badań przechowalniczych i ich wyników opisanych powyżej.

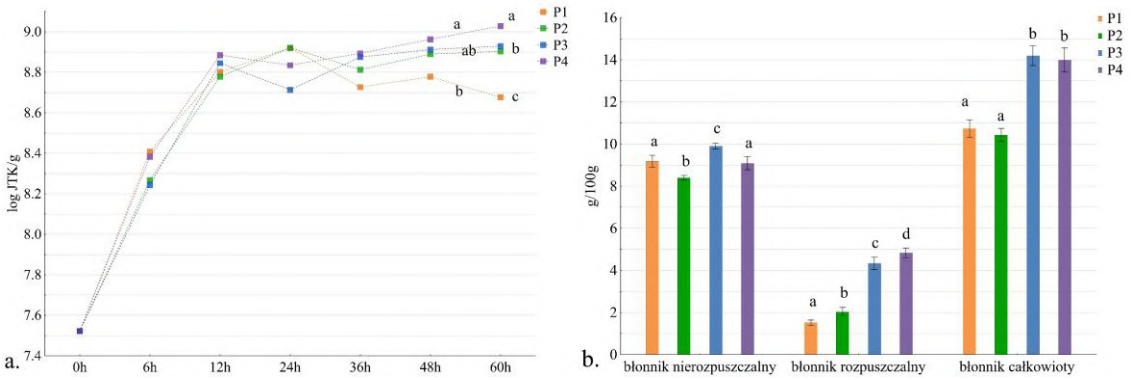
Tabela 1. Warianty próbek związane z eksperymentem o potencjalnych właściwościach synbiotycznych

Kod próbki	Skład g/100 g			
	Masa daktylowa	Jabłka suszone	Błonnik owsiany	Suszone wytłoki jabłkowe
P1	87,0	13,0	-	-
P2	83,6	13,0	3,6	-
P3	84,0	13,0	-	3,0
P4	80,4	13,0	3,6	3,0

Wyniki analiz potwierdziły różnice w zawartości błonnika pokarmowego i zwiększonego udziału frakcji rozpuszczalnej po dodatku wytłoków jabłkowych i błonnika owsianego do modelowych przekąsek (Ryc. 5 b).

Przygotowane warianty przekąsek przed zaszczepieniem bateriami probiotycznymi zhomogenizowano z wodą destylowaną i poddano sterylizacji. Opracowane typy przekąsek poddano sześćdziesięciogodzinnej fermentacji w 37°C z udziałem bakterii *L. rhamnosus* ATCC 53103 w celu analizy zmiany liczby bakterii pod wpływem różnych frakcji błonnika obecnych w dodanych surowcach do przekąsek. Zaobserwowano zjawisko przedłużonego wzrostu bakterii w próbkach z błonnikiem owsianym (P2), wytłokami jabłkowymi (P3) i obydwoma typami surowców (P4; Ryc. 5

a). W próbkach z dodatkiem surowców bogatych w błonnik, bakterie probiotyczne rozwijały się lepiej niż w próbce kontrolnej pozbawionej dodatków błonnikowych (P1). Wynik ten dowodzi obecności polisacharydów lub oligosacharydów fermentowanych po monosacharydach i innych łatwiej katabolizowanych substancjach chemicznych przez bakterie. Zgodnie z literaturą, po spożytkowaniu prostych źródeł energii, bakterie probiotyczne inicjują enzymatyczną degradację substancji prebiotycznych (Gänzle, 2015; Wang i in., 2021). Wytłoki jabłkowe są dobrze przebadanymi źródłami frakcji błonnika pokarmowego przede wszystkim pektyn oraz oligosacharydów pektynowych o zróżnicowanej strukturze molekularnej (Wei i in., 2021; Calvete-Torre i in., 2022). Frakcją prebiotyczną w owsie, który został zastosowanych jako drugi surowiec wysokobłonnikowy są β-glukany bezpośrednio stymulujące wzrost bakterii probiotycznych (Xu i in., 2021). Zaobserwowano, że połączenie dwóch źródeł włókna pokarmowego skutkowało większą stymulacją wzrostu bakterii probiotycznych. Dodatek wytłoków jabłkowych i błonnika owsianego do oddzielnych wariantów przekąsek skutkował wydłużeniem fazy stacjonarnej i żywotności bakterii w hodowlach. Natomiast, połączenie dwóch dodanych źródeł błonnika pokarmowego w jednej przekąsce spowodowało wydłużenie fazy logarytmicznego wzrostu bakterii probiotycznych.



Rycina 5 (a, b) Krzywe wzrostu *L. rhamnosus* ATCC 53103 w roztworach wodnych z próbek przekąsek z różnymi dodatkami błonnikowymi (a) i zawartość błonnika w badanych próbkach w podziale na frakcje rozpuszczalne i nierozpuszczalne (b); opisy skrótów próbek znajdują się w Tabeli 1; (n = 3); litery a, b i c oznaczają różnice statystyczne między próbkami w teście post hoc Tukeya po analizie ANOVA ($p < 0,05$), (a) różnice statystyczne odnoszą się do poszczególnych punktów czasowych; (b) różnice statystyczne odnoszą się do frakcji błonnika osobno; słupki błędów wskazują odchylenie standardowe; h – godziny

Przeprowadzone badania potwierdziły możliwość stworzenia przekąsek wzbogaconych wytłoki jabłkowe i bakterie probiotyczne o wysokiej zawartości błonnika pokarmowego. Optymalną formą bakterii probiotycznych był preparat liofilizowany

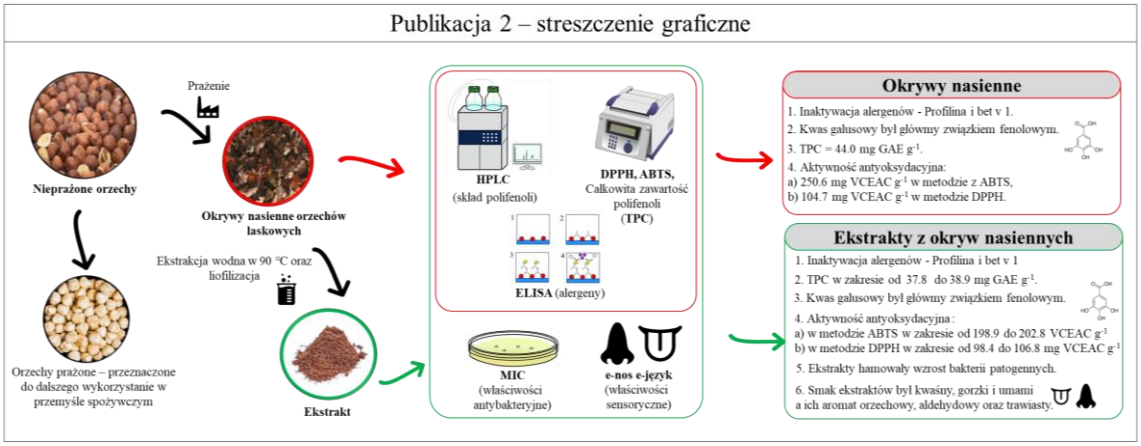
umieszczony w nadzieniu z pasty arachidowej o niskiej aktywności wody, co pozwoliło na utrzymanie rekomendowanego poziomu żywych bakterii przez okres do pięciu miesięcy w temperaturze pokojowej. Dodatek wytłoków jabłkowych miał kluczowe znaczenie dla podniesienia potencjalnych właściwości synbiotycznych opracowanych przekąsek. Wyniki te mają potencjał praktycznego wdrożenia w przemyśle spożywczym i wskazują na zasadność wprowadzania produktów ubocznych takich jak wytłoki jabłkowe do żywności funkcjonalnej.

Szczegółowy opis badań jest dostępny w P1.

Kruk, M., Lalowski, P., Hoffmann, M., Trząskowska, M., & Jaworska, D. (2024). Probiotic Bacteria Survival and Shelf Life of High Fibre Plant Snack—Model Study. Plant Foods for Human Nutrition.

4.2. Charakterystyka właściwości i składu chemicznego okryw nasiennych orzechów laskowych

Abstrakt graficzny dotyczący badania na temat okryw nasiennych orzechów laskowych przedstawiono na Rycinie 6.



Rycina 6. Abstrakt graficzny odnoszący się do Publikacji 2.

Celem przeprowadzonych badań była kompleksowa ocena składu chemicznego oraz właściwości bakteriostatycznych i sensorycznych okryw nasiennych orzechów laskowych jako potencjalnego składnika funkcjonalnego w przetwórstwie żywności i żywieniu człowieka.

Do ekstrakcji związków bioaktywnych zastosowano metodę wodnej ekstrakcji wspomaganej ultradźwiękami w temperaturze 90°C przez 10 minut. W badaniu uwzględniono dwa warianty ekstraktów różniące się stężeniem surowca. W pierwszym wariancie zastosowano koncentrację materiału ekstrahowanego 5% (E5) w stosunku do ekstrahenta, a w drugim 10% (E10). Po ekstrakcji próbki zostały zliofilizowane, co zapewniło ich trwałość i łatwość przechowywania.

Analiza chemiczna wykazała, że ekstrakty okryw nasiennych orzechów laskowych są wyjątkowo bogate w związki fenolowe, a całkowita zawartość polifenoli wynosiła średnio 38,4 mg GAE/g. Dominującym związkiem fenolowym w ekstraktach był kwas galusowy (średnio 3,5 mg/g). Wysoka aktywność przeciwutleniająca ekstraktów została potwierdzone metodą z wykorzystaniem rodników ABTS i DPPH. Średnie wartości uzyskane kolejno tymi metodami były w zakresie 198,9 – 202,8 mg VCEAC/g

(ABTS) i 98,4 – 106,8 mg VCEAC/g (DPPH). Te parametry sytuują okrywy nasienne orzechów laskowych wśród surowców o bardzo wysokim potencjale antyoksydacyjnym (Ceylan i in., 2023).

Kolejnym istotnym elementem badań była ocena alergenności okryw nasiennych orzechów laskowych. Wykorzystując przeciwciała specyficzne dla dwóch głównych grup białek alergennych orzechów laskowych (profiliny i Bet v 1) wykazano, że ich zawartość była poniżej progu detekcji w badanych próbkach. Rezultat ten wskazuje na skuteczną inaktywację powyższych grup alergenów na etapie przemysłowego prażenia orzechów i stanowi ważny wkład w wiedzę na temat bezpieczeństwa żywieniowego okryw nasiennych orzechów laskowych, pod względem alergennym. Istnieje potrzeba dalszej analizy innych grup alergenów, które wykazują wyższą stabilność termiczną. Za alergenność orzechów laskowych odpowiada kilka grup białek (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 14, Cor a 15 i Cor a TLP) (Costa i in., 2016; Nebbia i in., 2021; Camus-Ela i in., 2025). Cor a 1 należy do grupy alergenów Bet v 1, podczas gdy Cor a 2 należy do profilin. Obie te grupy białek wykazują zmniejszenie alergenności pod wpływem wysokich temperatur (powyżej 120°C) (Costa i in., 2016). Co ważne, ani prażenie, ani żadna inna obróbka nie sprawiają, że orzechy są bezpieczne dla alergików. W badaniach klinicznych udowodniono, że proces prażenia zmniejsza alergenność orzechów, a niektórzy badani nie doświadczyli reakcji alergicznej po ich spożyciu. Mimo to, efekt w tych badaniach nie został uzyskany u wszystkich członków badania lub efekt nie był klinicznie istotny (Hansen i in., 2003; Worm i in., 2009). Należy podkreślić, że cytowane prace skupiają się na alergenności orzechów laskowych, a nie ich okryw nasiennych. Niewątpliwie konieczna jest analiza okryw nasiennych orzechów pod kątem występowania alergenów z innych grup Cor. Można również przypuszczać, że okrywy nasienne to część orzechów laskowych najbardziej narażona na działanie czynników zewnętrznych podczas procesu przetwórczego i jednocześnie w tym produkcie ubocznym inaktywacja związków alergennych może być największa.

Jednym z kluczowych i nowatorskich aspektów pracy było wykazanie silnego działania bakteriostatycznego ekstraktów okryw nasiennych orzechów laskowych przeciwko szerokiemu spektrum bakterii patogennych, takich jak *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* czy *Escherichia coli*. Wyniki te przedstawiono w Tabeli 2. Minimalne stężenie hamujące dla większości

szczepów bakterii wynosiło 1,5–7,5 mg/g podłoża wzrostowego. Wiele badań przedstawia mechanizmy związków polifenolowych, które powodują działanie bakteriostatyczne lub bakteriobójcze (Borges i in., 2013; Kępa i in., 2018; Yuan i in., 2021). Kwasy fenolowe zidentyfikowane w naszych próbkach zakłócają szczelność i przepuszczalność błony komórkowej bakterii. Zakłócenie błony komórkowej jest tłumaczone jako miejscowe nadmierne zakwaszenie, które powoduje wzrost przepuszczalności błony komórkowej, a następnie denaturację białek wewnątrzkomórkowych i w rezultacie inaktywację komórki bakteryjnej (Borges i in., 2013; Kępa i in., 2018).

Tabela 2. Działanie bakteriostatyczne badanych ekstraktów okryw nasiennych orzechów laskowych (E5, E10) na różne szczepy bakterii w oparciu o MIC; n=3

Szczep bakterii	MIC E5	MIC E10
	mg/g	
Listeria monocytogenes ATCC 15131	2,5	2,5
Listeria monocytogenes ATCC 19111	2,5	2,5
Listeria monocytogenes ATCC 7644	2,5	2,5
Salmonella enterica ATCC 29631	2,5	2,5
Salmonella enterica ATCC 14028	2,5	2,5
Salmonella Hofit IFM 2318	5,0	5,0
Bacillus cereus ATCC 10876	1,5	1,5
Bacillus spizizenii ATCC 6633	2,5	5,0
Bacillus cereus ATCC 11778	1,5	1,5
Staphylococcus simulans CECT 4538	1,5	1,5
Staphylococcus aureus ATCC 6538	1,5	1,5
Staphylococcus aureus ATCC 25923	5,0	2,5
Escherichia coli o157:H7	5,0	5,0
Escherichia coli ATCC 15922	7,5	7,5
Escherichia coli ATCC 11775	5,0	7,5
Enterococcus faecalis ATCC 51299	5,0	5,0
Enterococcus faecalis ATCC 19433	5,0	5,0
Enterococcus faecalis ATCC 29212	5,0	5,0
Clostridium sporogenes ATCC 19404	5,0	5,0
Clostridium sporogenes ATCC 11437	5,0	5,0
Lactiplantibacillus plantarum 299v	>10,0	>10,0
Lacticaseibacillus rhamnosus ATCC 53103	>10,0	>10,0

Objaśnienia: E5 oznacza ekstrakt, w którym 5 g okryw nasiennych orzechów laskowych wyekstrahowano w 100 g wody, a E10 oznacza ekstrakt, w którym 10 g nasiennych orzechów laskowych wyekstrahowano w 100 g wody.

Mechanizm działania przeciwdrobnoustrojowego flawonoidów, takich jak katechiny, kemferol i kwercetyna, koncentruje się na zmianach w błonie komórkowej i ścianie komórkowej, ale także na hamowaniu syntezy DNA i zwiększonym stresie oksydacyjnym w komórce bakteryjnej. Flawonoidy prawdopodobnie uszkadzają warstwy fosfolipidowe, hamują łańcuch oddechowy lub syntezę ATP (Yuan i in., 2021). W innym badaniu zaobserwowano, że katechiny mogą zwiększać zawartość reaktywnych form tlenu w komórce i zakłócać gospodarkę antyoksydacyjną u bakterii *E. coli* (Xiong i in., 2017). Stwierdzono, że związki fenolowe mają działanie synergistyczne. Kwas galusowy ma znacznie większą aktywność przeciwdrobnoustrojową w połączeniu z hydroksytyrozolem i innymi polifenolami niż jako pojedynczy związek (Tafesh i in., 2011). Co istotne, ekstrakty nie wykazywały działania hamującego wobec bakterii probiotycznych (*L. plantarum* 299v, *L. rhamnosus* ATCC 53103), co sugeruje ich selektywny mechanizm działania w stosunku do bakterii patogennych. Montella i in. (2013) stwierdzili, że okrywy nasienne orzechów i ich ekstrakty są bogate w rozpuszczalny i nierozpuszczalny błonnik pokarmowy, który stymuluje wzrost bakterii fermentacji mlekowej. Przypuszcza się, że ekstrakty użyte w naszych badaniach zawierały frakcje błonnika rozpuszczalnego wpływając na wzrost bakterii probiotycznych. Co więcej, bakterie probiotyczne mogą katabolizować związki polifenolowe jako selektywne źródła energii (Sharma i in., 2022). Wykazano, że związki fenolowe w modelach jelita grubego *in vitro* lub ich spożycie w diecie powoduje wzrost liczby bakterii probiotycznych i komensalnych o pozytywnym działaniu na organizm człowieka z jednoczesnym zmniejszeniem rozwoju proteobakterii (Mithul Aravind i in., 2021; Plamada i Vodnar, 2022).

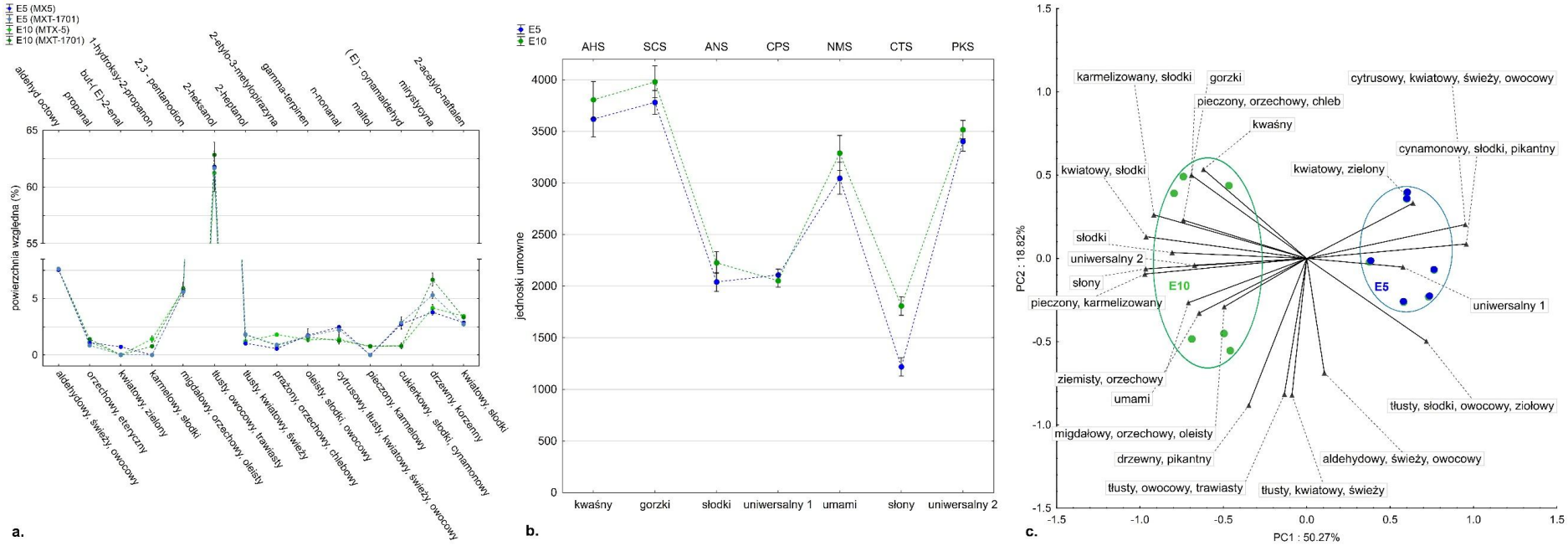
Dodatkowo w pracy tej zastosowano instrumentalne metody analizy sensorycznej (elektroniczny nos i język) do oceny profilu zapachowego i smakowego ekstraktów okryw nasiennych orzechów laskowych. Zidentyfikowano szereg lotnych związków zapachowych nadających ekstraktom charakterystyczne noty aromatyczne: tłuste, orzechowe, przyprawowe, owocowe i kwiatowe (Ryc 7. a, b, c). Z kolei profil smakowy obejmował głównie smaki gorzkie, kwaśne i umami. Analiza PCA pozwoliła wykazać różnice w profilu smakowo-zapachowym między próbkami E5 i E10, przypisując te rozbieżności konkretnym związkom lotnym, np. but-(E)-2-enal (nuty kwiatowe i zielone) obecnym wyłącznie w próbce E5. Co więcej w innych pracach naukowych autorzy sugerują, że okrywy nasienne orzechów laskowych mają duży potencjał do zastosowań

spożywczych. Okrywy nasienne orzechów laskowych dodawano do różnych matryc spożywczych, takich jak jogurty, burgery z kurczaka i batony bakaliowe (Dinkçi i in., 2021; Horoszewicz i in., 2022; Ceylan i in., 2023). Autorzy tych prac podkreślali, że materiał ten może być funkcjonalnym dodatkiem do żywności i w odpowiedniej dawce nie zmienia cech sensorycznych żywności, a nawet wpływa na nie pozytywnie. Ponadto w literaturze pojawiają się sugestie, że głębsze zrozumienie właściwości okryw nasiennych orzechów laskowych poprzez ich analizę na poziomie molekularnym przyczyni się do ich łatwego zastosowania w produktach żywnościowych (Ceylan i in., 2023).

Badanie dotyczące okryw nasiennych orzechów laskowych dostarczyło nowych danych na temat właściwości chemicznych, mikrobiologicznych i sensorycznych. Wykazano, że okrywy nasienne orzechów laskowych posiadają znaczny potencjał jako składnik funkcjonalny do zastosowania w żywności, dzięki wysokiej zawartości polifenoli, właściwościom przeciwutleniającym, przeciwbakteryjnym, obniżonej alergenności oraz złożonemu profilowi sensorycznemu. Praca otwiera nowe możliwości w zakresie zagospodarowania tego produktu ubocznego.

Szczegółowy opis badań jest dostępny w P2.

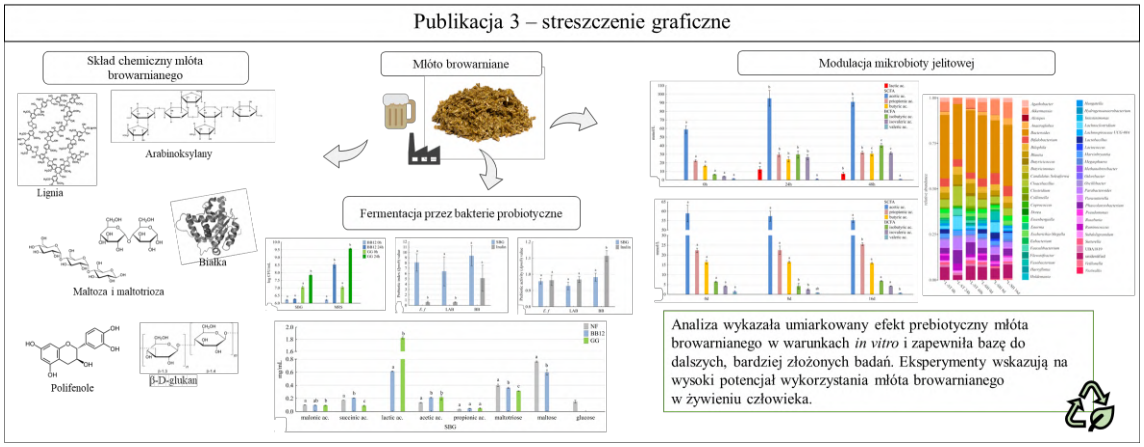
Kruk, M., Ponder, A., Horoszewicz, J., Popławski, D., Król, K., Leszczyńska, J., Jaworska, D., & Trząskowska, M. (2024). By-product hazelnut seed skin characteristics and properties in terms of use in food processing and human nutrition. Scientific Reports, 14(1), 18835.



Rycina 7. (a, b, c) Profil zapachowy analizowanych ekstraktów (E5, E10) na podstawie związków lotnych i ich wyróżników sensorycznych w analizie e-nosa wyniki przedstawiono jako procentowy udział w całkowitej ilości związków lotnych w zależności od kolumny chromatograficznej użytej do rozdziału (MTX-5, MXT-1701), n=3 (a); profil smakowy ekstraktów okryw nasiennych orzechów laskowych uzyskanych metodą e-języka, skróty przy górnej osi wykresu oznaczają czujniki odpowiedzialne za wykrywanie przypisanego im smaku; wartość wyrażona jest w jednostkach umownych bez zdefiniowanego zakresu maksymalnego, n=8 (b); analiza PCA instrumentalnej oceny sensorycznej ekstraktów przez e-nos i e-język, projekcja zmiennych (cech sensorycznych wykrytych związków lotnych przez e-nos i cech smaku wykrytych przez e-język) i przypadków (testowanych próbek) na płaszczyznę głównych składowych (c); E5 oznacza ekstrakt, w którym w 100 g wody wyekstrahowano 5 g okryw nasiennych orzechów laskowych a E10 oznacza ekstrakt, w którym w 100 g wody wyekstrahowano 10 g okryw nasiennych orzechów laskowych; słupki błędu przedstawiają przedział ufności ($p<0,05$); wyniki te potwierdzono testem t lub testem post hoc Tukeya po analizie ANOVA ($p<0,05$)

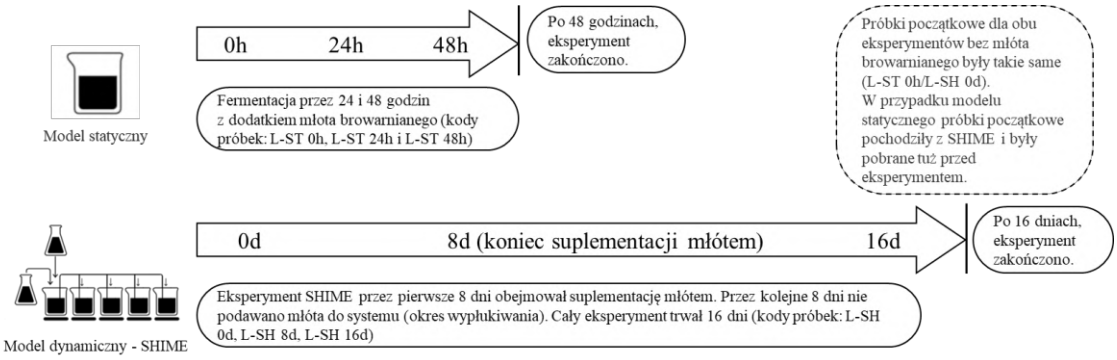
4.3. Właściwości prebiotyczne młóta browarnianego

Abstrakt graficzny dotyczący badania na temat młóta browarnianego przedstawiono na Rycinie 8.



Rycina 8. Abstrakt graficzny odnoszący się do Publikacji 3.

Celem pracy było zbadanie potencjału prebiotycznego młóta browarnianego, będącego najliczniej generowanym produktem ubocznym przemysłu piwowarskiego. Badanie przebiegało w trzech etapach. W pierwszym etapie przeprowadzono szczegółową analizę składu chemicznego młóta browarnianego, uwzględniając zawartość białka, frakcje błonnika pokarmowego z podziałem na polisacharydy nieskrobiowe, cukry resztkowe oraz związki polifenolowe. W drugim etapie, oceniono zdolność bakterii probiotycznych (*Lacticaseibacillus rhamnosus* ATCC 53103 (GG) oraz *Bifidobacterium animalis* subsp. *lactis* BB-12) do fermentacji młóta browarnianego. Przeanalizowano dynamikę wzrostu bakterii, zmiany pH i profil kwasów organicznych i cukrów. W trzecim etapie eksperymentu zastosowano dwa modele fermentacji charakterystycznej dla jelita grubego *in vitro*, były to, prosty model statyczny oraz dynamiczny model SHIME (Ryc. 9).

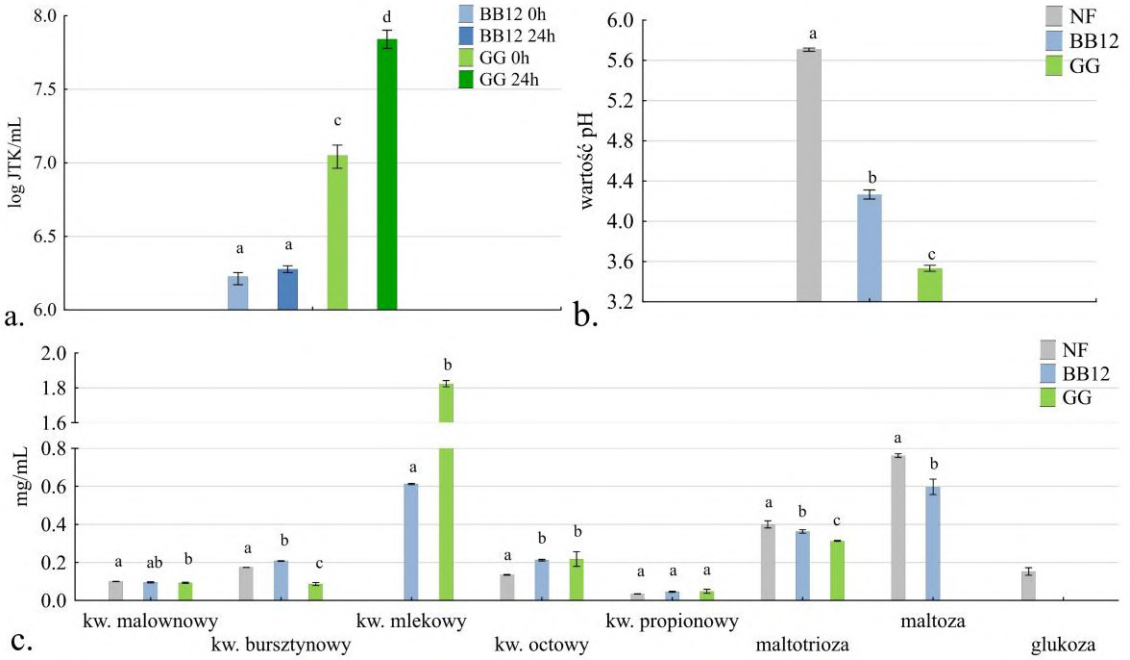


Rycina 9. Schemat eksperymentu obrazujący czas trwania, skróty próbek i warunki doświadczenia związane z modelem statycznym i dynamicznym jelita grubego *in vitro.*

Na podstawie analiz chemicznych stwierdzono, że młóto browarniane zawiera znaczny udział błonnika pokarmowego w tym największą jego frakcję stanowiły nieskrobiowe polisacharydy (29,7 g/100 g), do których zalicza się występujący w dużej ilości AX (16,7 g/100 g). Dodatkowo przeanalizowano, że młóto browarniane jest bogate w ligninę (9,0 g/100 g) i zawiera niewielkie stężenie β-glukanów (0,6 g/100g). Zawartość maltozy stanowiła 7,3 g/100 g, a maltotriozy 3,4 g/100 g. Zawartość związków polifenolowych ogółem była na poziomie 49,1 mg/100 g.

L. rhamnosus GG i *B. animalis* BB-12 wykazały zdolność do wzrostu w roztworze wodnym zawierającym 1% młóta browarnianego. Aktywność *L. rhamnosus* GG była istotnie większa niż *B. animalis* BB-12. Zmiany liczby bakterii, pH i profilu kwasów organicznych oraz cukrów po 24 godzina fermentacji w 37 °C i warunkach beztlenowych przedstawiono na Rycinie 10 (a, b, c). Całkowita zawartość cukrów uległa znacznemu obniżeniu po fermentacji. Maltotrioza została częściowo zdegradowana przez oba szczepy bakterii, podczas gdy *L. rhamnosus* GG wykazał większą zdolność do katabolizowania tego trisacharydu. Maltoza została całkowicie zredukowana z podłoża przez *L. rhamnosus* GG, natomiast tylko częściowo przez *B. animalis* BB-12. Katabolizm tych cukrów był związany z obecnością enzymów bakteryjnych α-1,4-glikozydazy i α-glukozydazy, które mogą rozkładać disacharydy i maltotriozę do cukrów prostych (Pokusaeva i in., 2011; Gänzle, 2015). Głównymi utworzonymi metabolitami były kwas mlekowy i octowy. Zmiany w profilu kwasów organicznych wskazują prawidłowość przemian metabolicznych badanych bakterii (Pokusaeva i in., 2011; Suissa i in., 2023). Kwasy malonowy i bursztynowy zgodnie ze szlakami metabolicznymi nie były wykorzystywane przez *B. animalis* BB-12. Inna sytuacja wystąpiła z *L. rhamnosus* GG,

gdzie kwas bursztynowy był metabolizowany przez dehydrogenazę bursztynianową w niepełnym cyklu kwasu cytrynowego (Suissa i in., 2023).



Rycina 10. (a, b, c) Dynamika fermentacji młóta browarnianego w oparciu o liczbę bakterii (a), zmiany wartości pH (b), profil kwasów organicznych i cukrów (c); „h" oznacza godziny, „kw." oznacza kwas, „NF" oznacza podłoże niefermentowane, „BB12" oznacza *Bifidobacterium animalis* subsp. *lactis* BB-12, „GG" oznacza *Lacticaseibacillus rhamnosus* GG; małe litery na wykresach oznaczają różnice statystyczne między próbkami w teście Tukeya po analizie ANOVA lub teście t ($p < 0,05$); słupki błędów na wykresie (a) oznaczają zakresy minimum-maksimum, w pozostałych przypadkach oznaczają odchylenia standardowe; n = 3.

Wyniki składu mikrobioty jelitowej oraz SCFA i BCFA przedstawiono w Rycinie 11. Wskaźniki bioróżnorodności alfa (Chao1, Shannon i Simpson) różniły się między próbkami, przy czym najwyższą wartość uzyskano dla próbek wyjściowych, co wskazuje, że bioróżnorodność bakterii na początku eksperymentu była największa (wyniki dostępne w P3). W próbce L-ST 24 h (model statyczny) nastąpiła znaczna redukcja bioróżnorodności alfa w porównaniu z próbką bazową. Pozostałe próbki charakteryzowały się podobnymi poziomami wskaźników bioróżnorodności alfa. Niemniej jednak, bioróżnorodność w próbkach z obu modeli zmniejszyła się w porównaniu do poziomu bazowego. Co ważne, zwykle powtarzającym się wynikiem w literaturze naukowej jest zmniejszenie bioróżnorodności alfa podczas suplementacji diety błonnikiem pokarmowym. Spowodowane jest to stymulacją specyficznych gatunków bakterii pod kątem ich rozwoju oraz brakiem dostarczenia nowej flory bakteryjnej różnicującej bioróżnorodność (Müller i in., 2020; Delzenne i in., 2025).

W modelu statycznym odnotowano istotny wzrost stężenia wszystkich analizowanych SCFA i BCFA w porównaniu ze stanem początkowym. Wyjątek stanowił kwas walerianowy, którego poziom pozostał stabilny przez okres trwania eksperymentu (Ryc. 11 a, b). Między 24 a 48 godziną fermentacji stężenie kwasu mlekowego uległo obniżeniu, a udział procentowy SCFA w ogólnej puli kwasów zmniejszył się na skutek zwiększenia udziału BCFA. Analiza mikrobioty wykazała, zmniejszenie względnej liczebności wielu grup bakterii, w tym m.in. *Bacteroides, Bifidobacterium* i *Lactobacillus*. Po 48 godzinach zaobserwowano częściową odbudowę społeczności mikrobioty - nastąpił wzrost udziału niektórych rodzajów bakterii (*Blautia, Eubacterium, Faecalibacterium, Subdoligranulum*) przy jednoczesnym utrzymującym się obniżeniu udziału innych (*Bacteroides, Bifidobacterium, Lactobacillus*) względem poziomu początkowego. Obecnie istnieją ograniczone dowody dotyczące zmian mikrobioty jelitowej, SCFA i BCFA pod wpływem suplementacji młótem browarnianym. Dotychczas badacze koncentrowali się głównie na właściwościach prebiotycznych wyizolowanych frakcji AX lub AXOS z młóta browarnianego lub innych materiałów zbożowych. Porównanie danych literaturowych na temat młóta browarnianego oraz AX z wynikami z tego badania ujawnia kilka podobieństw w doświadczeniach wykorzystujących model statyczny. Zaobserwowaliśmy wzrost względnej liczebności *Subdoligranulum, Faecalibacterium, Eubacterium* i *Phascolarctobacterium* oraz zmniejszenie udziału rodzajów *Bacteroides* i *Escherichia-Shigella*, co pozostaje zgodne z wynikami badań Harrisa i in. (2019) oraz Lyncha i in. (2021). Inne badania wykorzystujące fermentację statyczną potwierdziły podobną tendencję w zmianach SCFA i BCFA. Brak monitorowania pH podczas fermentacji kałowej prowadzi do intensywnej syntezy krótkołańcuchowych kwasów tłuszczowych (Reis i in., 2014; Gómez i in., 2015; Bonifácio-Lopes i in., 2022; Calvete-Torre i in., 2022).

W dynamicznym modelu SHIME w okresie ośmiodniowej suplementacji młótem browarnianym zaobserwowano stabilizację stężenia SCFA, przy jednoczesnym istotnym obniżeniu stężenia BCFA w porównaniu do okresu przed suplementacją (Ryc. 11 d, e). Po zakończeniu podawania młóta browarnianego (kolejne 8 dni - okres wymywania) stężenia BCFA powróciły do wartości wyjściowych, jedynie stężenie kwasu propionowego wzrosło powyżej poziomu początkowego. Procentowy udział SCFA w ogólnej puli kwasów tłuszczowych zwiększył się istotnie podczas suplementacji układu młótem browarnianym. W kwestii zmian SCFA i BCFA dotychczas nie opublikowano

innej pracy dotyczącej młóta browarnianego, która dostarczałaby danych z modeli zaawansowanych lub eksperymentów *in vivo*. Niemniej jednak, przegląd systematyczny dotyczący wpływu błonnika pokarmowego na metabolity bakteryjne przeprowadzony przez (Vinelli i in., 2022) nie wykazał istotnego wpływu włókna pokarmowego z nieprzetworzonej żywności na zmiany w zawartości SCFA. Liczne badania sugerują, że skład społeczności mikrobioty jelitowej jest modulowany przez błonnik pokarmowy bez wpływu na poziomy SCFA (Müller i in., 2020; O. Chen i in., 2021; Wilms i in., 2021; Vinelli i in., 2022). Zjawisko to przypisuje się przede wszystkim współzależnościom między mikroorganizmami mikrobioty jelitowej a ich metabolitami, w tym SCFA i BCFA. Oznacza to, że metabolity mikrobioty jelitowej utrzymującej homeostazę ulegają stałemu anabolizmowi i katabolizmowi przez mikroby (Peterson i in., 2022; Vinelli i in., 2022). Większą syntezę kwasów tłuszczowych zaobserwowano w badaniach wykorzystujących wyizolowane frakcje błonnika pokarmowego o wysokiej selektywności, które wpływały na wysoki poziom produkcji SCFA przez docelowe grupy mikroorganizmów (Vinelli i in., 2022). Kluczowymi czynnikami, które modulują syntezę SCFA, wydają się być dawka błonnika, rodzaj, stopień przetworzenia i jego struktura chemiczna (Yao i in., 2022).

Wpływ błonnika pokarmowego na produkcję BCFA jest różny, ale większość badań sugeruje, że diety bogate w błonnik obniżają poziom tych kwasów (Vinelli i in., 2022). Wyniki naszych badań są spójne z wynikami innych badaczy, biorąc pod uwagę redukcję zawartości BCFA w okresie suplementacji młóta browarnianego i stabilne poziomy SCFA w SHIME. Ważną obserwacją w naszym badaniu jest powrót zawartości BCFA do poziomu wyjściowego po zakończeniu suplementacji młóta browarnianego do SHIME. Udowadnia to bezpośredni efekt modulujący tego produktu ubocznego, który nie jest przedłużony po zakończeniu jego suplementacji. Aspekt kwasu propionowego, którego zawartość wzrosła po okresie wypłukiwania (L-SH 16d, Ryc. 11d), prawdopodobnie był spowodowany wzrostem udziału rodziny *Lachnospiraceae* i jego syntezy w szlaku akrylanowym przez *Akkermansia* (El Hage i in., 2019; Kirmiz i in., 2020; Zaplana i in., 2023).

Skład mikrobioty jelitowej w SHIME był odmienny niż w modelu statycznym. Po okresie ośmiodniowej suplementacji młótem browarnianym zaobserwowano wzrost udziału bakterii komensalnych korelowanych z korzystnym wpływem na organizm

człowieka. Były to rodzaje: *Bifidobacterium*, *Akkermansia*, *Lactobacillus*, czy *Butyricicoccus*. Jednocześnie obniżył się udział m.in. *Bacteroid*es oraz bakterii z grupy *Escherichia-Shigella*. Po zakończeniu okresu bez suplementacji młótem skład mikrobioty częściowo powrócił do stanu początkowego, choć niektóre zmiany utrzymały się do końca doświadczenia.

Istnieje wyraźna luka w dotychczasowej wiedzy na temat właściwości prebiotycznych młóta browarnianego w zaawansowanych, dynamicznych systemach *in vitro*, takich jak SHIME, modelach zwierzęcych lub badaniach z udziałem ludzi. Uzyskane przez nas wyniki badań z wykorzystaniem modelu SHIME znajdują częściowe odwzorowanie w pracy Lyncha i in. (2021), gdzie wykorzystano kontrolowane warunki pH w modelu statycznym. W tym badaniu zaobserwowano podobny wzrost względnej liczebności *Bifidobacterium*, *Lactobacillus* i *Enterococcus* oraz zmniejszenie udziału *Bacteroides*. Jednak podobieństwo pomiędzy układem statycznym a SHIME jest niewielkie ze względu na różnice w czasie trwania eksperymentu, parametrach systemu, dostarczaniu pożywki i materiału badawczego do systemu (Roupar i in., 2021; Isenring i in., 2023). Z drugiej strony udokumentowano obszerne dane na temat właściwości prebiotycznych AX i AXOS w dynamicznych systemach, modelach zwierzęcych i badaniach z udziałem ludzi. Badania te podkreślają zdolność AX i AXOS do promowania wzrostu *Bacteroides*, *Bifidobacterium*, *Blautia*, *Dorea*, *Eubacterium*, *Lactobacillus*, *Faecalibacterium*, *Prevotella*, *Roseburia* i *Enterococcus* (François i in., 2012; Walton i in., 2012; Zambrana i in., 2019; Kjølbæk i in., 2020; Schupfer i in., 2021, 2023). W innym podsumowaniu przeglądowym wskazano na znaczny potencjał redukcji *Campylobacter, Clostridium* i *Escherichia-Shigella* przez te frakcje błonnika (Schupfer i in., 2021). W kolejnych badaniach autorzy zanotowali istotny rozwój bakterii wytwarzających maślan pod wpływem AX i AXOS, których udział wzrósł również w naszych badaniach (*Butyricicoccus* , *Bilophila*, *Marvinbryantia*, *Odoribacter*, *Lachnoclostridium*) (Damen i in., 2011; Van den Abbeele i in., 2011; Schupfer i in., 2023). Podsumowując, badania dotyczące młóta browarnianego, AX i AXOS często wskazywały na umiarkowany efekt bifidogenny. Nie można jednak przypisać bezpośredniego działania AX i AXOS na powtarzającą się na przestrzenie badań modulację konkretnych taksonów bakteryjnych w mikrobiocie jelitowej. Kierunek zmian składu bakteryjnej mikrobioty i jej metabolitów zależy od struktury chemicznej AX i AXOS, co jest determinowane przez pochodzenie surowca (Neyrinck i in., 2018;

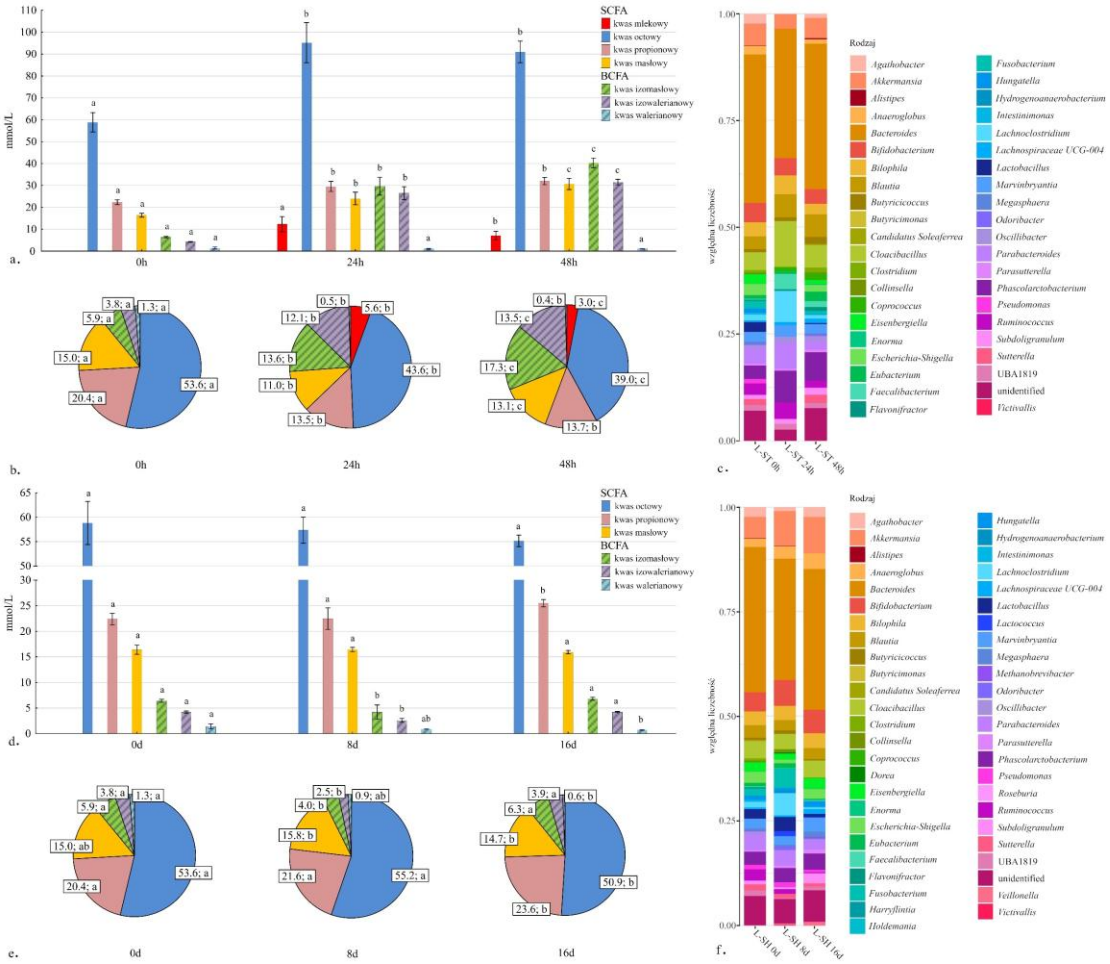
Z. Chen i in., 2019). Masa cząsteczkowa AX i liczba łańcuchów bocznych, które różnią się w zależności od surowca wpływają na te właściwości. Na przykład AX z jęczmienia ma wyższą masę cząsteczkową niż z ryżu, co skutkuje mniejszymi i mniej rozgałęzionymi cząsteczkami, które są łatwiej fermentowane przez mikrobiotę jelitową i powodują jej szybszą modulację (Z. Chen i in., 2019; Wang i in., 2021).

Porównanie obu systemów fermentacji wykazało, że wpływ młóta browarnianego na mikrobiotę jelitową i jej metabolity zależy od rodzaju modelu. W modelu statycznym nie stwierdzono efektu bifidogennego i stymulacji rozwoju bakterii kwasu mlekowego. W modelu dynamicznym młóto browarniane wywołało umiarkowany efekt prebiotyczny zwiększając udziału *Bifidobacterium, Akkermansia* i bakterii kwasu mlekowego. Ponadto system statyczny charakteryzował się wysoką niestabilnością w kwestii bioróżnorodności alfa, podczas gdy w systemie SHIME mikrobiota jelitowa pozostała bardziej stabilna. W rezultacie model dynamiczny okazał się bardziej adekwatny do badania właściwości prebiotycznych. Zaobserwowane różnice między stosowanymi systemami *in vitro* znajdują odzwierciedlenie w literaturze. Inni autorzy podkreślają różnice między modelami statycznymi a dynamicznymi. Najczęściej występujące problemy w modelach statycznych dotyczą niezdolności do stabilizacji mikrobioty, krótkoterminową fermentację, trudny do kontroli wpływ warunków środowiskowych (Roupar i in., 2021; Isenring i in., 2023).

Zebrane dane wskazują, że młóto browarniane może być uznane za produkt o umiarkowanym potencjale prebiotycznym. Efekty zaobserwowane w modelu SHIME sugerują, że młóto browarniane wpływa korzystnie na skład mikrobioty jelitowej oraz profil metabolitów. Wyniki te otwierają nowe perspektywy badań nad tym produktem ubocznym oraz propagują jego wtórne zagospodarowanie w żywieniu człowieka.

Szczegółowy opis badań jest dostępny w P3.

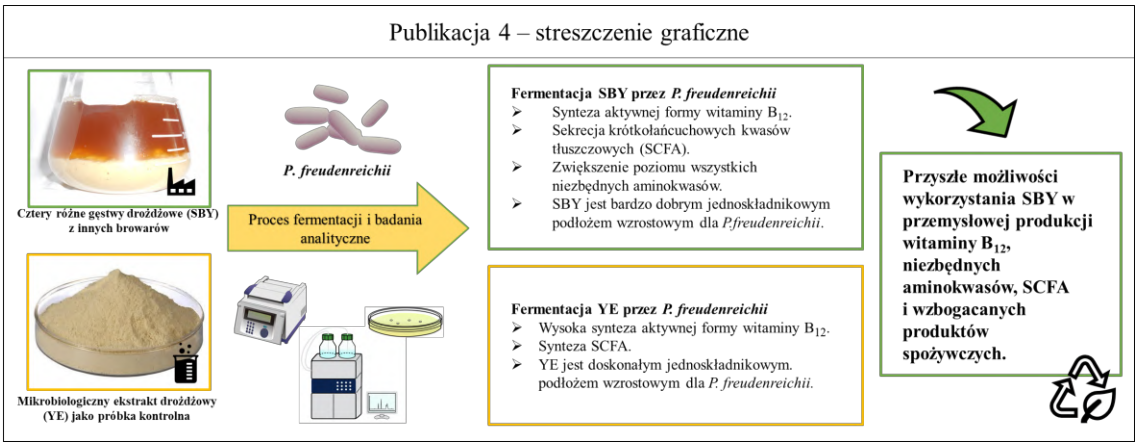
Kruk, M., Lalowski, P., Płecha, M., Ponder, A., Rudzka, A., Zielińska, D., & Trząskowska, M. (2025). Prebiotic potential of spent brewery grain – In vitro study. Food Chemistry, 463, 141254.



Rycina 11. (a, b, c, d, e, f) Zawartość SCFA, BCFA i kwasu mlekowego w modelu statycznym (L-ST) wyrażona w mmol/L (a) i udział procentowy (b) oraz zawartość w SHMIE (L-SH) w mmol/l (d) i udział procentowy (e) w różnych punktach czasowych eksperymentu; względna liczebność składu mikrobioty na poziomie rodzaju dla L-ST (c) i dla L-SH (f); „h" oznacza godziny, „d" oznacza dni; małe litery na wykresach oznaczają różnice statystyczne między próbkami w teście Tukeya po analizie ANOVA ($p < 0,05$), słupki błędów oznaczają odchylenia standardowe; n=3 (przy wynikach dotyczących SCFA i BCFA); n=1 (dla wyników z sekwencjonowania - 3 powtórzenia połączono w jedno i poddano sekwencjonowaniu).

4.4. Wzbogacanie odpadowej gęstwy drożdżowej w składniki odżywcze przez *Propionibacterium freudenreichii*

Abstrakt graficzny dotyczący badania na temat odpadowej gęstwy drożdżowej przedstawiono na Rycinie 12.



Rycina 12. Abstrakt graficzny odnoszący się do Publikacji 4.

Badanie miało na celu ocenę potencjału *Propionibacterium freudenreichii* do wzbogacania browarniczej odpadowej gęstwy drożdżowej poprzez wytwarzanie cennych metabolitów, przede wszystkim SCFA oraz witaminy B_{12}, a także analizę przydatności gęstwy drożdżowej jako podłoża hodowlanego dla tych bakterii. Eksperyment podzielono na trzy etapy. W pierwszym etapie scharakteryzowano skład chemiczny czterech próbek gęstwy drożdżowej pochodzących z różnych browarów i porównano go z próbą kontrolną, którą stanowił mikrobiologiczny ekstrakt drożdżowy (Tabela 3).

Tabela 3. Opis badanych odpadowych gęstw drożdżowych i próby kontrolnej

Skrót	Nazwa szczepu	Pochodzenie
SBY1	*Saccharomyces pastorianus* W34/70	Browar Ciechan
SBY2	*Sacharomyces cerevisiae* 3878	Browar Jabłonowo
SBY3	*Saccharomyces cerevisiae* US-05, *Saccharomyces cerevisiae* WLP066	Browar Palatum
SBY4	*Saccharomyces pastorianus* W34/70	Browar Perła
YE	Mikrobiologiczny ekstrakt drożdżowy	Gibco Thermofisher

Określono zawartość kluczowych pod względem badawczym związków chemicznych w próbkach gęstwy, w tym białka, cukrów (mono- i disacharydów), wolnych aminokwasów, kwasów organicznych, ryboflawiny, kobaltu i całkowitą zawartość polifenoli ogółem. Przed rozpoczęciem badań dotyczących fermentacji gęstw drożdżowych przez *P. freudenreichii*, materiał poddano ekstrakcji ultradźwiękowej oraz

filtracji próżniowej przez filtr o wielkości porów 0,22 μm w celu sterylizacji pożywki. Nie zdecydowano się na sterylizację termiczną uzyskanych ekstraktów z powodu na termolabilność niektórych składników odżywczych. W drugim etapie przeprowadzono fermentację *P. freudenreichii* na pożywkach przygotowanych z każdej z czterech próbek gęstwy drożdżowej oraz, dla porównania, na pożywce z ekstraktu drożdżowego. Fermentacje prowadzono przez 7 dni, wykorzystując dwa szczepy *P. freudenreichii* (DSM 4902 i DSM 20271). Monitorowano dynamikę wzrostu bakterii, zmiany wartości pH, oraz analizowano zmiany stężenia związków odżywczych (wolnych aminokwasów, zawartość cukrów oraz kwasów organicznych w tym SCFA). Celem etapu drugiego była ocena, w jakim stopniu poszczególne próbki gęstwy drożdżowej pozwalają na wzrost *P. freudenreichii*. W tym etapie również wybrano gęstwę drożdżową o najlepszych parametrach fermentacyjnych do ostatniego etapu. W etapie trzecim skupiono się na syntezie witaminy B_{12}. Przeprowadzono fermentację na wybranej próbce gęstwy drożdżowej w warunkach zoptymalizowanych pod kątem syntezy witaminy B_{12} (Deptula i in., 2017). Równolegle prowadzono fermentację kontrolną na pożywce YE. Po fermentacji oznaczono stężenie witaminy B_{12} (w formie cyjanokobalaminy) oraz dokonano pomiarów parametrów hodowli (pH, OD, liczba komórek, stężenia cukrów i kwasów organicznych) w celu identyfikacji czynników wpływających na biosyntezę B_{12}.

Wyniki pierwszego etapu wykazały, że badane próbki gęstwy drożdżowej miały zbliżony skład chemiczny między sobą, natomiast ekstrakt drożdżowy zawierał istotnie większe ilości analizowanych związków chemicznych (Tabela 4). Wszystkie próbki gęstwy drożdżowej zawierały cukry resztkowe, co tłumaczy się częściowym rozpadem cukrów zapasowych drożdży (trehalozy, glikogenu) podczas autolizy (François i Parrou, 2001). Kwas propionowy nie był obecny w żadnej próbce, ze względu na niezdolność drożdży browarniczych do jego syntezy (Gonzalez-Garcia i in., 2017; Ding i in., 2022). Istotne różnice odnotowano w zawartości ryboflawiny i kobaltu. Analiza całkowitej zawartości polifenoli wykazała znaczne zróżnicowanie między próbkami, związane było to prawdopodobnie z różnicami w procesie warzenia piwa i ilością użytego chmielu przy przygotowaniu brzeczki piwnej. Jest to istotne, ponieważ polifenole chmielowe są znane ze swojego działania antybakteryjnego (Bryant i Cohen, 2015). Profile wolnych aminokwasów w niefermentowanych próbkach gęstwy drożdżowej były podobne

względem siebie i zgodne z danymi literaturowymi (wyniki dotyczące składu aminokwasów są dostępne w P4).

Tabela 4. Zawartość wybranych związków chemicznych w badanym materiale

		SBY1	SBY2	SBY3	SBY4	YE
Białko	g/100g	$40,3^{a}$	$38,9^{ab}$	$38,6^{b}$	$31,3^{c}$	$64,6^{d}$
Disacharydy		$1,7^{a}$	$0,9^{b}$	$0,7^{b}$	$5,0^{c}$	$8,5^{d}$
Glukoza		$1,4^{a}$	$1,2^{a}$	$1,1^{a}$	$2,8^{b}$	$0,9^{a}$
Kw. bursztynowy		$0,2^{a}$	$0,2^{a}$	$0,3^{a}$	$0,1^{a}$	$1,6^{b}$
Kw. mlekowy		$1,5^{a}$	$1,9^{b}$	$2,7^{c}$	$2,4^{c}$	$2,1^{b}$
Kw. octowy		nd	nd	$0,3^{a}$	$0,3^{bc}$	$0,3^{c}$
Kw. propionowy.		nd	nd	nd	nd	nd
TPC	mg GAE/100g	$7,6^{a}$	$3,3^{b}$	$12,7^{c}$	$0,7^{d}$	$1,8^{e}$
Ryboflawina	mg/100g	$0,6^{a}$	$0,7^{a}$	$0,7^{a}$	$0,5^{a}$	$7,9^{b}$
Kobalt	µg/100g	8,8	8,3	14,2	9,2	127,8

Objaśnienia: litery a, b, c, d i e oznaczają różnicę statystyczną między próbkami w teście Tukeya post hoc ($p<0,05$); nd – nie wykryto; n=3; Co, n=1.

Fermentacja z *P. freudenreichii* spowodowała istotne zmiany w składzie analizowanych gęstw drożdżowych (Ryc. 13). We wszystkich próbkach gęstwy drożdżowej i próbie kontrolnej odnotowano zmniejszenie stężenia niektórych aminokwasów (np. asparaginy, glutaminy, GABA). Jednocześnie fermentacja doprowadziła do zwiększenia stężenia aminokwasów egzogennych dla człowieka. Wzrost zawartości po fermentacji wystąpił we wszystkich analizowanych próbkach gęstwy drożdżowej i próbce kontrolnej (z wyjątkiem treoniny w próbce kontrolnej). Ten efekt przypisuje się peptydazom i zdolnościom metabolicznym bakterii propionowych a także proteolitycznym enzymom drożdżowym, które zostały uwolnione z komórek drożdżowych z gęstw (Thierry i in., 2011; Aburjaile i in., 2016; Jacob i in., 2019).

Równolegle fermentacja z *P. freudenreichii* wpłynęła na przemiany cukrów i kwasów organicznych w analizowanym materiale (Ryc. 13). Bakterie propionowe wykorzystywały metabolicznie kwas bursztynowy i mlekowy we wszystkich próbkach. *P. freudenreichii* katabolizuje kwas mlekowy, przekształcając go w pirogronian, który wchodzi do cyklu Wooda-Werkmana (Thierry i in., 2011). Również kwas bursztynowy został włączony do tego szlaku metabolicznego (Feng i in., 2010). Konsekwencją aktywności *P. freudenreichii* był znaczny przyrost stężenia kwasu octowego i propionowego w pożywkach (Thierry i in., 2011). Najwyższą sekrecję tych kwasów

odnotowano w próbce SBY4 oraz w próbie kontrolnej (YE). We wszystkich próbkach gęstwy drożdżowej stwierdzono także wzrost stężenia wolnej glukozy po fermentacji, co jest nietypowe dla metabolizmu *P. freudenreichii*. Najprawdopodobniej glukoza pochodziła z hydrolizy cukrów zapasowych drożdży przez enzymy autolityczne (Rautio i Londesborough, 2003; Piddocke i in., 2009). Same bakterie propionowe nie dysponują pełnym zestawem enzymów do rozkładu sacharydów obecnych w gęstwie drożdżowej (trehalozy, maltozy czy oligosacharydów słodowych), choć potrafią metabolizować niektóre trisacharydy (np. maltotriozę) (Cardoso i in., 2007; Piwowarek i in., 2018). W pożywce kontrolnej *P. freudenreichii* całkowicie zdegradowało glukozę (jej stężenie spadło do poziomu poniżej progu detekcji), zaś ilość dwucukrów w YE praktycznie nie uległa zmianie. Wynika to z faktu że, jedynym dostępnym cukrem w YE była prawdopodobnie sacharoza, którą *P. freudenreichii* wykorzystuje w ograniczonym stopniu (Piwowarek i in., 2018). Drugi etap badania wykazał dodatkowo że *P. freudenreichii* jest w stanie zwiększać liczbę komórek w pożywce przygotowanej wyłącznie z gęstw drożdżowych. Najlepsze parametry wzrostu bakterii propionowych uzyskano na próbie SBY4 oraz na pożywce YE, natomiast wyraźnie słabsze w pozostałych (Ryc. 13). Wyniki te sugerują, że obecność polifenoli w podłożach z gęstw drożdżowych hamowała wzrost *P. freudenreichii*. W próbkach o najwyższej zawartości polifenoli uzyskano niższą liczbę bakterii i mniejszą ich aktywność metaboliczną. Natomiast, w próbce SBY4, która zawierała niskie stężenie polifenoli, bakterie wzrastały istotnie lepiej, co czyniło ją dobrym kandydatem do dalszych badań nad produkcją witaminy B_{12}.

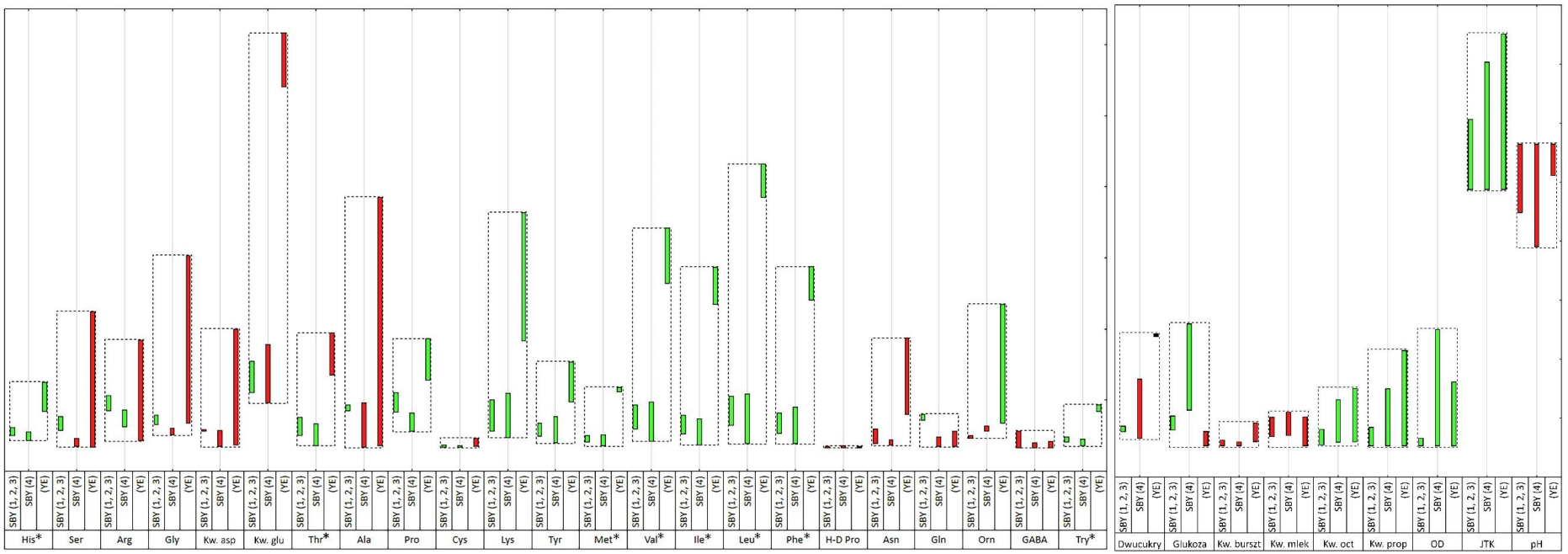
W trzecim etapie wykorzystano próbki SBY4 i YE do fermentacji ukierunkowaną na biosyntezę witaminy B_{12}. Zastosowano w tym celu zoptymalizowane warunki hodowli, znane z wcześniejszych badań jako sprzyjające wytwarzaniu kobalaminy przez *P. freudenreichii* (Deptula i in., 2017). Wyniki pokazały, że gęstwa drożdżowa może służyć jako podłoże umożliwiające produkcję biologicznie aktywnej formy witaminy B_{12}, choć wydajność tej syntezy była niższa niż w próbce kontrolnej. W próbie SBY4 po fermentacji stwierdzono stężenie witaminy B_{12} na poziomie ok. 25 ng/mL, podczas gdy w hodowli na pożywce YE bakterie wytworzyły aż ok. 200–215 ng/mL. Kontrolny ekstrakt drożdżowy okazał się około ośmiokrotnie lepszym medium pod kątem syntezy witaminy B_{12} niż gęstwa drożdżowa. Analiza parametrów hodowli i składu chemicznego rzuciła światło na czynniki ograniczające i promujące biosyntezę witaminy B_{12} w podłożu

z gęstwy drożdżowej (Ryc. 14). Po pierwsze, potwierdzono kluczowe znaczenie dostępności kobaltu i ryboflawiny. Po drugie, zaobserwowano niekorzystne obniżenie pH podczas fermentacji w SBY4 wskutek intensywnej produkcji kwasów organicznych. W trakcie fazy fermentacji *P. freudenreichii* szybko przetwarzało dostępne cukry (w SBY4 było ich stosunkowo dużo) do kwasu octowego i propionowego, powodując zakwaszenie środowiska do pH w przybliżeniu 4,5. Tak niska wartość niekorzystnie wpływa na syntezę witaminy B_{12} (Deptula i in., 2017). W przypadku próbki kontrolnej taki efekt zakwaszenia nie wystąpił, środowisko miało wyższą pojemność buforową (przez większą zawartość białek i peptydów) i zawierało mniej cukrów prostych, dzięki czemu pH pozostało w okolicach obojętnego, a nawet nieco wzrosło na skutek fermentacji (pH 8). Jednocześnie poprzez ograniczony dostęp do glukozy bakterie były w stanie wtórnie metabolizować powstały kwas propionowy i octowy jako źródło węgla (Dank i in., 2021, 2022). Te przemiany podwyższyły pH i stworzyły sprzyjające warunki do kontynuacji syntezy witaminy B_{12} w pożywce kontrolnej. Niższa synteza witaminy B_{12} w gęstwie drożdżowej wynikała ze złożonej kombinacji kilku czynników. Głównym jednak mechanizmem, który był kluczowy przy ograniczeniu syntezy w tym medium była wysoka zawartość glukozy, która poprzez jej metabolizm w komórkach bakteryjnych spowodowała wzmożone zakwaszenie środowiska (Ryc. 14). Pomimo tych ograniczeń, wynik analiz potwierdza zdolność *P. freudenreichii* do produkcji witaminy B_{12} w podłożu z odpadowej gęstwy drożdżowej i stanowi punkt odniesienia do dalszych badań.

Podsumowując, uzyskane wyniki potwierdzają duży potencjał waloryzacji odpadowych gęstw drożdżowych przy udziale *P. freudenreichii*. Gęstwy drożdżowe po fermentacji *P. freudenreichii* charakteryzowały się podwyższoną zawartością wolnych aminokwasów egzogennych, obecnością korzystnych dla zdrowia SCFA, a także zawartością aktywnej formy witaminy B_{12}. Co więcej, kontrolny ekstrakt drożdżowy może służyć jako jednoskładnikowe i wydajne podłoże hodowlane dla *P. freudenreichii*.

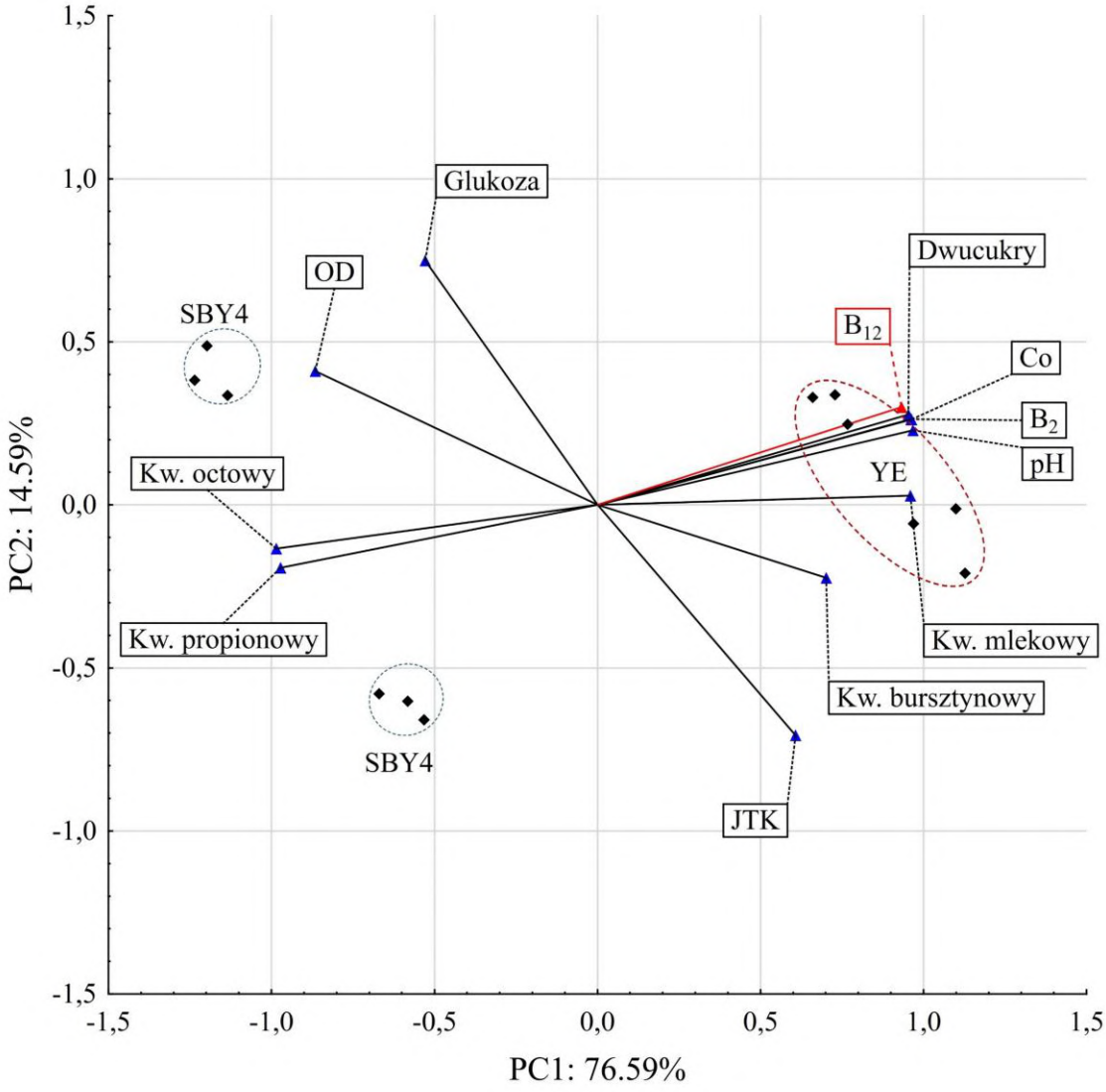
Szczegółowy opis badań jest dostępny w P4.

Kruk, M., Varmanen, P., Edelmann, M., Chamlagain, B., & Trząskowska, M. (2024). Food by-product valorisation in nutrients through spent brewer's yeast bioprocessing with Propionibacterium freudenreichii. Journal of Cleaner Production, 434, 140102.



Rycina 13. Wykresy zmienności badanych związków chemicznych i parametrów w wyniku fermentacji *P. freudenreichii* oddzielnie dla próbek SBY4, YE i wspólnie dla próbek SBY (1, 2, 3) odnoszący się do drugiego etapu badań.

Objaśnienia: Każdy słupek na wykresie wskazuje zmienność analizowanego parametru lub związku chemicznego. Im dłuższy słupek, tym większa zmienność względem wartości początkowej oraz im większy dystans między słupkami tym większa różnica pomiędzy wartościami w obrębie próbek. Położenie słupka na wykresie wskazuje względną zawartość lub poziom danego parametru w badanych materiałach. Jeśli słupek jest zielony, oznacza to, że zawartość badanej substancji lub wartość parametru wzrosła istotnie po okresie fermentacji. Czarny słupek oznacza, że nie nastąpiła istotna zmiana, a czerwony słupek oznacza, że wartość istotnie zmniejszyła się. Dane użyte do przygotowania wykresu zostały znormalizowane. Skala obowiązująca na wykresie jest niezdefiniowana. * - aminokwasy egzogenne; kw. burszt – kwas bursztynowy; kw. mlek – kwas mlekowy, istotność statystyczna na poziomie $p<0,05$ w analizie ANOVA z testem Tukeya.



Rycina 14. Analiza głównych składowych, wybranych związków chemicznych i badanych parametrów wpływających na syntezę witaminy B_{12} w próbkach SBY4 i YE Objaśnienia: projekcja zmiennych (analizowanych związków chemicznych i parametrów) oraz przypadków (badanych próbek przedstawionych jako okręgi) na płaszczyznę głównych składników (PC1 i PC2).

5. Wnioski końcowe

Zrealizowane badania w ramach niniejszej pracy potwierdziły, że produkty uboczne przemysłu spożywczego, takie jak młóto browarniane, odpadowa gęstwa drożdżowa, skórki orzechów laskowych i wytłoki jabłkowe, posiadają istotny potencjał jako źródła składników bioaktywnych. Analizy ich składu chemicznego wskazują na możliwości wtórnego wykorzystania produktów ubocznych w biotechnologii, przemyśle spożywczym i żywieniu człowieka. Zastosowane podejścia analityczne umożliwiły ocenę zawartości związków bioaktywnych i ich właściwości. Uzyskane wyniki badań wskazały również na możliwości praktycznego wdrożenia tych surowców jako dodatków funkcjonalnych żywności, czynnika prebiotycznego i substratów fermentacyjnych do syntezy substancji odżywczych. W każdym z analizowanych przypadków potwierdzono możliwość nadania produktom ubocznym wartości dodanej, zarówno przez wykorzystanie ich naturalnego składu, jak i poprzez ich modyfikację przy udziale mikroorganizmów.

Pomimo uzyskanych obiecujących wyników, każde z przeprowadzonych badań posiadało istotne ograniczenia, które należy uwzględnić przy planowaniu dalszych analiz. W przypadku produktów z dodatkiem wytłoków jabłkowych ograniczeniem był brak oceny ich wpływu na mikrobiotę jelitową, nawet w warunkach *in vitro*, co uniemożliwia jednoznaczne określenie ich potencjału prebiotycznego. Dodatkowo, konieczne jest przeprowadzenie pogłębionej analizy chemicznej wytłoków jabłkowych, ze szczególnym uwzględnieniem struktury molekularnej błonnika. W badaniu dotyczącym okryw nasiennych orzechów laskowych, wskazuje się na potrzebę dalszej oceny ich właściwości sensorycznych w różnych matrycach żywnościowych, a także konieczność pełnej charakterystyki alergenności ekstraktów. Ograniczenia badania nad młótem browarnianym dotyczyły przede wszystkim zastosowania jednego typu surowca oraz relatywnie krótkiego czasu suplementacji w modelu SHIME, przez co uzyskane wyniki nie odzwierciedlają długofalowych efektów modulacji mikrobioty jelitowej pod wpływem tego surowca. W badaniu dotyczącym odpadowej gęstwy drożdżowej i jej fermentacji z udziałem *P. freudenreichii*, ograniczenie związane było z wykorzystaniem tylko jednego rodzaju gęstwy do etapu badań powiązanych z biosyntezą witaminy B_{12}. W celu uzyskania pełniejszego obrazu, niezbędne jest porównanie różnych typów gęstwy drożdżowej oraz dalsza optymalizacja warunków fermentacji. Dodatkowo w przypadku

odpadowej gęstwy drożdżowej zauważono że nie wszystkie rodzaje gęstwy drożdżowej posiadają właściwości klasyfikujące je do wtórnego zagospodarowania w aspekcie omawianym w pracy. Wysoka zawartość związków polifenolowych pochodzenia chmielowego jest czynnikiem ograniczającym wzrost *P. freudenreichii* i musi być rozpatrywana przy klasyfikacji użyteczności tego produktu ubocznego.

Zoptymalizowane zagospodarowanie produktów ubocznych może obniżyć skalę strat żywności. Ponadto, umiejętne zagospodarowanie produktów ubocznych przemysłu spożywczego może przyczynić się do rozwoju żywności ukierunkowanej na szczególne wspieranie zdrowia człowieka. Istotne jest przy tym indywidualne podejście do każdego surowca, uwzględniające jego skład chemiczny, właściwości sensoryczne i możliwe ryzyko zdrowotne wynikające ze specyfiki surowca. W świetle przeprowadzonych badań można stwierdzić, że zagospodarowanie produktów ubocznych przemysłu spożywczego stanowi realny kierunek rozwoju dyscypliny technologii żywności i żywienia, integrujący podejścia środowiskowe, zdrowotne i ekonomiczne. Wyniki badań uzyskane w ramach tej rozprawy doktorskiej otwierają nowe perspektywy badawcze, zwłaszcza w obszarze badań *in vivo* w celu potwierdzenia fizjologicznego działania związków chemicznych wchodzących w skład produktów ubocznych przemysłu spożywczego.

Podsumowując, wyżej opisane wyniki badań własnych pozwalają pozytywnie zweryfikować hipotezę, że produkty uboczne z przetwórstwa spożywczego posiadają właściwości klasyfikujące je do dalszego wykorzystania w przemyśle spożywczym i żywieniu człowieka, a także mogą być wykorzystywane w celu podnoszenia wartości zdrowotnej żywności.

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7. Kopie artykułów naukowych wchodzących w skład rozprawy doktorskiej

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RESEARCH

Probiotic Bacteria Survival and Shelf Life of High Fibre Plant Snack - Model Study

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Abstract

The study aimed to develop plant-based model snacks that are high in fibre, contain probiotic bacteria and are convenient for long-term storage. The research focused on selecting a suitable form of probiotic bacteria (active biomass, microencapsulated, freeze-dried), inoculation method (in the base mass or in the filling of a snack) and appropriate storage conditions (4°Cor 20 °C). The potential synbiotic properties were evaluated. The microencapsulated bacteria had the highest survival rate at 4 °C, while the freeze-dried bacteria showed better survival rates at 20 °C. Probiotics had a higher survival rate when enclosed inside snacks with a low water activity ($a_w = 0.27$) peanut butter filling than in snacks without filling ($a_w = 0.53$). Enclosing the probiotics in a low a_w filling ensures their survival at ambient temperature for 5 months at a count higher than 6 log CFU/g. The snacks exhibited high antioxidant capacity (average 300 mg ascorbic acid equivalent/100 g), polyphenol content (average 357 mg gallic acid equivalent/100 g) and high fibre content (average 10.2 g/100 g). The sensory analysis showed a high overall quality of the snacks (average 7.1/10 of the conventional units). Furthermore, after six months of storage, significant changes were observed in the antioxidant properties, polyphenol content and texture of the snacks, while their sensory quality remained unchanged. Moreover, a potential synbiotic effect was observed. The method used to assess bacterial growth indicated significantly higher values in the model snacks compared to a control sample. Therefore, this study has effectively addressed the gap in knowledge regarding the survival of probiotics in snacks of this nature.

Keywords Probiotic bacteria · No-dairy products · Antioxidants · bacteria survival · By-products

Abbreviations

ABTS	diammonium 2,2′-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate] radical
CFU	colony formation units
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
F-C	Folin–Ciocalteu
GAE	gallic acid equivalent
PBS	phosphate-buffered saline
PCA	principal components analysis
QDP	Quantitative Descriptive Profiling
TPC	total polyphenol content
VCEAC	ascorbic acid equivalent

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Introduction

Modern life often exposes individuals to significant stress, leading to increased consumption of fast food and low-nutrient sweet snacks. A well-balanced diet is a critical determinant of health [1]. Fibre, an integral component of a balanced diet, significantly impacts the beneficial microbial fractions in the gut microbiota, which in turn affects the well-being of the host [2]. It is imperative to ensure the effective delivery of fibre and beneficial bacteria to the diet for the maintenance of homeostasis in the digestive system [1, 3]. Therefore, combining fibre and probiotic bacteria as a synbiotic is important to ensure appropriate modulation of the host gut microbiota and the development of probiotic bacteria supplied with food [2]. Fermented dairy products are a primary source of beneficial lactic acid bacteria [4]. However, the consumption of these products is limited because of dietary restrictions prohibiting dairy, short shelf life of products, or specific sensory characteristics that do not meet the sensory expectations of users [5]. Thus, there is a need to diversify the market for probiotic products that

do not contain the above exclusions, provide dietary fibre, and have high consumer acceptance [6].

According to scientific literature, selecting the appropriate probiotic bacteria and fibre sources is critical when formulating synbiotic products. Factors in probiotic strain selection include storage temperature, form and method of bacterial inoculation, and viability of the probiotic strain in the final product [7]. In addition, introducing different sources of fibre into potentially synbiotic snacks is important. Mixing fibre fractions from various raw materials has been proven to increase the positive effect on the probiotic bacteria [8]. Therefore, to ensure proper differentiation of the prebiotic fibre fractions, authors decided to use various raw materials in this study such as dates, oat fibre, peanut butter and apple pomace. As an additional benefit, apple pomace is a waste material from the juice pressing industry and its waste level is still concerning because 25–30% is used as animal feed or fertilizer [9]. Incorporating apple pomace into synbiotic snack development could help reduce waste and have a positive environmental effect [10]. Moreover, snacks containing probiotics with a long shelf life have recently appeared on the market. However, there is a lack of scientific literature on the survival of these bacteria in such products and the methods for maintaining high bacterial counts in the finished product.

Consequently, the research aimed to create non-dairy snacks rich in fibre, fortified with probiotics, and enhanced with apple pomace. The investigation centred on three main aspects: (1) identifying the most suitable form of probiotic bacteria (whether biomass, freeze-dried, or microcapsules), (2) determining the optimal method for inoculation of the probiotics into the product, and (3) assessing the feasibility of storing the snacks under ambient conditions. The analysis encompassed evaluations of microbiological, sensory, physical, and chemical attributes. Furthermore, the study explored the potential synbiotic properties of the samples.

Materials and Methods

This section is presented in the supplementary materials.

Results and Discussion

In terms of basic chemical composition, two types of samples were analysed: samples with filling (C-F) and samples without filling (C-W). The contents of total fat, total fibre and its fraction are shown in Table 1. The total fat content was significantly higher in the C-F sample ($p < 0.05$). The fibre content was higher in the C-W sample due to the lower amount of fat in the composition ($p < 0.05$). Literature and reference data on fat and fibre content in dates, apples, peanut butter and apple pomace are similar to the results obtained [11, 12]. According to the manufacturer, the oat fibre used for the experiment contained 19 g /100 g of fibre, including 9 g /100 g of β-glucans.

Table 1 Total fat and fibre fraction content in tested samples ($n = 3$)

	C-W	C-F
	g/100 g	
Total fat	$4.5^{a} \pm 0.1$	$11.5^{b} \pm 0.2$
Insoluble fibre	$8.7^{a} \pm 0.0$	$8.4^{b} \pm 0.1$
Soluble fibre	$2.0^{a} \pm 0.0$	$1.4^{b} \pm 0.2$
Total fibre	$10.6^{a} \pm 0.1$	$9.8^{b} \pm 0.1$

Explanations: C-F- filled samples, C-W - samples without filling, letters a, and b show a statistical difference in the t-test ($p < 0{,}05$); descriptions of samples abbreviations are in the supplementary materials (Table S1); the value after the ± sign indicates the standard deviation, the statistical differences refer to compounds separately.

The total viable count of bacteria, moulds and yeasts was below 10 CFU/g in all tested samples. These results are presented in Supplementary Table S4. The micro-organisms were probably inactivated during the pasteurisation process of the raw materials.

The results of probiotic bacterial survival in model snacks are shown in Fig. 1a. During the 6 months of storage, the survival rate was similar in all variants stored under refrigerated conditions (4 °C). A significant decrease in probiotic survival was observed in samples stored at 20 °C ($p < 0.05$). After 2 months (20 °C), no probiotic bacteria were detected in the samples without peanut butter filling, regardless of the form in which they were added, i.e. biomass, freeze-dried or microcapsules. Probiotics survived better when peanut butter was used as a filling ($p < 0.05$). The initial water activity of the snack without filling was 0.53 ± 0.02 (Fig. 1b). Meanwhile, in the filled samples, the peanut butter enclosing the bacteria had a starting water activity of 0.27 ± 0.01. During storage, the water activity value of the samples without filling and filling from peanut butter did not change significantly regarding time and temperature ($p > 0.05$). Location bacteria in the peanut butter filling inside the snacks resulted in the viability above 6 log CFU/g after 4 months of storage (20 °C) in the samples M-F-20, M-W-20 and B-F-20. Nevertheless, after 6 months of storage, the number of probiotics decreased below the acceptable limit of 6 log CFU/g. Statistical analysis revealed that the time, storage temperature and type of sample were significant factors influencing the number of bacteria in the products ($p < 0.05$). The storage temperature had the greatest effect on reducing the survival rate of bacteria in the samples. Moreover, the sample type was an equally important factor influencing the survival of bacteria in products stored at ambient temperature ($p < 0.05$). After 6 months of storage in the samples, the bacteria were still alive, and the genetic sequencing exhibited its belonging

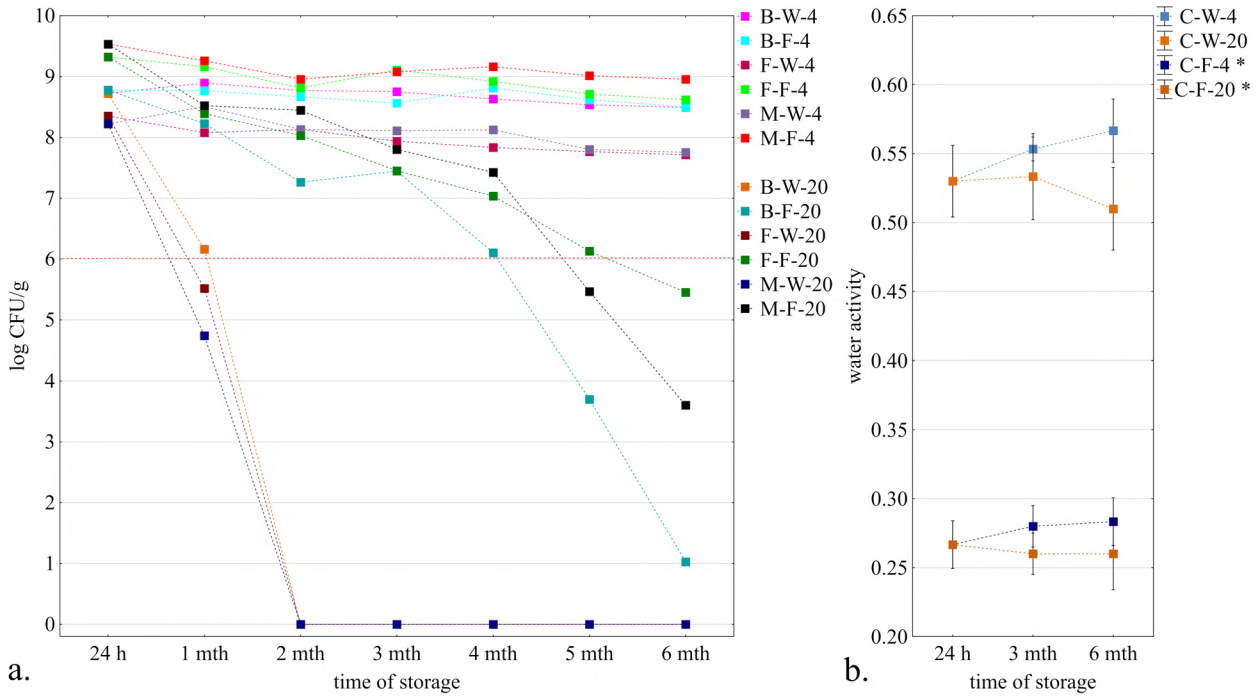

Fig. 1 **a**, **b** Survival of bacteria in samples (**a**) and the water activity of the snacks and peanut butter filling (**b**) during storage; descriptions of samples abbreviations are in the supplementary materials (Table S1); * - water activity for peanut butter filling inside the sample, the water activity value for the date mass base did not differ significantly from the C-W sample; ($n=3$); the red dashed line indicates 6 log CFU/g of the product; error bars indicate the standard deviation; h - hours; mth – month

to the species *L. rhamnosus* from all tested samples (supplementary materials - Table S3). This proves the lack of cross-contamination with other bacteria during storage. The literature indicates that storage temperature directly affects the rate of biochemical and molecular reactions in bacterial cell ageing. The higher the temperature, the more destructive the effect on cells [13]. For this reason, the reduction in bacteria population was greater in samples stored at 20 °C than at 4 °C. Furthermore, variations in bacterial survival were observed depending on the form of bacteria present in a peanut butter-filled snack stored at ambient temperature. It has been reported that spray-drying microencapsulation can damage bacterial cell membranes, ribosomes and regulatory proteins, thereby reducing their viability. In contrast, freeze-drying does not have such a detrimental effect on the cells, allowing them to remain viable [14]. Water activity also affects the viability of bacterial cells. Bacteria in the high range (1.0–0.9) show good metabolic activity, and nutrient transfer and excretion activities through a cytoplasmic membrane and cell wall function properly [15]. At lower water activities (0.85 to 0.35), inactivation and cessation of reproduction of microorganisms occurs [15]. This phenomenon was observed in the samples stored at 20 °C without peanut butter filling ($a_w=0.57$), where all bacteria were inactivated after two months of storage. When water activity is less than 0.30, bacterial cells are dormant and metabolic activity is almost inhibited [16]. This property is used in food and industry to prolong the storage of bacteria, e.g. in freeze-dried form [16]. Peanut butter has low water activity due to its high fat and low moisture content [17]. A combination of low temperature and low water activity allows the long-term survival of microorganisms [17]. This mechanism was also observed in samples where probiotics were placed inside the peanut butter filling ($a_w=0.27$). Therefore, the success of our study is the high survival of the probiotic in products with peanut butter filling stored, especially at room temperature.

Regarding the findings on the potential effect of synbiotic snacks (Fig. 2). The consumption of synbiotics (probiotics + prebiotics) has a positive effect on homeostasis of human microbiome and it is essential to include them in the daily diet [2]. To demonstrate the synbiotic potential of a food, it must contain probiotics and prebiotics that stimulate their growth [2]. In this study, the analysis of dietary fibre content was divided into soluble and insoluble fractions (Fig. 2b). To observe the influence of these fractions on the change in the growth number of *L. rhamnosus* ATCC 53103, a 60-hour incubation was applied. This was because the fibre prebiotic fraction is catabolized by bacteria after the reduction of easily metabolised molecules such as monosaccharides or disaccharides [18]. The phenomenon of prolonged bacterial growth was observed in the samples with oat fibre (P2),

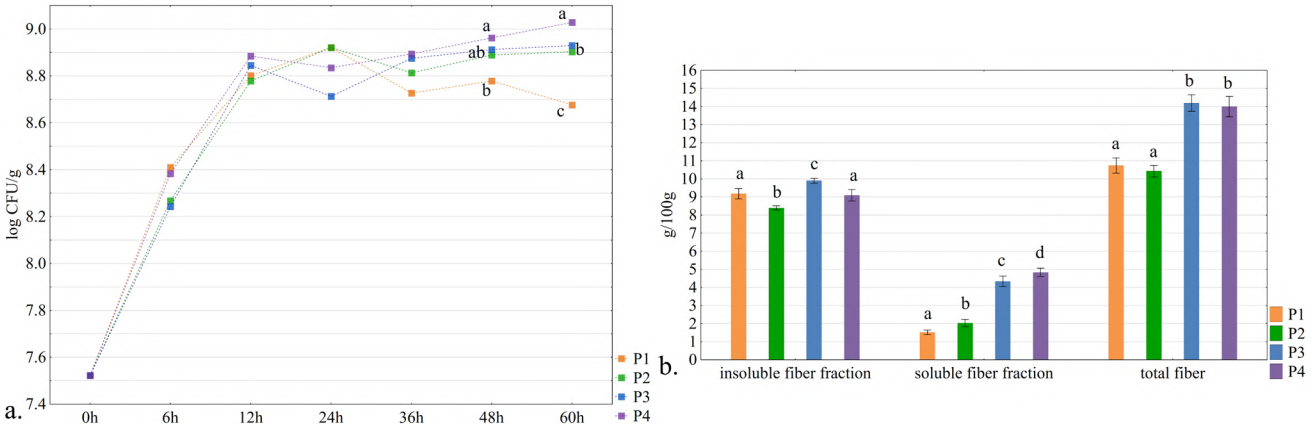

Fig. 2 **a**, **b** Growth curves of *L. rhamnosus* ATCC 53103 in extracts from snack samples with various additional ingredients as a source of prebiotic substances (**a**) and the fibre content in tested samples divided into soluble and insoluble fractions (**b**); descriptions of samples abbreviations are in the supplementary materials (Table S2); ($n = 3$); letters a, b and c mean the statistical difference between the samples in the post-hoc Tukey's test ($p < 0{,}05$), a. statistical differences relate to individual time points; b. the statistical differences refer to the fibre's fractions separately; error bars indicate the standard deviation; h – hours

apple pomace (P3) or both (P4), where the probiotic bacteria grew better than in the control sample (P1) (Fig. 2a). This result proves the presence of polysaccharides fermented after monosaccharides and other more easily catabolized energetic molecules. According to the literature, after metabolising simple energy sources probiotic bacteria initiate the enzymatic degradation of prebiotic substances [19]. Oat fibre and apple pomace are well-studied sources of prebiotic or potentially prebiotic fibre fractions. The prebiotic substance in oats is the β-glucan fraction, which directly stimulates the growth of probiotic bacteria [20]. Apple pomace contains potential prebiotic pectins with different molecular structures [21]. It was observed that mixing the prebiotic substances resulted in a higher stimulation of the growth of probiotic bacteria [8]. The combination of fibre fractions from two different sources prolonged the stationary (P2, P3) or logarithmic (P4) phase in the culture of probiotic bacteria.

The results of TPC and antioxidant activity are shown in Fig. 3. TPC and antioxidant activity were higher in the samples without peanut butter filling. Probiotics addition to the snacks did not significantly affect the TPC and antioxidant capacity ($p > 0.05$). However, the sample with the added biomass (B-W) had higher antioxidant properties in the DPPH evaluation than the other products ($p < 0.05$). The samples with a peanut butter filling and probiotics differed from those without filling and had lower TPC and antioxidant activity ($p < 0.05$). Storage time and temperature significantly decreased the polyphenol content and antioxidant capacity. The decrease of TPC and antioxidants in samples stored at 4 °C was nonsignificant ($p > 0.05$). The changes in the content of the compounds analysed were greater in samples stored at 20 °C ($p < 0.05$). Probiotic bacteria did not preserve polyphenols and other antioxidants in the samples ($p > 0.05$). The content of antioxidant compounds in peanut

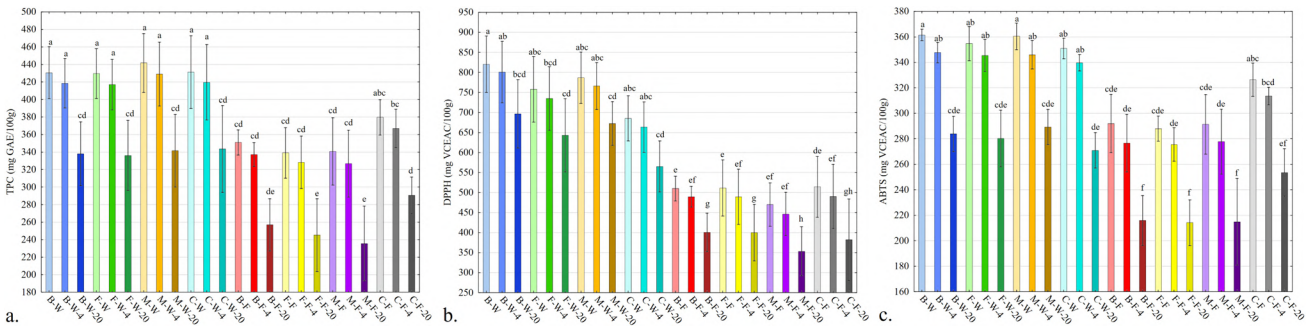

Fig. 3 **a**, **b**, **c** Total polyphenol content (TPC) (**a**), and antioxidant activity expressed by scavenging radicals, DPPH (**b**), ABTS (**c**) of the tested snacks samples; descriptions of samples abbreviations are in the supplementary materials (Table S1); ($n = 4$); TPC results are expressed as gallic acid equivalent (GAE)**;** ABTS and DPPH results are expressed as ascorbic acid equivalent (VCEAC); error bars indicate the standard deviation; letters a, b, and c mean the statistical difference between the samples in the post-hoc Tukey's test ($p < 0.05$)

butter is lower than in dates and apple pomace. For this reason, the introduction of an extra dose of peanut butter in the filled samples decreased the content of polyphenols and antioxidant compounds. The high lipid and low polyphenol content effectively decreased the TPC and antioxidant capacity of the snacks with an additional portion of peanut butter filling [22, 23]. Concerning the scavenging of ABTS and DPPH radicals, different values (Fig. 3) were obtained in these methods. However, the difference between the methods was also found in other works and is caused by different reactions generated by ABTS and DPPH and differences in their sensitivity to other types of antioxidants [24]. Bacteria in freeze-dried or encapsulated form have extremely limited metabolism. As a result, the production of substances with antioxidant properties is virtually inhibited. This phenomenon is caused by depriving bacterial cells of access to water, which regulates enzyme activity, protein synthesis and cell reproduction [25]. Also, the probiotic carriers like maltodextrin, cellulose and sodium alginate during the freeze-drying and microencapsulation process are not substances with antioxidant properties, so they will not enhance these properties [26]. In the B-W sample (samples without filling), highly active metabolic biomass of probiotic bacteria was introduced into a stressful environment with a destructive water activity effect (aw = 0.53). Subsequently, the intracellular substances, including antioxidant molecules, were probably released from the bacterial cells into the environment. The water activity (aw = 0.53) indicated cellular stress and damage to the bacterial cell. It probably changes the permeability and integrity of the cell membrane due to the osmotic stress effect [27]. As a result, intracellular metabolites leaked out of the cell, causing the higher antioxidant capacity of the B-W sample. Probably, this phenomenon did not occur in the sample with peanut butter filling and bacterial biomass (B-F). It was due to the low water activity of peanut butter (aw = 0.27), which supports cell protection against osmotic and other environmental stresses. The mechanism of water activity for bacterial cells was discussed in detail in section 3.2. The changes in TPC and antioxidant capacity of the tested samples during 6 months of storage were typical for food products [28]. The higher the temperature, the greater the decrease in the value of polyphenols and other antioxidants in the treated materials [28]. It has been suggested in the literature that higher temperatures during storage accelerate the radical and oxidative degradation of compounds in food matrices, whereas low temperatures slow down the reactions [29]. This mechanism was observed in the present study.

Texture measurement results are shown in Table 2. Storage time significantly increased the cutting force and decreased the degree of deformation ($p < 0.05$). Storage temperature affected cutting force and deformation between C-W-20 and C-W-4 samples ($p < 0.05$). All the values obtained were higher in the samples without filling (C-W). The peanut butter filling caused a noticeable softening of the products. However, after 6 months all samples became harder than at the beginning. The low temperature limited water evaporation and allowed slower changes in hardness. In contrast, a temperature of around 20 °C favours water vaporisation and hardening of the samples [30]. The observed changes in texture are typical for the ageing of foods from this area [30, 31]. The changes in textural parameters agree with the sensory analysis results, where an increase in sample hardness was observed after storage (Table 3 and Fig. 4a, b).

Regarding the sensory study, the QDP analysis of the samples without peanut butter filling (C-W, Fig. 4a) shows that the tested material changed significantly in colour intensity, gloss, softness and stickiness during 6 months of storage at 4 °C. Storing at 20 °C changed the qualitative profile of the samples and reduced the sensory quality ($p < 0.05$). This was mainly due to the greater decrease in gloss and softness of the samples stored at 20 °C. The sensory quality of stored C-W samples was similar during 3 months, regardless of storage conditions. The same tendency was observed

Table 2 Texture measurements results of cutting force and deformation ($n = 3$)

Sample	Storage time	Cutting force [N]	Deformation [mm]
C-W	0 mth	$13.5^{a} \pm 0.9$	$24.6^{a} \pm 0.4$
C-W-20	6 mth	$63.3^{b} \pm 2.8$	$9.5^{b} \pm 0.9$
C-W-4		$52.5^{c} \pm 1.5$	$7.6^{c} \pm 1.5$
C-F	0 mth	$8.5^{d} \pm 0.1$	$10.2^{b} \pm 0.4$
C-F-20	6 mth	$56.4^{c} \pm 0.7$	$7.4^{c} \pm 0.5$
C-F-4		$49.7^{c} \pm 1.3$	$7.0^{c} \pm 1.3$

Explanations: descriptions of samples abbreviations are in the supplementary materials (Table S1); letters a, b, c, and d mean the statistical difference between the samples in the post-hoc Tukey's test ($p < 0.05$); the value after the ± sign indicates the standard deviation; mth – month.

Table 3 Consumer acceptance of samples ($n = 40$)

	C-W	C-F
	9-point hedonic scale (1–9)	
Appearance	$5.7^{a} \pm 1.6$	$5.8^{a} \pm 1.6$
Consistency	$6.6^{a} \pm 1.5$	$7.1^{a} \pm 1.2$
Flavour	$6.4^{b} \pm 1.4$	$7.5^{a} \pm 1.4$
Overall liking	$6.5^{b} \pm 1.3$	$7.3^{a} \pm 1.2$

Explanations: C-W - samples without filling, C-F- filled samples, letters a, and b show a statistical difference in the t-test ($p < 0.05$); full descriptions of samples abbreviations are provided in the supplemental materials (Table S1); the value after the ± sign indicates the standard deviation, the statistical differences refer to sensory attributes separately.

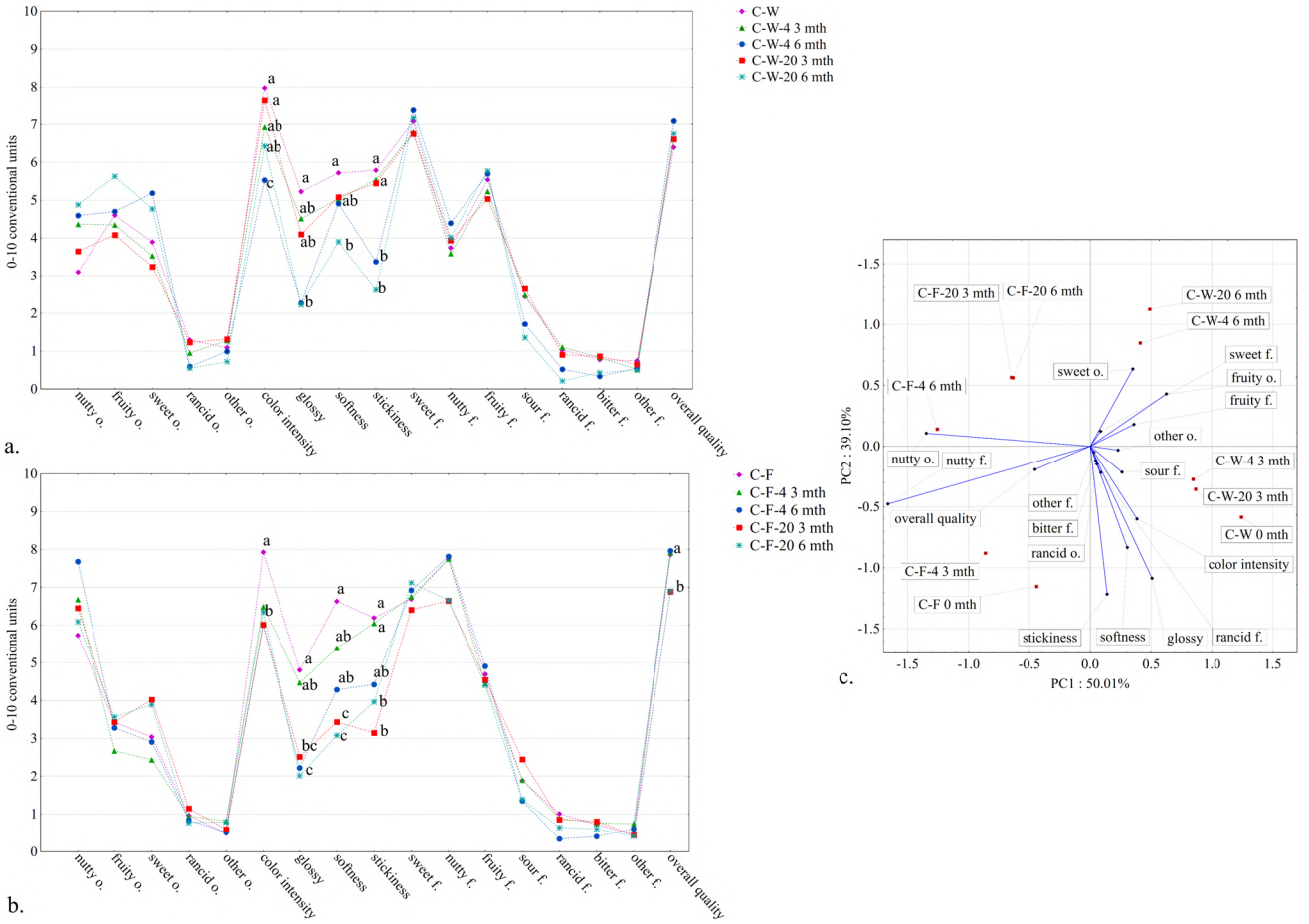

Fig. 4 **a**, **b**, **c** Sensory profiles of the C-W (control sample without filling) (**a**) and C-F (control sample with the peanut butter filling) (**b**), after production ($n = 16$), after 3 and 6-months storage and, PCA of variables (sensory discriminants) and cases (tested samples) onto the plane of the principal components (PC1 and PC2) sensory discriminants and cases (tested samples) onto the plane of the principal components (PC1 and PC2) (**c**); full descriptions of samples abbreviations are provided in the supplemental materials (Table S1); letters a, b, and c mean the statistical difference between the samples in the post-hoc Tukey's test ($p < 0{,}05$), statistical differences relate to individual sensory discriminants; o.: odour; f.: flavour; mth – month

for the peanut-filled samples (C-F). Storage for 6 months at 4 °C resulted in fewer changes in the sensory profile of C-F samples (Fig. 4b) than C-W samples. The PCA analysis (Fig. 4c) showed that the overall quality of the snack was strongly and positively correlated with the nutty smell and taste. Softness was the most important attribute characterising freshness. In the PCA plot, the placement of filled snacks stored at 4 °C for 6 months indicates high sensory quality (close to the "overall quality" vector) (Fig. 4c). Furthermore, the closer position of C-F samples to the overall quality vector compared to C-W samples indicates their higher quality.

The consumer acceptance results (Table 3) show that the stuffed samples (C-F) were characterised by higher overall liking ($p < 0.05$) than the unstuffed samples (C-W). This resulted from a higher liking of the flavour ($p < 0.05$). Liking of appearance was at a similar level and not statistically different. There was no significant difference in consistency liking. As consumer demands and preferences are constantly changing, the sensory quality of food products plays a crucial role in measuring consumer response. A high sensory quality of food is one of the most important characteristics that determine the success of the product in the market and the consumer's liking of it [32]. The mean value of more than 7 points (on a points scale from 1 to 9) obtained for the samples with fillings can predict the acceptance of the food products. In this study, the addition of peanut butter filling had a direct effect on the liking and sensory quality of the product. Sithole et al. [33] found that peanut butter positively affected the textural properties, odour, taste and overall quality of the food. Moreover, the peanut butter filling varied the texture of the snacks. It is defined that the complex texture of food products increases their overall sensory quality by intensifying the sensations resulting from consumption [34]. Adding

peanut butter also increased the fat content of the snacks, which improved the flavour [35]. The higher consumer ratings of the product with a peanut butter filling and no major sensory changes during storage (QDP) may facilitate the easy introduction of the snack to the market. The filling improved sensory quality and allowed the product to be stored at room temperature for five months while still containing the recommended number of probiotic bacteria.

Conclusion

The results demonstrated the possibility of developing a plant snack with high fibre content and probiotic bacteria. The findings suggest that the freeze-dried form of probiotic bacteria is optimal. However, their viability was highest when introduced into a snack with low water activity ($a_w = 0.27$). Storage conditions were identified as critical, with bacteria surviving at room temperature for up to five months at levels exceeding 6 log CFU/g. This required the probiotics to be in a low water activity environment and freeze-dried form; otherwise, survival rates diminished. Notably, snacks exhibited a high antioxidant content, dietary fibre, and superior sensory quality, with robust sensory including textural stability. Moreover, a positive probiotic growth response was observed in a model assessing growth stimulant presence, suggesting potential synbiotic properties.

Whilst yielding valuable insights, the study faces several limitations requiring future attention. Future investigations should delve into modulating intestinal microbiota through human studies and advanced in vitro models. Moreover, research is needed to assess levels of peroxides and other chemical compounds formed during storage, alongside exploring alternative probiotic strains with potentially higher survival rates. However, this study has effectively addressed the gap in knowledge regarding the survival of probiotics in snacks of this nature. Its findings hold potential for straightforward implementation within food processing.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11130-024-01196-5.

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Data Availability Data will be made available upon request to the corresponding author.

Declarations

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

Ethical Approval Number 11/2022 of the resolution of the ethics committee for research involving human subjects at the Institute of Human Nutrition Sciences of the Warsaw University of Life Sciences.

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By-product hazelnut seed skin characteristics and properties in terms of use in food processing and human nutrition

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The hazelnut seed skins (HSS) are by-products from roasting or blanching hazelnuts without direct second utilization. The generation of HSS creates an economic and environmental problem. The object of the study was a comprehensive analysis of the properties for reuse of HSS. Water extraction of industrial HSS was applied (water with sonication of the HSS for 10 min at 90 ℃). The extracts obtained were freeze-dried to facilitate analysis and future application. The HSS and their extracts were analysed. Polyphenols, antioxidants, allergens, antimicrobial properties and instrumental sensory analysis were examined. The total polyphenol content in the samples was 37.8–44.0 mg gallic acid equivalent g^{-1}. Gallic acid was the major phenolic compound. The antioxidant capacity of the samples was 198.9–250.6 mg VCEAC g^{-1} (vitamin C equivalent) according to the ABTS method and 98.4–106.8 mg VCEAC g^{-1} in the DPPH method. The extracts inhibited all tested strains of pathogenic bacteria. Allergen content was reduced in HSS and the extracts. Instrumental sensory analysis showed differences between taste parameters and odour profile samples. HSS can be reused in food production as a bacteriostatic, antioxidant additive and sensory-creating factor due to various chemical compounds corresponding with taste and odour.

Keywords Residues, Polyphenols, Antimicrobial properties, Allergens, Instrumental sensory analysis, Bioactive compounds

The seed skins are a by-product generated by roasting or blanching hazelnuts. The hazelnut seed skins (HSS) are material which does not have direct second utilization[1]. Hazelnuts (*Corylus avellana L.*) produce approximately 1 million tons of shelled seeds[2]. HSS are a by-product of the confectionery industry. They are generated by roasting or blanching hazelnuts. Even though it constitutes about 1% of the weight of the whole nut, it is produced in large quantities and poses an economic and environmental disposal problem[1]. However, HSS have low moisture content. It makes it effortless to transport and store, which is usually a major problem in the processing of food by-products[3]. It is worth noting that the polyphenols in hazelnuts are mostly concentrated in seed skins and the largest part are flavonoids and phenolic acids[4,5]. Scientific studies have shown the beneficial effects of polyphenols from nuts in maintaining well-being and preventing chronic diseases through their antioxidant, anti-inflammatory, cardioprotective, and hypolipidemic properties[6]. Moreover, polyphenolic compounds show bacteriostatic properties against a wide spectrum of pathogenic and saprophytic bacteria[7,8]. Within food products, polyphenolic compounds exert their activity by scavenging free radicals, significantly contributing to the prevention of lipid oxidation, thereby extending a product's shelf life and preserving sensory quality[9].

Currently, more and more application works are being created. Mainly, concern the addition of HSS to yoghurts, sweet snacks, pasta or meat products to increase their health-promoting properties and stabilisation of the transformation of nutrients[4,10–12]. Moreover, in the latest scientific reports, HSS are used to feed animals

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to improve meat quality or as an element of compost when growing plants[13,14]. However, understanding of the properties of HSS at the molecular level is needed. Up to now, there are several works describing properties of HSS other than the polyphenol content. Importantly, hazelnuts are among the food products that most often cause allergies. There is data that technological processes can reduce the allergenicity of hazelnuts[15]. Thus, the content of allergens contained in HSS should be assessed. Furthermore, the sensory properties of HSS and the main chemical compounds responsible for them have not been specified so far. Analysing the sensory properties of the food ingredients is extremely important because it can modulate the holistic sensory profiles of the food product. However, HSS have a mainly unfavourable impact on the sensory properties of food products[1]. It is necessary to characterize this material to use it in food without introducing negative sensory characteristics. Therefore, developing an efficient and sustainable extraction process is necessary. It will allow for easy application of this material into food products. Further, the chemical composition of HSS, including the high polyphenols content, can be a bacteriostatic and preservative factor that protects food against pathogens and oxidation. Therefore, there is a need for research on these properties of HSS.

The application of HSS can contribute to the by-products management from the food industry and increase the nutritional value of food products and safety. Nonetheless, it is required to evaluate the mentioned properties and analyse the advantages and risks of HSS[1]. Moreover, food industry residue management can support environment protection and introduce sustainability practices by reusing generated by-products in the food system[16,17].

The hypothesis of the work was that HSS had properties that would improve food quality and safety if added to it. The objective of this study was a comprehensive analysis of the potential reuse of HSS in food processing, in particular to determine the possibility of HSS extraction, polyphenolic compounds content analysis, assessment of antioxidant capacity, estimation of bacteriostatic properties, analysis of allergen content and evaluation of sensory properties using instrumental methods.

Results and discussion

Extraction efficiency

The selected extraction method was used due to its environmental non-invasiveness and ease of use. No extractants other than water were used, which means there is no additional chemical contamination or generation of chemical waste. This approach is important in recent green sustainable chemistry[18]. The analysis of the dry matter shows that extraction efficiency was higher in the E5 sample than in the E10 ($p < 0.05$) (Table 1; description of the abbreviations E5 and E10 can be found in the materials and methods section). The acquired results demonstrate that the adaptation extraction method to the HSS allowed for a lower extraction yield than in the original paper where the extraction method was first described[19]. Regarding this phenomenon, extraction efficiency depends on the soluble fraction content that can migrate to the solvent[20]. The HSS is composed of insoluble fibre (46.5–67.1 g per 100 g), lipids (11.0–21.2 g per 100 g), and approximately 4.3–8.6 g water per 100 g[1,21]. Therefore, the chemical composition of HSS limited the achievement of higher extraction yields due to the high content of non-water-soluble molecules such as insoluble fibre and lipids. However, recovery from HSS was quite effective considering the amount of insoluble compounds in this by-product. Significantly higher values were obtained in the case of the E5 sample and the conditions used in this sample can be applied for efficient extraction of HSS. Also, it has been shown that a smaller amount of extracting material allows for a higher level of extraction. This phenomenon is caused by better penetration of the solvent into the extracted material and, consequently, greater extraction efficiency. As stated in a comprehensive review of extraction methods by Zhang et al.[20], the higher the solvent-to-solid ratio, the higher the extraction yield. For this reason, the extraction efficiency was higher for sample E5 than E10.

Antioxidant properties, the polyphenols content and composition

The antioxidant properties and the content of the polyphenols in the HSS and their extracts are presented in Table 1. The antioxidant properties of E5 and E10 extract were similar to the HSS. The statistical difference between the used methods of the antioxidant activity (ABTS, DPPH) and total polyphenols content (TPC) by Folin–Ciocalteu (F–C) method of the HSS, E5 and E10 was not significant ($p > 0.05$). However, the differences between HSS and their extracts in the sum of the polyphenols were observed in the HPLC method ($p < 0.05$). The total content of the polyphenols was lower in the E5 and E10. It shows that the water high-temperature extraction had a detrimental effect on the polyphenols in extract samples[22,23].

The antioxidant activity of extracts and HSS is directly related to the content of polyphenols in them—the higher antioxidant capacity and polyphenol content had HSS in comparison with E5 and E10 (Table 1). Generally, the antioxidant activity of the analysed samples was similar to those already described[5,24]. However, the authors of the cited works emphasize that the laboratory conditions and the origin of the samples have a considerable impact on antioxidant activity. For this reason, the differences between the obtained results of the analyses in the same type of materials were noticed.

Polyphenol analysis by HPLC showed that gallic acid was present in the highest concentration in all tested samples (Table 1). The results are consistent with those reported in the articles, where gallic acid and catechin are the predominant phenolic compounds found in HSS[11,25]. The content of the rest of the phenolic compounds was various. Some polyphenols were lost during aqueous extraction of HSS to obtain the E5 and E10, i.e. caffeic acid, p-coumaric acid, ferulic acid, glycoside-3-O-kaempferol, quercetin, and kaempferol. It was due to high-temperature treatment during E5 and E10 preparation (90 ℃, 10 min, water extraction). Literature data reported that polyphenols are sensitive compounds for temperature processing which can cause their degradation or change chemically and biologically active form[25]. Nevertheless, based on the data obtained and the literature, the water extraction method was effective and easy to employ[18,19].

Analysed properties	Sample type			Units
	HSS	E5	E10	
Extraction efficiency	na	5.2 ± 0.4^{a}	3.7 ± 0.4^{b}	g 100 g^{-1}
Antioxidant properties				
ABTS	250.6 ± 6.9^{aA}	202.8 ± 11.8^{bA}	198.9 ± 6.0^{bA}	mg VCEAC g^{-1}
DPPH	104.7 ± 8.8^{aB}	106.8 ± 4.0^{aB}	98.4 ± 3.8^{aB}	
Polyphenols				
TPC (F–C)*	44.0 ± 2.5	38.9 ± 0.9	37.8 ± 1.6	mg GAE g^{-1}
TPC (HPLC)	9.9 ± 0.9^{a}	$4.2 \pm 0{,}2^{b}$	4.6 ± 0.3^{b}	mg g^{-1}
Gallic acid	8623.0 ± 336.6^{a}	3197.3 ± 188.4^{a}	3868.4 ± 121.5^{c}	µg g^{-1}
Chlorogenic acid	18.5 ± 1.2^{a}	78.5 ± 0.7^{b}	80.5 ± 0.3^{b}	
Caffeic acid	535.9 ± 40.7	nd	nd	
p-coumaric acid	150.4 ± 2.5	nd	nd	
Ferulic acid	53.1 ± 5.8	nd	nd	
Catechin	184.2 ± 10.9^{a}	108.1 ± 0.2^{b}	160.2 ± 1.9^{c}	
Eigallocatechin gallate	nd	439.8 ± 21.0^{a}	107.1 ± 1.0^{b}	
Epigallocatechin	33.5 ± 0.2^{a}	95.3 ± 11.0^{b}	120.4 ± 8.6^{b}	
Glycoside-3-O-kaempferol	234.4 ± 11.1	nd	nd	
Quercetin	57.1 ± 7.1	Nd	nd	
Glycoside-3-O-Quercetin	29.9 ± 1.3^{a}	291.6 ± 7.1^{b}	419.5 ± 13.1^{c}	
Kaempferol	45.4 ± 1.6	nd	nd	
Allergens				
Profiline	> 1.2 (LOQ)	> 1.2 (LOQ)	> 1.2 (LOQ)	ng mL^{-1}
Bet v 1	> 0.8 (LOQ)	> 0.8 (LOQ)	> 0.8 (LOQ)	

Table 1. Characteristics of antioxidant properties, the content of individual phenolic compounds and allergens in hazelnut seed skins (HSS) and their extracts (E5, E10) and extraction efficiency, (n = 3 for HPLC and extraction efficiency, n = 8 for colorimetric methods). Explanations note: E5 means extract where 5 g of HSS was extracted in 100 g of water, and E10 means extract where 10 g of HSS was extracted in 100 g of water, full explanations on the preparation of extracts are in section "Extraction method and extraction efficiency"; lowercase letters, a and b indicate statistical differences between samples in the t-test or ANOVA and post-hoc Tukey test ($p < 0.05$); capital letters A and B mean differences between antioxidant activity (ABTS, DPPH) within one sample in the t-test ($p < 0.05$); * means no statistical differences between samples within the analysed parameter ($p > 0.05$); the values after the ± indicate the standard deviation; TPC, total polyphenols content; F–C, Folin–Ciocalteu method; VCEAC, ascorbic acid equivalent; GAE, gallic acid equivalent; na, not analyzed; LOQ, less than the limit of quantification; nd, not detected.

It can be stated that the content of polyphenols in hazelnut seed coats varies depending on the origin of the raw material, the method of roasting the nuts and the temperature of this process[24]. The literature presents various levels of the content of polyphenolic compounds in HSS and their extracts. For example in the paper by Del Rio et al. and Taş and Gökmen[5,26], the researchers using the F–C method obtained results ranging from 41 mg (gallic acid equivalent) GAE g^{-1} to 203 mg GAE g^{-1}. The results presented in this study coincide with those described in the literature. Similar results to ours, regarding the polyphenols content and composition in the HSS are presented by Pelvan et al.[25]. Also, in the study by Król et al.[27] based on the HPLC analysis were detected similar content and composition of polyphenols. Even though the obtained research results are consistent with other studies, it should be emphasized that the available data contain a significantly wide range of outcomes regarding the content of polyphenols and their composition. The selection of the polyphenol extraction method for analysis is important[22]. The authors indicated the direct influence of the origin of hazelnuts and their seed coats on the content of polyphenols. In these studies, the influence of the hazel variety on the content of these compounds was also analysed in the hazelnut's skins[5,26,27]. Important is that the research material in this study was industrial HSS, which makes the obtained results reflect the content of bioactive compounds found in this material to industrial conditions.

Allergens content

Hazelnuts are one of the most common food allergens. Therefore, an allergen analysis was conducted and the results are shown in Table 1. The analysis of profilins and Bet v 1 showed that they were inactivated as a result of thermal processes which were subjected to HSS and tested extracts. The content of allergens was below the detection level of the applied research method. This means that profilines and Bet v 1 have been inactivated already at the stage of hazelnut processing in the industry. Other groups of allergens in HSS and its extracts need to be investigated in the future. Several identified and described proteins are responsible for this allergenicity (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 14, Cor a 15 and Cor a TLP)[28,29]. Cor a 1 belongs to the Bet v 1 allergen group, while Cor a 2 belongs to profilines. Both of these groups of proteins show a decrease

in allergenicity under the influence of high temperatures (above 120 ℃)[28]. Based on the performed analysis, it can be concluded that roasting as a high-temperature treatment is sufficient to inactivate these two groups of hazelnut allergens. Similar results were obtained by López et al.[15] who, by autoclaving hazelnuts, reduced their allergenicity. Studies have already shown that roasting and other food processing can directly affect the allergenicity of nuts. It should be emphasized that neither roasting nor any other treatment makes the nuts safe for allergy sufferers and loss of allergenicity was achieved only in a few cases[30]. In clinical trials, it has been proven that the roasting process reduces the allergenicity of nuts and some of the test subjects did not experience an allergic reaction. Despite this, the effect in these studies was not obtained on all study members or the effect was not clinically significant[31,32]. However, all the cited research results focus on the allergenicity of hazelnuts and not HSS. It is undeniably necessary to test HSS for the occurrence of allergens from other Cor groups, as such data have not been published yet. Referring again to López et al.[15], it was noted that the autoclaving process significantly inactivated allergens. It can be concluded that HSS are the parts of the hazelnut most exposed to heat during roasting, and at the same time, the inactivation of allergenic compounds in this by-product may be the greatest.

Bacteriostatic activity

The bacteriostatic activity of the extracts is presented in Table 2. The minimum inhibitory concentration (MIC) level was achieved for all tested pathogenic and saprophytic bacterial strains. *Lactiplantibacillus plantarum* 299v and *Lacticaseibacillus rhamnosus* ATCC 53,103 did not have sensitivity to the tested concentrations range so the MIC is under 10 mg g^{-1} and it has not been achieved. The E5 and E10 had a similar effect on the tested bacteria. Usually, the E5 and E10 extracts inhibited the growth of the same strain of tested bacteria. There were a few exceptions where E5 had better bacterial inhibitory activity. It was in the case of the *Bacillus spizizenii* ATCC 6633 and *Escherichia coli* ATCC 11775. The E10 had only a better inhibitory effect on *Staphylococcus aureus* ATCC 25923. Many studies present the mechanisms of polyphenolic compounds that cause bacteriostatic or bactericidal effects[7,33]. The phenolic acids identified in our samples interfere with the tightness and permeability of the bacterial cell membrane. The disruption of the cell membrane is explained as local hyperacidification, which causes an increase in the permeability of the cell membrane and then denaturation of intracellular proteins and, as a result, inactivation of the bacterial cell[7,33]. The mechanism of antimicrobial action of flavonoids such as catechins, kaempferol and quercetin is focused on the changes in the cell membrane, and cell wall but also the inhibition of DNA synthesis and increased oxidation stress in the bacterial cell. The effect of flavonoids, on cell membranes, is different than in the case of phenolic acids. They probably damage phospholipid bilayers, inhibition of the respiratory chain or ATP synthesis[34]. Studies indicate that the action of flavonoids on the cell

Bacterial strain	MIC E5	MIC E10
	mg g^{-1}	
Listeria monocytogenes ATCC 15,131	2.5	2.5
Listeria monocytogenes ATCC 19,111	2.5	2.5
Listeria monocytogenes ATCC 7644	2.5	2.5
Salmonella enterica ATCC 29,631	2.5	2.5
Salmonella enterica ATCC 14,028	2.5	2.5
Salmonella Hofit IFM 2318	5.0	5.0
Bacillus cereus ATCC 10,876	1.5	1.5
Bacillus spizizenii ATCC 6633	2.5	5.0
Bacillus cereus ATCC 11,778	1.5	1.5
Staphylococcus simulans CECT 4538	1.5	1.5
Staphylococcus aureus ATCC 6538	1.5	1.5
Staphylococcus aureus ATCC 25,923	5.0	2.5
Escherichia coli o157:H7	5.0	5.0
Escherichia coli ATCC 15,922	7.5	7.5
Escherichia coli ATCC 11,775	5.0	7.5
Enterococcus faecalis ATCC 51,299	5.0	5.0
Enterococcus faecalis ATCC 19,433	5.0	5.0
Enterococcus faecalis ATCC 29,212	5.0	5.0
Clostridium sporogenes ATCC 19,404	5.0	5.0
Clostridium sporogenes ATCC 11,437	5.0	5.0
Lactiplantibacillus plantarum 299v	> 10.0	> 10.0
Lacticaseibacillus rhamnosus ATCC 53,103	> 10.0	> 10.0

Table 2. Bacteriostatic effect on different species of the bacteria of the tested hazelnut seed skins extracts (E5, E10) based on the minimum inhibitory concentration (MIC); n = 3. Explanations note: E5 means extract where 5 g of HSS was extracted in 100 g of water, and E10 means extract where 10 g of HSS was extracted in 100 g of water, full explanations on the preparation of extracts are in section "Extraction method and extraction efficiency".

wall causes disturbances in its integrity and, as a result, lyses the cell wall and releases the cytoplasmic contents of the bacteria[35]. On the other hand, it was found that quercetin was able to inhibit the function of DNA gyrase, thus leading to the disruption of DNA synthesis[36]. In another study, it was observed that catechins may increase the content of reactive oxygen species in the cell and disrupt the antioxidant economy of *E. coli* cells[37]. This property can be linked to the reaction between catechins and oxygen present in the culture, which results in the formation of hydrogen peroxide and then highly reactive hydroxyl radicals. The activity of these radicals leads to irreversible changes in bacterial cells, through the oxidation of proteins, disturbances in DNA synthesis[38]. It has been found that phenolic compounds have a synergistic effect when used as mixes gallic acid is shown to have significantly greater antimicrobial activity when combined with hydroxytyrosol and other polyphenols than as a single compound[39]. In our previous work, we showed that HSS powders have bacteriostatic potential. HSS without extraction showed lower bacteriostatic activity compared to the results obtained in this study[11]. In other studies, extracts from the seed coats of peanuts were analysed. Klompong and Benjakul[40] obtained high microbiological activity against *S. aureus*, *E. coli* and *B. cereus*. Microscopic images, cell damage and the release of intracellular substances into the external environment were observed, which indicates an analogy to the mechanisms described above. An important result is the lack of effect of the extracts used on the tested probiotic bacteria. Other studies tested the effect of HSS on the growth of *L. plantarum* P17630 and *Lactobacillus crispatu* P17631. Montella et al.[41] found that HSS and their extracts are rich in soluble and insoluble dietary fibre, which stimulates the effect on the growth of probiotic bacteria. It is assumed that the extracts used by our research team contained soluble fibre. The high fibre content in this case could significantly influence the high metabolic activity of probiotic bacteria and stimulate their growth. Moreover, probiotic bacteria can metabolize polyphenolic compounds as prebiotics and have a highly adapted enzymatic apparatus for their catabolism[42]. It has been shown that polyphenolic compounds addition to in vitro large intestine models or their dietary consumption cause an increasing number of probiotic bacteria and a simultaneous reduction in the development of proteobacteria[8].

Instrumental sensory analysis

Instrumental sensory methods allow the detection of small differences between the tested samples and permit the analysis of products that are not usually consumed, such as by-products. The volatile molecules in the tested HSS extracts obtained using e-nose are presented in Fig. 1a and the supplementary materials in Table S1. Both extracts (E5 and E10) contained the highest proportion of 2-hexanol (61.1–62.8%), acetaldehyde (7.6–7.6%), 2.3—pentanedione (5.7–6.1%) and myristicin (3.8–6.8%). These compounds were mainly responsible for the fatty, fruity, woody, spicy, aldehydic and nutty aromas. An important feature of the E5 extract is that it contained aromatic compounds that did not occur in the E10 extract. It was but-(E)-2-enal in the E5 sample with a floral and green sensory characteristic. The analysis of the taste by e-tongue (Fig. 1b) shows that bitter, sour, umami and universal taste are the highest intensity in the tested samples. The taste profiles of the tested extracts were consistent and similar to each other. The statistical difference between the e-tongue sensor response occurred only in the saltiness sensor (CTS) ($p < 0.05$). The PCA analysis defined the impact of individual sensory descriptors contributed by the identified compounds and the obtained intensity of taste on the sensory profile. The amount

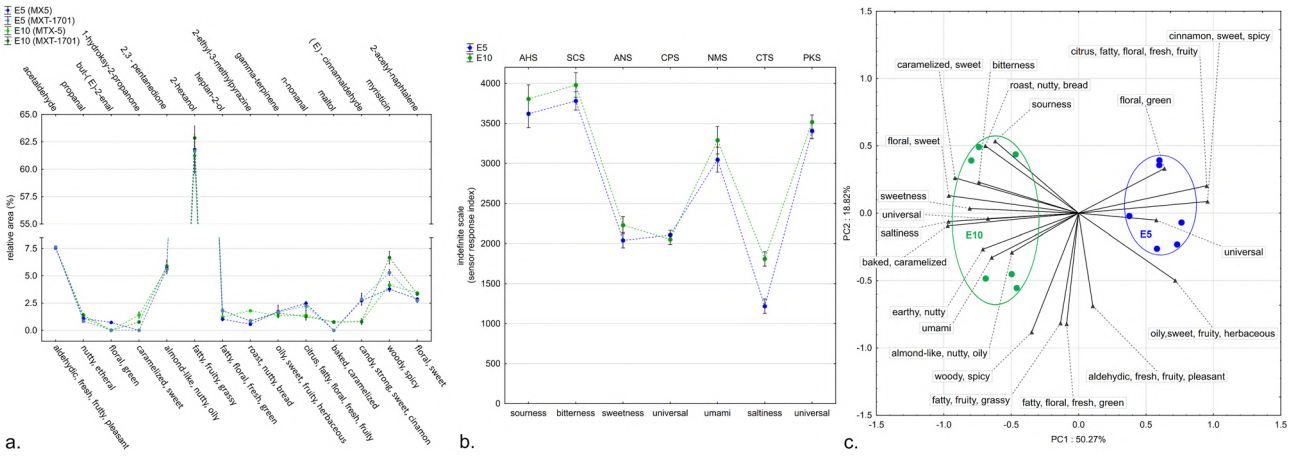

Figure 1. **(a-c)** The odour profile of the analysed extracts (E5, E10) based on volatile compounds and their sensory descriptors in e-nose analysis, the percentage share in the total volatile compounds depending on the chromatographic column (MTX-5, MXT-1701) used for the separation, n = 3 (**a**); the taste profile of the hazelnut seed skins extract obtained by e-tongue, the abbreviations mean the sensors that are responsible for detecting the taste assigned to them; the value is expressed in units of the scale of indefinite taste intensity, n = 8 (**b**); E5 means extract where 5 g of HSS was extracted in 100 g of water, and E10 means extract where 10 g of HSS was extracted in 100 g of water, full explanations on the preparation of extracts are in section "Extraction method and extraction efficiency".; principal component analysis of the instrumental sensory evaluation by e-nose and e-tongue of extracts, projection of variables (sensory descriptors of detected volatile compounds by e-nose and taste descriptors detected by e-tongue) and cases (tested samples) onto the plane of the principal components (**c**); error bars represent the confidence interval ($p < 0.05$), these results were confirmed by t-test or Tukey's HSD post hoc test after ANOVA analysis ($p < 0.05$).

of HSS in the extraction process affected the sensory profile of the obtained extract. The E10 extract was more bitter, salty, sour, baked, caramelized, roasted, nutty, almond, earthy and nutty. The E5 extract was more floral, fatty and fresh. These properties were directly associated with the content of the but-(E)-2-enal with floral and green sensory descriptors and it was identified only in sample E5. The E5 extract had also a higher amount of the n-nonanal, (E)—cinnamaldehyde and 2-acetyl-naphtalene which had a citrus, floral, candy and sweet aroma. The PCA analysis has shown the dependency between these selective aromas (Fig. 1c). The vectors which are longer and situated closer themselves have a bigger correlation to each other. Cases representing test samples, marked with green or blue points and ellipses, are significantly distant from each other in the plane of the coordinate system and correlated with other sensory descriptors represented by black lines (Fig. 1c). This result indicates sensory differences in the tested samples. The samples mainly contained the same aroma compounds and had similar taste profiles with the comparable intensity of the analysed tastes. Nonetheless, thanks to instrumental sensory methods and statistical analysis like PCA, it is possible to obtain and present the sensitive difference between tested samples if they are similar[43,44].

So far, a similar sensory analysis has not been published. Nevertheless, the aromatic and taste profile of HSS extracts is other than that of hazelnuts. However, both contain similar features such as nut, fatty or sweet aromas. Noteworthy, HSS is removed during roasting, therefore a large part of the published studies concern the impact of the roasting process on the aromatic parameters of nuts[1,45]. For this reason, the results are difficult to compare with each other. On the other hand, many studies suggest that HSS and their extracts have a high potential for food applications. HSS was added to various food matrices such as yoghurts, chicken burgers, and date bars[1,10,11]. The authors of these works consistently conclude that this material can be a functional food additive, and in appropriate dosage, it did not change the sensory characteristics of food. In addition, there are suggestions in the literature that a deeper understanding of the properties of HSS and their extracts from the molecular sensory characteristics will contribute to their facile application to food in the future[1].

Conclusion and future perspectives

A comprehensive study of HSS and their extracts yielded new knowledge about these residues. It has been demonstrated that HSS contain a high concentration of polyphenolic and antioxidant compounds. Additionally, HSS exhibit broad bacteriostatic and sensory properties that do not discriminate against their use in further processing in the food industry. Another significant finding is the reduced level of allergenicity, which has been proven.

This holistic analytical approach paves the way for further scientific research under HSS. Consequently, the allergenic activity of HSS and their extracts must be assessed across all allergen groups. Alternative methods of extracting bioactive compounds may provide further insight into the properties of this material. It will be essential to apply the bacteriostatic properties of HSS in various food matrices. The sensory properties of HSS should be evaluated by classical quantified descriptive analysis to establish the addition of the HSS on an acceptable level in food. Future investigation of toxic substances formed during high-temperature roasting is essential. Studies on HSS stability, sensory properties, and safety in different food matrices will be crucial.

The research indicates that HSS extracts have significant potential for use in food products. The study also provides a diverse and broad knowledge base that can be utilised by other researchers or industries seeking various substances with a natural bacteriostatic effect, creating sensory properties, high concentrations of bioactive substances and reduced content of allergens.

Materials and methods

The scheme of the experiment is presented in Fig. 2.

Hazelnut seed skins

The research material was HSS (Ferrero; Belsk Duży, Poland), a by-product material remaining after the roasting of hazelnuts and water extracts of this material were used for the research. The HSS before extraction and all analyses were ground to obtain a powder by laboratory grinder (Chemland; Stargard; Poland). Material was stored at − 20 °C.

Extraction method and extraction efficiency

The extraction method was adapted according to Ferysiuk et al.[19]. Two types of samples were researched, with different amounts of the HSS substrate used for extraction. The 5 g HSS per 100 g of water (E5) and 10 g HSS

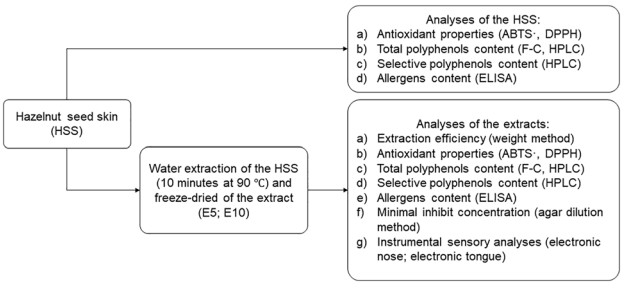

Figure 2. The scheme of the experiment.

per 100 g of water (E10) were weighed separately into the 100 mL tubes. The tubes were filled with 90 ℃ water and sonicated in the ultrasound bath (IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland) for 10 min at 90 ℃. Next, the extracts were centrifugated (Eppendorf Centrifuge 5804 R; Hamburg, Germany) at 6000 rpm and 4 ℃ for 15 min. The extracts were filed into a separate beaker and froze at − 80 ℃. Next, samples were freeze-dried using Labconco freeze-drier (− 45 ℃, 0.11 mBar) for 6 days. Freeze-dried the extracts were stored tightly closed at − 20 ℃.

Extraction level efficiency was obtained by the weight method. Liquid extracts were used in this evaluation. Empty glass weighing dishes were weighed, filled extracts (E5 and E10) and weighed again. Samples were dried at 100 °C, at atmospheric pressure, for 24 h in a laboratory dryer. After this time samples were cooled and put to the exicator to stabilise weight for 24 h. Samples were weighted on the laboratory scale (AS 60/220.R2; Radwag; Radom; Poland). Weighting was performed accurately to within 0.00001 g. Extraction level efficiency was calculated like g dry mater extracted from 100 g of the HSS. Experiments were performed in 3 independent biological replicates.

Total polyphenol content, individual polyphenols and antioxidant capacity

The 100 mg of HSS or HSS extracts (freeze-dried) were mixed with 5 mL of 80% methanol (Chempur, Piekary Śląskie, Poland). The samples were extracted in an ultrasonic bath (IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland) at 30 °C for 10 min. The samples were centrifuged using an Eppendorf Centrifuge 5804 R (Eppendorf; Hamburg, Germany) for 10 min at 10,000 rpm and 0 °C. The extract was stored at − 20 °C in the dark until use.

Total polyphenol content (TPC)

The Attard[46] method was used to determine TPC. The extract of the sample was diluted in ultrapure water (HPL 20UV; Hydrolab; Straszyn, Poland). 20 µL of the prepared sample dilution was poured into the 96-well plate (NEST Biotechnology; Wuxi, China), and 100 µL of F–C (Folin-Ciocalteu's phenol reagent; Chempur, Piekary Śląskie, Poland) was added. The plate was left for 5 min at room temperature (20 °C) in the dark. 80 µL of the (7.5 g 100 g^{-1}) sodium carbonate (Chempur, Piekary Śląskie, Poland) solution in H_2O was poured into the wells and mixed at 150 rpm for 5 min. The samples were left for 2 h in the dark. The absorption was measured at wavelength λ = 750 nm using the SpectraMax iD3 reader (Molecular Devices, San Jose, CA, USA). Before the measurement, the plate was shaken for 1 min by the auto-shaker of the SpectraMax iD3 reader. Experiments were performed in 8 independent biological replicates.

HPLC polyphenol determination

Polyphenols according to Król et al.[27]. Shimadzu equipment was used for analysis (USA Manufacturing Inc, USA, two LC-20AD pumps, a CBM-20A controller, a CTD-20AC oven, a SIL-20AC autosampler, and a UV/Vis SPD-20AV detector). The samples were filtered before being analysed by 0.22 µm. 20 µL of the sample was injected into the HPLC Synergi Fusion-RP 80i Phenomenex column (250 mm × 4.60 mm; Torrance, CA, USA). Polyphenols were separated under gradient conditions with a flow rate of 1 mL min^{-1}. As liquid phases were employed an aqueous solution of 10 mL 100 mL^{-1} acetonitrile (phase A) (Sigma-Aldrich, Poznań, Poland) and 55 mL 100 mL^{-1} acetonitrile (phase B) (Sigma-Aldrich, Poznań, Poland. Both phases were acidified by ortho-phosphoric acid (Sigma-Aldrich, Poznań, Poland) to pH 3.0. The analysis lasted 38 min. The phases changed as follows: 1.00–22.99 min 95% phase A and 5% phase B, 23.00–27.99 min 50% phase A and 50% phase B, 28.00–35.99 min 80% phase A and 20% phase B, and 36.00–38.00 min 95% phase A and 5% phase B. The wavelengths used for detection were 250 nm for flavonols and 370 nm for phenolic acids. Identifying individual phenolics was established on Sigma–Aldrich and Fluka external standards with a 99.00–99.99% purity. Experiments were performed in 3 independent replicates.

ABTS·+ (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ABTS·+ (Sigma-Aldrich, Poznań, Poland) was prepared 24 h before the determination. The powder ABTS·+ radicals (7 mM L^{-1}) were mixed with $K_2S_2O_8$ salt (2.45 mM L^{-1}) (Sigma-Aldrich, Poznań, Poland) in deionized water. The solution was stored at room temperature in the dark. Before the determination, the ABTS·+ solution was diluted with PBS (phosphate-buffered saline; Sigma-Aldrich, Poznań, Poland), and the absorbance of the radicals was adjusted to 0.7 ± 0.02 at 734 nm. The samples were diluted in demineralised water. 50 µL of each of the test sample solutions and 150 µL of the ABTS·+ radical solution were poured into a well of a 96-well plate (NEST Biotechnology; Wuxi, China). The reaction was performed for 6 min and then immediately measured at 734 nm with a SpectraMax iD3 reader (Molecular Devices, San Jose, CA, USA). The determination was carried out with limited access to light. Experiments were performed in 8 independent replicates.

DPPH (1,1-Diphenyl-2-picrylhydrazyl)

The antioxidant activity was determined using the radicals DPPH (Sigma-Aldrich, Poznań, Poland). The inactive powdered radicals were mixed with pure < 99.5% methanol (Chempur, Piekary Śląskie, Poland). The solution was diluted to the absorbance of 1.1 ± 0.05 using methanol at the wavelength λ = 517 nm. The DPPH solution was stored in the dark. The samples were diluted in demineralised water. The tested sample contained 150 µL of DPPH solution and 2 µL of the sample extract. The absorbance was measured after 30 min from the initiation of the reaction. The sample was stored in the dark with constant shaking at 150 rpm throughout the incubation period. Absorbance was measured at a wavelength of λ = 517 nm using a SpectraMax iD3 reader (Molecular Devices, San Jose, CA, USA). The determination was carried out with limited access to light. Experiments were performed in 8 independent replicates.

Allergens content

The determination was performed according to Słowianek et al.[47]. The following reagents were used to assess the allergenic potential of the samples: mouse antibodies against Bet v1 (Dendritics, Dardilly, France), rabbit antibodies against profilin (Dendritics, Dardilly, France), the conjugate of antibodies against mouse immunoglobulins with alkaline phosphatase (Sigma-Aldrich, Poznań, Poland), antibodies against the rabbit immunoglobulin conjugate with alkaline phosphatase (Sigma-Aldrich, Poznań, Poland). 10 µl of each of the above-mentioned reagents was suspended in 10 ml of deionized water. Commercial skim milk solution (3 g 100 g^{-1}) in deionized water and pNPP (p-Nitrophenyl Phosphate; Sigma-Aldrich, Poznań, Poland) as the substrate for the alkaline phosphatase, and 3 M NaOH (Sigma-Aldrich, Poznań, Poland) as the stop reagent was used. As a washing solution, the PBS with 0.1 mL 100 mL^{-1} Tween 20 (Sigma-Aldrich, Poznań, Poland) was used. The total protein extraction kit for plant tissues was used to obtain the extracts of the samples. The standard solution was prepared by serial dilutions of 0.01 mg per 1 ml Bet v1 stock solution or profilin for the standard curve. First, each well of the microplate (SPL Lifesciences, Geumgang-ro, Korea) was filled with 100 µL of the standard solution (reference curve) or the samples of the extracts diluted ten times in the carbonate buffer. The plate was then incubated at 4 °C for 12 h. Next, the wells were washed 4 times with 350 µL of the PBS solution. The plate was incubated for 2 h, and after that 400 µL of 3 g 100 g^{-1} skim milk in PBS solution was added to the wells. The wells were rinsed again 4 times with 350 µl PBS solution. Then, 100 µL of the antibodies against Bet v1 (1000 × diluted) or against profilin (1000 × diluted) was added and incubated for 1 h at room temperature. After that, the plate was washed 4 times with 350 µL of the washing buffer. Next, 100 µL of the anti-mouse antibody in the case of analogues Bet v 1 determination (or anti-rabbit in the case of profilin determination) conjugated to alkaline phosphatase (diluted 5000×) was added to each well and incubated for one hour. The plate was washed 4 times with 350 µL of the washing buffer. Finally, 100 µL of the substrate pNPP for the enzyme was added. After 30 min, the yellow colour was observed, and the reaction was stopped by adding 100 µL of the stopping NaOH solution. The plate was read at 405 nm by a Multiscan RC microplate reader (Labsystems, Vantaa, Finland). The results were calculated using a standard curve prepared with the Bet v 1 allergen (the range of concentration 0.5–50 ng mL^{-1}) or profilin (range of concentration 0.5–100 ng mL^{-1}). Bet v 1 limit detection was 0.88 ng mL^{-1}, for profilin it was 1.2 ng mL^{-1}. Experiments were performed in 8 independent replicates.

Bacteria growth inhibition

Determination of MIC (minimal inhibitory concentration) of the HSS extracts was carried out according to Wiegand et al.[48]. Each strain was activated from frozen (− 80 °C) cultures by incubation overnight at 37 °C in nutrient broth (Oxoid, Basingstoke, UK) except LAB lactic acid bacteria (LAB), *Enterococcus faecalis* and *Clostridium* strains. LAB and *Enterococcus faecalis* were activated with MRS broth (De Man, Rogosa, Sharpe; Biokar Diagnostic, Allonne, France). *Enterococcus faecalis* strains were incubated at 30 °C. LAB was incubated at 37 C. *Clostridium* strains were activated in the cooked meat medium (Becton, Dickinson and Company; Maryland, USA) at 37 °C. *Clostridium* strains were incubated in anaerobic conditions with the of use an absorbing oxygen bag (Thermo Fisher Scientific; Waltham, MA, USA). After overnight incubation, 1 mL of all strains were separately transferred to the new broth medium (9 mL) and incubated for another 24 h. MH agar (Mueller–Hinton; Bio-Rad; Hercules, CA, USA) was used for the MIC determination. Tested microorganisms are listed in Table 2. To obtain the growth of LAB and *E. faecalis*, 1 g of glucose (Sigma-Aldrich; Poznań, Poland) for 100 g MH medium was added. HSS extracts were mixed with sterile distillate water to 100 mg g^{-1} concentration. Water HSS extracts were diluted in a molten MH at 40 °C to receive tested concentration i.e., 1—10 mg g^{-1}. After adding the extract solution, the MH agar was immediately poured onto sterile plates and cooled. Bacterial cultures were diluted in buffered peptone water (Biocorp; Warsaw, Poland). The tested strain with a density of 10^4 colony forming units mL^{-1} was spread on a growth medium. The samples were incubated for 24 h under the above conditions selected for the bacteria species. The positive control was MH agar inoculated with the bacteria without the extracts. Not inoculated plates containing a tested concentration of extracts were negative control. The MIC level was established when there was no eye-visible bacterial growth. In analysing growth on agar media, single bacterial colonies were omitted in establishing the MIC. The analysis was performed in 3 replicates.

Electronic nose

Volatile compounds were identified using the Heracles Neo ultrafast gas chromatograph (Alpha M.O.S., Toulouse, France). The e-nose instrument has an autosampler and two capillary chromatography columns with different polarities—MXT-5 (non-polar; 10 m × 18 µm, Restek) and MXT-1701 (slightly polar; 10 m × 18 µm, Restek) and two Flame Ionization Detectors (FID). For analysis, 1 g of each HSS extract was dissolved in 100 mL of deionized water. The sample solution (1 mL) was transferred into the 20 mL headspace vials and closed with a treflon-faced silicon rubber cap. Each sample was incubated in the autosampler for 20 min at 50 °C with constant shaking speed at 500 rpm. After incubation, the headspace was collected and injected into GC. The injection volume was 1.0 cm^3, the speed was 125 cm^3 s^{-1}, and the temperature was 200 °C. The temperature of the injector was 200 °C, and the detector (FID1 and FID2) was 260 °C. The content of volatile molecules is expressed as the percentage of the relative area which is the area of the chromatogram peaks. The method was calibrated using an alkane solution (C6–C16) to convert retention time in Kovats indices and identify the volatile compounds using the AroChemBase database. The AlphaSoft v 16.0 software was used to process the data. The analysis was performed in 3 replicates.

Electronic tongue

The taste profiles of the water extracts of the HSS were measured using an Astree electronic tongue (Alpha MOS, Toulouse, France). The e-tongue has an autosampler and seven potentiometric chemical sensors for the

detection of individual tastes (sensor set: CTS—saltiness; ANS—sweetness; PKS—universal taste; AHS—sourness; SCS—bitterness; CPS—universal taste; NMS—umami taste), a reference electrode of Ag/AgCl and data acquisition software. The potentiometric difference between each electrode and the Ag/AgCl reference electrode in the equilibrium state was recorded as a response signal. The electronic tongue sensor was pre-conditioned and calibrated with 0.01 mol L^{-1} hydrochloric acid solution. The diagnostic started after calibration. For diagnostic purposes, a 0.01 mol L^{-1} hydrochloric acid solution, monosodium glutamate and sodium chloride were used to evaluate the sensitivity and discrimination of electronic tongue sensors. The sourness, saltiness, and umami were measured using 0.1 M HCl, 0.1 M NaCl, and 0.1 M monosodium glutamate as reference materials. Samples of the HSS extracts were prepared by dissolving 1 g of each extract in 100 mL of deionized water. The solutions were transferred into the electronic tongue sample beaker. The signal of each electrode was recorded per second. The detection time was set for 120 s to ensure the sensors acquired enough signal information for the sample. Between sample analyses, the sensors were rinsed in ultrapure water for 10 s to stabilize them. Eight replicate measurements were conducted for each sample, and five of the most stable measurement points were used for data processing.

Statistical analysis

Statistical analysis was performed using Statistica 13.3 (StatSoft, Cracow, Poland) and Microsoft Excel 2019 (Microsoft; Redmond, WA, USA). Mean as well as standard deviation analysis was calculated. The homogeneity of variance and the normality of the distribution of results were checked. ANOVA was performed with Tukey's post hoc test. If the number of grouping variables was too low to analyse them by the ANOVA, Student's t-test was applied. Principal components analysis (PCA) was also performed based on the covariance matrix.

Data availability

Data will be made available upon request to the corresponding author.

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Author contributions

MK: Conceptualization; Data curation; Formal analysis; Investigation; Methodology, Software; Validation; Visualization; Writing—original draft; AP: Methodology; Writing—review & editing; JH: Formal analysis; DP: Formal analysis; KK: Formal analysis; Methodology; JL: Methodology; DJ: Conceptualization; Writing—review & editing; MT: Supervision; Writing—review & editing

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Competing interests

The authors declare no competing interests.

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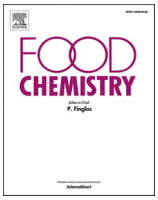

Prebiotic potential of spent brewery grain – *In vitro* study

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ABSTRACT

Spent brewery grain (SBG) is a by-product of the brewery industry. The study aimed to investigate the prebiotic potential of SBG. The chemical composition and fermentation capacity of SBG were checked. The gut microbiota response to SBG was assessed in two *in vitro* models (batch fermentation and dynamic system). Substances with prebiotic properties, including arabinoxylans (16.7 g/100 g) and polyphenols (49.1 mg/100 g), were identified in SBG. Suitable growth and fermentation by probiotic bacteria were observed. The modulatory effect of gut microbiota depends on the *in vitro* system used. In batch fermentation, there was no stimulation of *Bifidobacterium* or lactic acid bacteria (LAB), but short-chain fatty acid (SCFA) and branched short-chain fatty acids (BCFA) synthesis increased. In dynamic, SBG exhibited a moderate bifidogenic effect, promoting *Akkermansia* and LAB growth while reducing *Bacteroides* and *Escherichia-Shigella*. SCFA stabilisation and reduction of BCFA content were noted. Moderate prebiotic effects were observed.

1. Introduction

The problem of food waste and loss is one of the greatest challenges facing modern food systems (Willett et al., 2019). According to the Food and Agriculture Organization, approximately 30 % of food is lost or wasted each year worldwide (FAO, 2019). In the European Union, about 20 % of food production is wasted each year, according to Eurostat. Most waste occurs at the household level (53 %), followed by the processing sector (19 %), production (11 %), catering (12 %) and retail (5 %) (EUROSTAT, 2021). Losses and waste directly contribute to greenhouse gas emissions, agricultural overproduction, energy and economic losses as well as excessive resource consumption (Willett et al., 2019). An additional problem is the by-products of food production and their recycling. Selected by-products are used for further processing in the food industry, some are used as feed for livestock, others are utilised in different sectors of the economy but some are not recycled at all (Van Raamsdonk et al., 2023). However, many by-products have the potential to be recycled as food for human. Several by-products have promising nutritional and processing properties, and the health risk of consumption can be eliminated by appropriate processing (Comunian et al., 2021).

One such by-product is SBG generated during beer production. Approximately 20 kg of SBG is produced for every 100 L of beer (Lynch et al., 2016). The main direction of SBG reuse is feeding livestock. However, the scale of beer production often results in surpluses of SBG that are not utilised and wasted (Nyhan et al., 2023). SBG has a short shelf life in unprocessed form due to its high moisture, protein and fermentable residual sugars (Jackowski et al., 2020). However, the microbiological quality of SBG immediately after production is satisfactory due to the high-temperature treatment during the wort mashing and filtration process (around 85 °C). Therefore, rapid processing of SBG can ensure high microbiological quality and shelf life (Lao et al., 2020;

Abbreviations: Apreb, prebiotic activity; AX, arabinoxylans; AXOS, arabinoxylan-oligosaccharides; BCFA, branched short-chain fatty acids; CFU, colony formation units; GC, gas chromatography; HPLC, high performance liquid chromatography; *Iprob*, prebiotic index; ISAPP, International Scientific Association for Probiotics and Prebiotics; LAB, lactic acid bacteria; S-AX, soluble arabinoxylans; S-NSP, soluble non-starch polysaccharides; I-AX, insoluble arabinoxylans; I-NSP, insoluble non-starch polysaccharides; NSP, non-starch polysaccharides; PCoA, Principal Coordinate Analysis; SBG, spent brewery grains; SCFA, short-chain-fatty acids; SHIME®, Simulator of Human Intestinal Microbial Ecosystem.

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Zeko-Pivač et al., 2022). Moreover, the uses of SBG are broad and include biotechnological, agricultural and nutritional (high-fibre, functional foods and supplement development) applications (Bianco et al., 2020). SBG contains about 20 % protein, 5–8 % fat and a high fibre content of 40–65 % in dry matter. SBG is also a rich source of phenolic compounds (ferulic, p-coumaric, vanillic, caffeic, and sinapic acids), which are increasingly reported to have bifidogenic and LAB stimulation effects (Alves-Santos et al., 2020). Fibre in SBG is formed mainly of non-soluble parts of cellulose, lignin and hemicellulose, the main component of which is AX. AX alone accounts for 20 to 40 % of SGB (Ikram et al., 2017; Lynch et al., 2016). The prebiotic properties of AX and AXOS have been extensively studied recently. It was pointed to the properties that modulate the intestinal microbiota focused on bacterial community, the bifidogenic effect, the synthesis of SCFA, the regulation of the immune response, the increase in the synthesis of intestinal mucus, the regulation of the frequency of bowel movements and other health indicators (Hall et al., 2023; Nguyen et al., 2020; Schupfer et al., 2021). However, studies using a high-fibre diet that included foods rich in AX and AXOS, rather than isolates of these fractions, did not show a direct selective prebiotic effect but a complex influence connected to long-term consumption. The effect of consuming unprocessed high-fibre foods occurs with regular consumption. The scientific community suggested that the efficacy of the intervention was due to a moderate bifidogenic effect and an effect on the SCFA and BCFA profiles (Gill et al., 2021; Vinelli et al., 2022; Yao et al., 2022). Therefore, there is a recent need to investigate the prebiotic potential of unprocessed high-fibre food ingredients, because their effect on the intestinal microbiota is not fully understood (Gill et al., 2021; So et al., 2018; Vinelli et al., 2022). Hence, SBG contains a complex of substances that may have potential prebiotic properties, but there is limited scientific evidence in this area.

The ISAPP defines a prebiotic as a substrate selectively used by host microorganisms to provide a health benefit. In addition, a prebiotic must be resistant to digestion and fermented by the gut microbiota (Gibson et al., 2017). Research focusing on SBG prebiotic activity concerns only on batch fermentation *in vitro* systems of the colon, which is the simplest scheme to study the response of the intestinal microbiota (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023; Lynch et al., 2021; Reis et al., 2014). However, nowadays more advanced *in vitro* digestive systems are accessible. Therefore, further studies of SBG are necessary in other models, such as the SHIME®, which is closer to *in vivo* studies. Moreover, dynamic *in vitro* systems can provide a better understanding of SBG properties compared to batch fermentation (Isenring et al., 2023).

The present study aimed to assess the prebiotic potential of SBG. To achieve this, we investigated the content of NSP, AX, proteins, sugars, and polyphenols. Also, the fermentation capacity, *Ipreb* and *Apreb* of SBG were assessed. Additionally, the gut microbiota response to SBG supplementation was examined in two *in vitro* models to compare their response to potential prebiotics.

2. Materials and methods

2.1. Experiment design

The study included three main steps. The first experiment investigated the chemical composition of SBG, analysing its protein, NSP, polyphenols, polysaccharides, lignin, and sugar content. The second step examined the fermentation dynamics of SBG with probiotic bacteria strains, assessing bacteria number, pH value, and changes in metabolites and sugars before and after fermentation, as well as measuring the *Ipreb* and *Apreb* of SBG. The third part focused on changes in microbiota composition and their metabolites under SBG supplementation in two *in vitro* models: batch fermentation and the SHIME®.

2.2. Spent brewery grains

SBG was obtained from Kampania Piwowarska Dojlidy Brewery (Bialystok, Poland) during the malting process of Żubr lager beer, which uses only barley malt. After brew filtration, SBG was collected, frozen at −20 °C, dried at 95 °C to a stable weight, cooled, and ground to a flour consistency. It was then stored at 4 °C in a vacuum.

2.3. Chemical composition of spent brewery grains

2.3.1. Total protein content

Nitrogen in SBG was determined using the Kjeldahl method (ISO 1871:2009, 2009) with the Digestor 20 AutoLift mineralisation system, scrubber, and Kjeltec 8200 distillation unit (FOSS, Denmark). Results were converted to total protein content by multiplying the nitrogen content by 6.25.

2.3.2. Non-starch polysaccharides, β-glucan and lignin content

NSP content was determined by GC using the standard AACC 32–25, AOAC 994.13 method (AOAC, 1999). The NSP content is the sum of sugars: arabinose, xylose, mannose, galactose and glucose. The S-NSP and I-NSP fractions and the polysaccharide composition of both fractions were determined. The AX content of each fraction was calculated as the sum of arabinose and xylose. After treatment according to AACC 32–25, AOAC 994.13, the samples were separated with 96 % ethyl alcohol (Poch, Polnad) and centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 10 min, 5000 rpm) to obtain soluble (supernatant) and insoluble (pellet) fractions. Each fraction was hydrolysed to monosaccharides using 1 M H_2SO_4 (100 °C, 2 h) and then converted to volatile alditol acetates according to Brunton et al. (2007). Samples were separated on a Clarus GC (Perkin Elmer, MA, USA) equipped with an RTX-225 quartz capillary column (0.53/30 m), an autosampler, a split injector and a flame ionisation detector (FID). Carrier gas for analysis: helium. The separation was performed at 225 °C, with an injector and detector temperature of 275 °C. Separation, detection and quantification were performed according to Fraś et al. (2016). The determination was performed in an accredited laboratory, The Plant Breeding and Acclimatization Institute - National Research Institute (PBAI-NRI).

The determination of (1–3)(1–4)]-β-D-glucan content was performed according to 995.16 AOAC, 32–23 AACC with β-Glucan Assay Kit K-BGLU (Megazyme, Neogen, MI, USA).

Lignin was determined according to Dence (1992). Samples (50 mg) were treated with 72 % (*w/v*) sulfuric acid (0.75 mL) for 3 h at ambient temperature. Then, 9 mL of water was added, mixed and incubation continued for 2.5 h at 100 °C. The residues were recovered by filtration through sintered glass funnels under vacuum. The solid fraction was washed three times with water to remove the acid. The glass filters were dried at 50 °C in an oven until constant weight was obtained.

2.3.3. Organic acids and sugars

Samples were diluted in Milli-Q water (200 mg/5 mL) and extracted twice by sonication for 30 min at 30 °C (IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland). After the first sonication, the sample was centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 5000 rpm, 15 °C, 10 min). The extraction was repeated twice. The supernatant was filtered through a 0.45 μm syringe filter into the vials. Organic acids and sugars were analysed using Shimadzu HPLC with LC-20 CE pump, CBM-20 A controller, CTD-20 AC oven, SIL-20 AC autosampler, RID-10 A detector and UV/Vis SPD-20AV detector (Kyoto, Japan). An Aminex HPX-87H column 300 × 7.8 mm (Bio-Rad, Warsaw, Poland) was used with an isocratic flow of 0.5 mL/min of 10 mM H_2SO_4 at 40 °C. Quantification was based on detection at 210 nm wavelength UV/Vis for organic acids, RI for sugars and external standard curves of 0.10–60 μg per injection of each analyte. All external standards were purchased from Sigma Aldrich (Poznan, Poland) with a purity of 99 %, except for maltotriose with a purity of 95 %.

2.3.4. Polyphenols content

SBG (200 mg) was extracted with 5 mL of 80 % (*v*/v) methanol using an ultrasonic bath (15 min, 30 °C; IS-6; 35 kHz; Inter Sonic; Olsztyn, Poland). The samples were centrifuged (Eppendorf Centrifuge 5804 R; Hamburg, Germany, 10 min, 5000 rpm, 0 °C). Extraction was repeated twice. The supernatant was transferred to a vial and filtered through a 0.22 um synergy filter. The polyphenol content was determined according to Kazimierczak et al. (2020). An HPLC system described in 2.3.3 equipped with a Fusion-RP 80 A column (250 mm × 4.60 mm, 4 μm, Phenomenex, CA, USA) was used. Acetonitrile (Poch; Poland) with MiliQ standard water was used as the mobile phase (phase A was 10 % C_2H_3N (v/v) in H_2O and phase B was 55 % (v/v) C_2H_3N in H_2O). The analysis time was 42 min. The gradient flow of 1 mL/min was applied as follows 1.00–22.99 min, 95 % phase A and 5 % phase B; 23.00–27.99 min, 50 % phase A and 50 % phase B; 28.00–32.99 min, 80 % phase A and 20 % phase B; and 33.00–42.00 min, 95 % phase A and 5 % phase B. The single run lasted 42 min. The wavelength range used was 270–360 nm. For the identification of phenolic compounds, external standards from Fluka and Sigma-Aldrich (Poznań, Poland) with purities of 95.0–99.9 % were used. The concentrations of phenolic compounds were calculated from the standard curves.

2.4. Fermentation dynamic of SBG and prebiotic scores

2.4.1. SBG medium preparation

To prepare growth media, 1 g of SBG was mixed with 100 mL of distilled water, autoclaved at 121 °C for 15 min and cooled. MRS broth (NeoGen, MI, USA) was used as a control medium for fermentation.

2.4.2. Fermentation conditions and number of bacteria

Lacticaseibacillus rhamnosus ATCC 53103 (GG) and *Bifidobacterium animalis* subsp. *lactis* BB-12 were activated from frozen cultures (−80 °C) on MRS agar (NeoGen, MI, USA) and incubated anaerobically at 37 °C for 48 h. A selected colony was then transferred to MRS broth (NeoGen, MI, USA) and incubated anaerobically for 24 h at 37 °C. The culture was then centrifuged for 10 min at 5000 rpm, and washed with PBS (Sigma Aldrich, Poznań, Poland). Next, centrifuged again under the same conditions and suspended in 10 mL of fresh PBS. The SBG medium and the MRS broth were inoculated by adding 1 % (100 uL/10 mL) of the bacterial suspension. Incubation was 24 h at 37 °C under anaerobic conditions.

2.4.3. pH value

The pH value was measured with an Orion Star A211 (Thermo Fisher, Massachusetts, USA) at 20 °C before and after fermentation.

2.4.4. Organic acids and sugars

Samples were diluted in Milli-Q water, centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5804 R; Hamburg, Germany), and the supernatant was filtered through a 0.22 μm syringe filter into vials. Analysis conditions are detailed in section 2.3.3.

2.4.5. Prebiotic index and prebiotic activity

Ipreb and *Apreb* were analysed according to Huebner et al. (2007) and Palframan et al. (2003). *Escherichia coli* ATCC 10536, *E. coli* ATCC 11775, *E. coli* ATCC 25922, *E. faecalis* ATCC 51299, *E. faecalis* ATCC 29212, *E. faecalis* 29,433, *L. rhamnosus* GG, *Lactoplantibacillus plantarum* 299v, *Lactobacillus acidophilus* ATCC 4356, *B. animalis* subsp. lactis BB-12 and *B. infantis* 35,624 were used. All bacteria were activated from frozen (−80 °C) cultures. *E. coli* was cultured on nutrient agar (Oxoid, UK), while other bacteria were cultured on MRS agar (NeoGen, MI, USA). After 48 h incubation at 37 °C, a selected colony was transferred to culture broths. Liquid cultures were performed at 37 °C for 24 h. *E. coli* was cultured in nutrient broth (Oxoid, UK) and the remaining bacteria in MRS broth (NeoGen, MI, USA).

To investigate the prebiotic scores, cultures of strains from the same species of bacteria were mixed in equal proportions. The culture medium was prepared with 5 g/L casein peptone, 3 g/L yeast extract, and glucose as the control at 10 g/L or 10 g/L of SBG or inulin (positive prebiotic control). The medium was inoculated with 1 % (10uL/1 mL) bacterial suspension. Plate cultures for numbering bacteria were performed immediately after inoculation and after 24 h of anaerobic incubation at 37 °C. Culturing was performed according to Naghili et al. (2013). Briefly, bacterial suspensions were serially diluted in buffered peptone water (Oxoid, UK) and immediately inoculated onto the appropriate selective media. *E. coli* was cultivated on TBX agar (Biokar Diagnostics, France), *Lactobacillus* at MRS agar, *Bifidobacterium* at BSM agar (Millipore, MA, USA), *E. faecalis* at COMPASS® *Enterococcus* Agar (Biokar Diagnostics, France). Bacterial cultures were incubated under anaerobic conditions at 37 °C for 24 h for *E. coli* and 48 h for the rest of the bacteria. After this time the colonies were counted.

The *Ipreb* is a ratio of bacterial growth in the medium with the addition of the tested substance (potentially prebiotic) compared to their growth in the control sample with glucose. The *Ipreb* was calculated using the Eq. (1).

$$Ipreb = \frac{CFU\ of\ bacteria\ in\ samples\ with\ SBG\ or\ inulin}{CFU\ of\ bacteria\ in\ samples\ with\ glucose} \quad (1)$$

Equation 1. *Ipreb* calculating formula.

Apreb reflects a given substance's ability to support a probiotic or beneficial bacteria's growth relative to enteric bacteria and relative to growth on a non-prebiotic substrate, such as glucose. The *Apreb* was calculated using the Eq. (2).

$$Apreb = \frac{(LogP24 - LogP0)\ SBG\ or\ inulin}{(LogP24 - LogP0)\ glucose} - \frac{(LogE24 - LogE0)\ SBG\ or\ inulin}{(LogE24 - LogE0)\ glucose} \quad (2)$$

Equation 2. *Apreb* calculating formula; *LogP24*- decimal logarithm of the tested bacteria number of CFU in the sample after 24 h incubation; *LogP0*- decimal logarithm of the tested bacteria number of CFU in the sample initially; *LogE24*- decimal logarithm of *E. coli* CFU in the sample after 24 h incubation; *LogE0*- decimal logarithm of *E. coli* CFU in the sample initially.

2.5. In vitro intestinal systems

2.5.1. Faecal human microbiota inoculum

The human microbiota was obtained from a healthy 39-year-old female volunteer who had not taken antibiotics for 12 months prior. The participant gave written consent to take part in the study. Consent for the use of this material in the study was obtained from the Research Ethics Committee (decision KE-U/12/2022).

2.5.2. The simulator of human microbial intestinal ecosystem (SHIME®) dynamic in vitro model

An instrumental model SHIME® (ProDigest, Gent, Belgium) was applied in the study. The experiment was run in a MULTISHIME® setup focused on the distal colon where according to the literature the major part of AX is fermented (Z. Chen et al., 2019; Salden et al., 2018). All conditions and all reagents used were according to the SHIME® manual from ProDigest (Gent, Belgium). Briefly, the study was performed regarding the intestinal lumen. The standard nutritional medium (PD-NM001B, ProDigest, Gent, Belgium) in this design was sequentially flowing through three separated digestive compartments stomach/duodenum joint vessels, proximal colon bioreactors, distal colon bioreactors (three bioreactors connected to a single proximal colon - three repetitions of this colon section). The instrument was inoculated with faecal microbiota. The fresh faecal sample was suspended in a phosphate buffer (1:5 ratio), homogenising and centrifuging for 2 min at 500 rpm. The supernatant was injected into SHIME® filled with standard nutrient medium (proximal and distal colon) and left overnight for initial

stabilisation. Afterwards, a two-week stabilisation period was conducted, in which the standard nutritional medium (210 mL) and pancreatic liquid (90 mL) were dozed automatically three times a day to the system. During the experiment, the standard media were supplemented with 5 g/L SBG. The SBG was added before autoclaving of media (121 °C, 15 min). The feeding with SBG lasted 8 days (Fig. 1). After that, the 8-day washing was applied with medium without SBG supplementation. Sampling was after a stabilisation period (point 0 0d), after 8 days (8d) of SBY supplementation and after 8 days of washing (16d). The SHIME® feeding program is in the repository dataset (RepOD, University of Warsaw, Poland) connected to this paper.

2.5.3. Batch fermentation in vitro model

The static system was performed according to Pérez-Burillo et al. (2021) with modification. The intestinal microbiota inoculum was collected from the SHIME® system from the descending colon after the stabilisation period at 0 point time (L-ST 0 h). 25 mL of intestinal fluid was poured into 50 mL falcons with previously weighted sterile SBG (addition was the same as in the SHIME® system). Fermentation specific to the colon lasts 24 (L-ST 24 h) and 48 h (L-ST 48 h) under anaerobic conditions at 37 °C (Fig. 1).

2.5.4. SCFA and BCFA analyse

Samples from SHIME® and batch fermentation were diluted in Milli-Q water, centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5804 R; Hamburg, Germany), and the supernatant was filtered through a 0.22 μm syringe filter into vials. Analysis conditions are detailed in section 2.3.3.

2.5.5. Bacterial DNA extraction and metabarcoding

Total genomic DNA was extracted using Genomic Mini AX Bacteria+ (A & A Biotechnology, Gdansk, Poland) according to the manual. After isolation DNA quality was checked by running the sample on 1 % agarose gel and template quantity was measured by fluorimetry using Qubit 2.0 and High Sensitivity Picogreen reagents (Thermo Fisher Scientific, MA, USA). Amplification and sequencing of conserved bacterial 16S rRNA gene fragments covering V3 and V4 regions were performed externally by DNA Sequencing and Oligonuleotide Synthesis Laboratory IBB PAS (Warsaw, Poland). For amplification of 450 bp long fragments following primers were used: 16S_V3-F 341-357F: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG 3′ and 16S_V4-R 785-805R: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC 3′ (Klindworth et al., 2013).

Obtained amplicons were checked on 1 % agarose gel, purified by Ampure XP magnetic beads (Beckman Coulter, CA, USA). Amplicon libraries were pooled in equimolar ratios and indexed according to Nextera indexing strategy by PCR and sequenced on MiSeq instrument paired-end mode using 600 cycle v3 chemistry kit (Illumina, CA, USA. Further analyses were performed locally. Obtained sequencing reads were quality-controlled using FastQC (Andrews, 2010) and accepted 16S rDNA amplicon sequences were classified using Qiime2 (Bolyen et al., 2019) with dada2 pipeline and taxonomic assignment based on Naïve Bayes classifier trained on Silva database v. 138 as downloaded in April 2024, presenting the bacterial community composition (OTUs - operational taxonomic units). The alpha-diversity (Shannon, Chao1, and Simpson indexes) were calculated using the phyloseq R version 1.22.3 package (McMurdie & Holmes, 2013). For plotting, ggplot2 R version 3.3.5 package was used.

2.6. Statistical analysis

Statistical analyses were performed in Statistica 13.3 (StatSoft, Poland) and Prism 10 (GraphPad Software, MA, USA). Basic descriptive analyses were performed. Percentage and proportional data were subjected to compositional data transformation. The Shapiro-Wilk test, Brown-Forsythe test and Levene test were performed to assess the parametricity of the data. ANOVA and *t*-tests were performed. The correlation matrix was prepared using Pearson's correlation. Bray–Curtis distance matrix was used for PCoA. Statistical significance was set at $\alpha = 0.05$ for all analyses.

3. Results

3.1. Chemical composition of spent brewery grains

The protein content in SBG was at 20.1 g/100 g (Table 1). Fibre in SBG was mainly rich in I-NSP fractions, including I-AX and lignin. S-AX and β-glucans were at a low level (Table 1). A relatively high content of maltose and maltotriose was found in SBG. The sum of polyphenols identified in SBG was 49.1 mg/100 g using the HPLC method. The highest amounts were kaempferol-3-glucoside, quercetin, kaempferol, myricetin and chlorogenic acid.

3.2. Fermentation dynamic of SBG and prebiotic scores

The results of the SBG fermentation, *Ipreb* and *Apreb* are presented in Fig. 2. The intensity of bacterial growth (Fig. 2A) was better in the MRS medium. However, in the SBG a significantly increased number of *Lacticaseibacillus rhamnosus* GG was observed compared to the initial number ($p < 0.05$). *Bifidobacterium animalis* subsp. *lactis* BB-12 in SBG showed a slight increase in live cells after fermentation ($p > 0.05$). The initial pH of the growth media was similar between SBG and MRS (Fig. 2B). A decrease in pH was observed in all samples analysed after fermentation. The decrease in pH caused by L. *rhamnosus* GG was greater in SBG than in MRS. In both media, *B. animalis* BB-12 had a lower ability

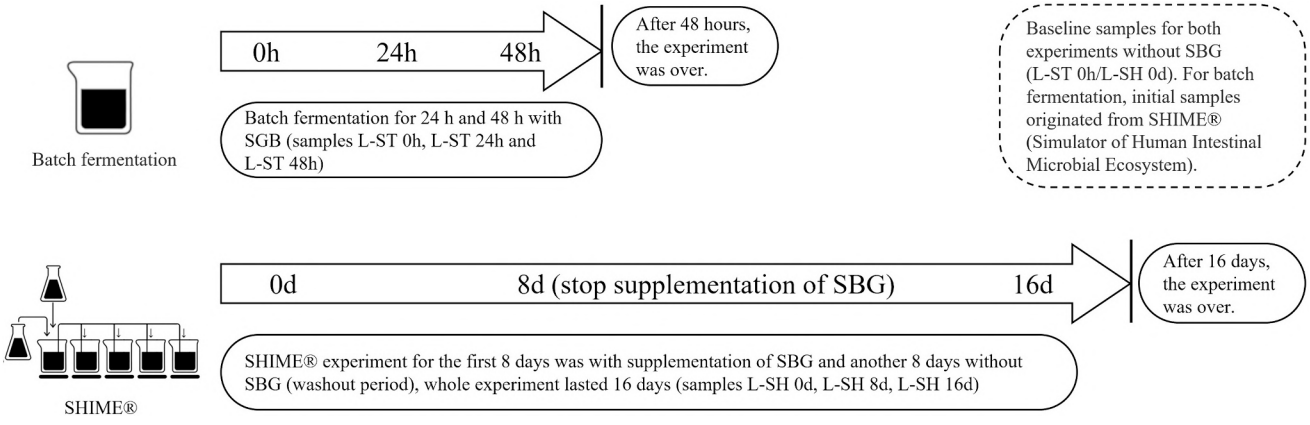

Fig. 1. Batch fermentation and SHIME® experiment scheme.

Table 1
Content of selected chemical compound in the analysed spent brewery grains; $n = 3$.

Proteins	20.1 ± 0.3	
Fiber fractions		
Total NSP	29.7 ± 0.2	
I-NSP	28.2 ± 0.3	
S-NSP	1.5 ± 0.1	
Total AX	16.7 ± 0.2	
I-AX	16.2 ± 0.2	g/100g
S-AX	0.5 ± 0.1	
Lignin	9.0 ± 0.2	
β-glucans	0.6 ± 0.0	
Water-soluble carbohydrates		
Maltotriose	3.4 ± 0.3	
Maltose	7.3 ± 0.4	
Glucose	0.6 ± 0.1	
Polyphenols		
Chlorogenic acid	4.0 ± 0.1	
Caffeic acid	2.6 ± 0.0	
p-coumaric acid	1.8 ± 0.0	
Vanillic acid	3.1 ± 0.4	
Salicylic acid	2.7 ± 0.4	
Sinapic acid	1.3 ± 0.1	mg/100g
Quercetin-3-o-rutinoside	3.5 ± 0.6	
Kaempferol 3-glucoside	12.2 ± 1.3	
Myricetin	5.3 ± 0.8	
trans-Cinnamic acid	0.4 ± 0.0	
Quercetin	6.8 ± 2.0	
Kaempferol	5.4 ± 0.5	

to acidify the environment than L. *rhamnosus* GG.

The content of organic acids and sugars in the samples subjected to incubation with each probiotic depended on the type of medium and the bacteria (Fig. 2 C, D). The total sugar content was significantly reduced after incubation ($p < 0.05$). In the SBG medium, maltotriose was partially degraded by both bacterial strains, while L. *rhamnosus* GG showed a greater ability to catabolize this trisaccharide ($p < 0.05$). Maltose was completely reduced from the medium by L. *rhamnosus* GG, and partially by *B. animalis* BB-12 ($p < 0.05$). Glucose was catabolised best from all the sugars by both strains regardless of media ($p < 0.05$). Organic acid and sugar content was lower in SBG before fermentation than in the MRS medium. Also, after fermentation, the concentrations of organic acids were lower in SBG ($p < 0.05$). The observed trends in metabolite changes were similar for both the tested media and the bacteria. The main metabolites formed in both media and by both probiotics were lactic and acetic acid. In addition, *B. animalis* BB-12, increased significantly the content of succinic acid content in SBG ($p < 0.05$) but not in MRS ($p > 0.05$). On the other hand a slight reduction of malonic acid content was observed in media incubated with L. *rhamnosus* GG, but not *B. animalis* BB-12. The propionic acid content did not change significantly across media and probiotics ($p > 0.05$).

Ipreb values (Fig. 2E) were higher for SBG samples than for inulin for all tested bacterial groups, but for *Bifidobacterium*, the difference was not significant ($p > 0.05$). The *Apreb* (Fig. 2F) of SBG was higher than 0.9 and comparable to inulin ($p > 0.05$). Only in the case of *Bifidobacterium*, the value of *Apreb* of SBG was significantly lower than that of inulin ($p < 0.05$). The high values for the *Ipreb* indicate good growth of the tested bacteria with SBG. Moreover, *Apreb* values demonstrated that LAB, *Bifidobacterium* and *E. faecalis* growth was better with the addition of SBG than *E. coli* (Fig. 2F).

3.3. Gut microbiota, SCFA and BCFA modulation

The levels of lactic acid, SCFA (acetic acid, propionic acid, butyric acid) and BCFA (isobutyric acid, isovaleric acid, valeric acid) in the batch fermentation (Fig. 3A) were changing over time more dynamically than in SHIME® (Fig. 3D). In the batch fermentation, the levels of all compounds tested increased significantly after 24 and 48 h ($p < 0.05$), except for valeric acid, which remained at a similar level throughout the experiment. The lactic acid content decreased significantly between 24 and 48 h in the batch fermentation system. The percentage of SCFAs in batch fermentation (Fig. 3B) showed a decreasing trend due to the increase in BCFAs.

No lactic acid was detected in the SHIME® (Fig. 3D). By supplementing SBG to SHIME® nutrient medium (from 0d to 8d), a significant decrease in the content of isobutyric and isovaleric acids was observed ($p < 0.05$), while valeric acid remained at a constant level. Simultaneously, SCFA levels were stable during SBG supplementation (Fig. 3D). After 16 days (L-SH 16d) of the experiment, and thus after an 8-day washout period without SBG, a significantly higher concentration of propionic acid was observed. However, isobutyric acid and isovaleric acid levels returned to near initial concentrations. In addition, valeric acid was significantly lower than at the beginning of the experiment. In SHIME®, the percentage of all SCFAs increased significantly ($p < 0.05$) within 8 days of SBG supplementation. After 16 days (8 days without SBG supplementation), the percentage of acetic acid decreased significantly due to the increase of propionic acid ($p < 0.05$). The remaining acids returned to initial levels, except for valeric acid, the percentage of which decreased significantly ($p < 0.05$).

Sequencing data were deposited in the RepOD repository. All data regarding sequencing statistics and biodiversity indices are available in Supplementary files 1 and 2. 640,902 paired-end raw sequencing reads for five samples were obtained with the mean number of reads per sample 128,180. After filtration and denoising, the average number of reads for the sample was 55,163 (Supplementary 1). The alpha-diversity indices (Chao1, Shannon, and Simpson) differ between the samples, with the highest value for the time 0 sample (0 was the same for both batch fermentation and SHIME®), indicating that bacterial diversity at the start of the experiment was the most diverse (sample deriving from faeces after the stabilisation period). In the L-ST 24 h sample (batch fermentation), an extensive reduction compared to the baseline sample in alpha-biodiversity occurred. The rest of the samples present similar levels of alpha-diversity indicators. Nevertheless, alpha-biodiversity in samples from both models was reduced compared to the levels at the beginning of the experiment (time 0).

In the batch fermentation after 24 h (L-ST 24 h) a decrease in the relative abundance of many genera of bacteria was observed (Fig. 3C), including: *Agathobacter, Akkermansia, Anaeroglobus, Bacteroides, Bifidobacterium, Cloacibacillus, Collinsella, Escherichia-Shigella, Eubacteriaceae, Faecalibacterium, Fusobacterium, Holdemania, Hungatella, Lactobacillus, Megasphaera, Pseudomonas, Sanguibacteroides, Subdoligranulum, Sutterella* and *Victivallis* Those observations are in line with the decrease of alpha-diversity indices. After 48 h of batch fermentation (L-ST 48 h), a partial return of alpha-diversity and the increase of relative abundance of bacteria to the initial state was observed. However, there were noticeable changes in the microbiota composition between the baseline and L-ST 48 h sample. The relative abundance of *Blautia, Butyricicoccus, Cloacibacillus, Coprococcus, Eubacterium, Faecalibacterium, Flavonifractor, Lachnospiraceae* UCG-004, *Phascolarctobacterium, Subdoligranulum* increased notably. A decrease in the relative abundance of *Agathobacter*, *Bacteroides*, *Bifidobacterium*, *Eisenbergiella*, *Escherichia-Shigella*, *Fusobacterium*, *Lactobacillus*, *Parabacteroides*, and *Pseudomonas* was also observed after 48 h (L-ST 24 h).

In SHIME®, the dynamics of changes in microbiota composition (Fig. 3F) were different than in batch fermentation. With SBG supplementation (L-SH 8d), the relative abundance of *Akkermansia, Anaeroglobus, Bifidobacterium, Butyricicoccus, Collinsella, Eubacterium, Fusobacterium, Lachnoclostridium, Lactobacillus, Lactococcus, Odoribacter, Veillonella, Victivallis* increased. The relative abundance of *Agathobacter, Bacteroides, Clostridium, Eisenbergiella, Escherichia-Shigella, Faecalibacterium, Flavonifractor, Intestinimonas, Ruminococcus, Subdoligranulum* and UBA1819 decreased. After cessation of SBG supplementation (L-SH 16d), the composition of the microbiota changed compared to previous periods. The relative abundance of some of the bacterial taxa returned to the initial state (*Agathobacter, Bacteroides, Cloacibacillus, Eisenbergiella,*

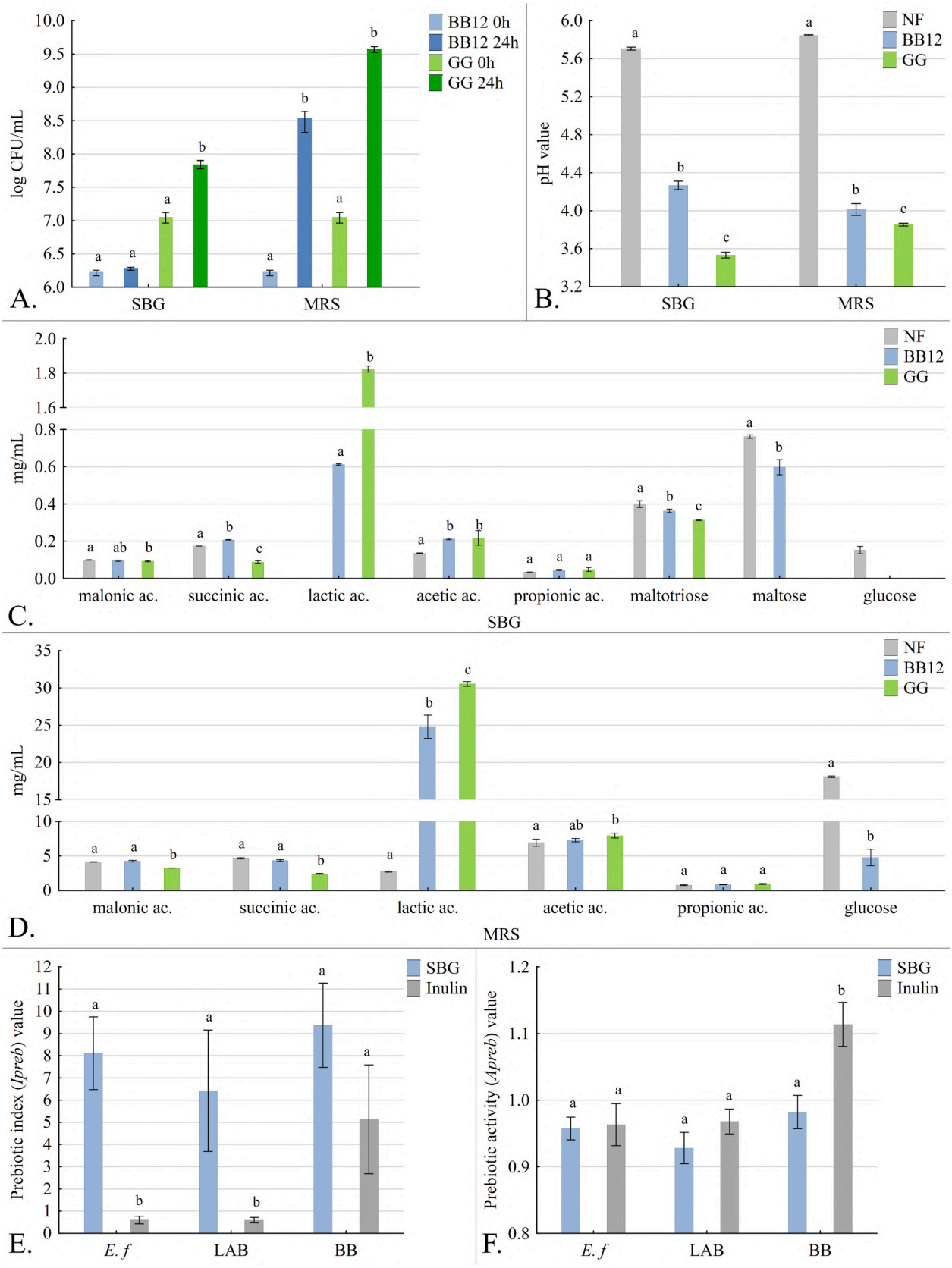

Fig. 2. Fermentation dynamic based on the bacterial count (A), pH values changes (B), organic acid and sugars variation in spent brewery grains (SBG; C) and MRS medium (D); prebiotic index (E) and prebiotic activity (F) of the SBG and inulin; "h" means hours, "ac." means acid, "NF" means not fermented medium, "BB12" means *Bifidobacterium animalis* subsp. *lactis* BB-12, "GG" means *Lacticaseibacillus rhamnosus* GG (GG), "*E.f* " means *Enterococcus faecalis* strains mix, "LAB" means lactic acid bacteria strains mix, "BB" means *Bifidobacterium* strains mix; lowercase letters indicate statistical differences between samples in Tukey's test after ANOVA analysis or *t*-test ($p < 0.05$); error bars in chart A indicate the minimum-maximum ranges, in the rest they indicate standard deviations; n = 3.

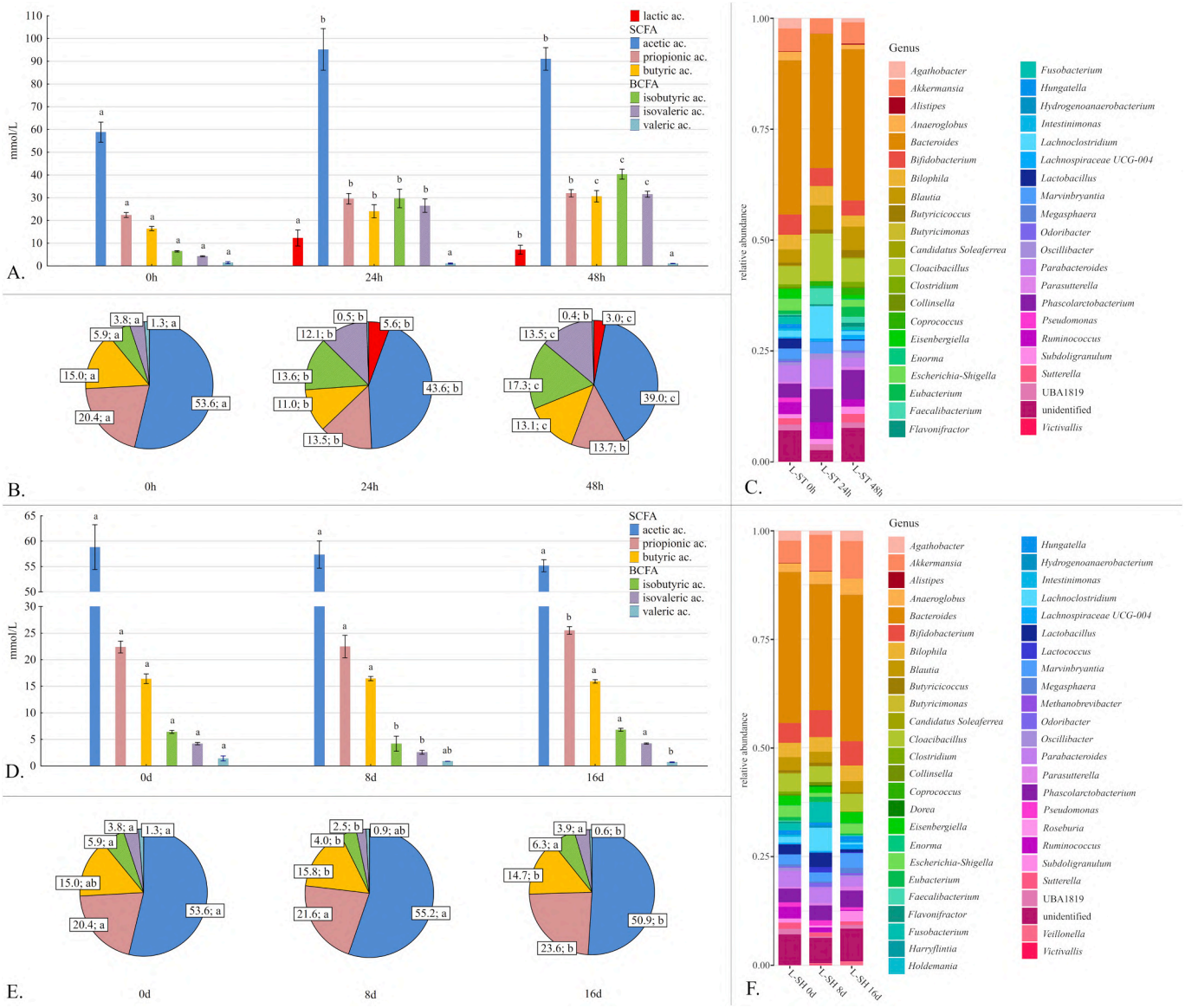

Fig. 3. Lactate, short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) in the batch fermentation (L-ST): content in mmol/L (**A**) and in percentage share (**B**) and in the SHMIE (L-SH): content in mmol/L (**D**) and percentage share (**E**) in different times of the experiment; relative abundance of microbiota composition at the genus level for L-ST (**C**) and for L-SH (**F**); "ac." means acid; "h" means hours, "d" means days; lowercase letters indicate statistical differences between samples in Tukey's test after ANOVA analysis ($p < 0.05$), error bars indicate standard deviations; for SCFA and BCFA, n = 3; for sequencing samples from 3 replications were merged into one ($n = 1$).

Escherichia-Shigella and *Faecalibacterium*). On the other hand, *Akkermansia, Anaeroglobus, Bifidobacterium, Hungatella, Marvinbryantia, Megasphaera, Phascolarctobacterium, Subdoligranulum, Veillonella* and unidentified bacteria were more abundant than at initial state. *Bilophila, Blautia* and *Eubacteriaceae* remained at similar levels of abundance compared to that after the SBG supplementation period. When comparing the batch fermentation system and SHIME®, differences in SCFA and BCFA and its microbiota taxonomic composition were noticed. The changes observed in batch fermentation were more varied, while the changes in the SHIME® system were balanced.

The correlation between the relative abundance of bacterial genera and metabolites was determined by Pearson's correlation (Fig. 4A, B and Supplementary 3), where significant ($p < 0.05$) pars were marked. Batch fermentation (Fig. 4A) and SHIME® exhibit (Fig. 4B) different correlations between metabolites and microbial groups. The correlation in batch fermentation was directly related to the loss of biodiversity in the system and the reduction in the relative abundance of certain taxa. Noteworthy, the strong positive correlation between *Blautia, Butyricicoccus, Cloacibacillus, Coprococcus, Eubacterium, Faecalibacterium, Lachnoclostridium, Lachnospiraceae* UCG-004, *Oscillibacter, Parasutterella, Subdoligranulum* and SCFAs and BCFAs except valeric acid. These bacterial genera during incubation were probably mainly responsible for the synthesis and transformation of fatty acids.

In SHMIE® (Fig. 4B), other pairwise correlation values were observed between taxonomic groups and SCFAs and BCFAs than in batch fermentation. Bacterial genera strongly positively correlated with acetic acid where *Alistipes*, *Clostridium*, *Coprococcus*, *Enorma*, *Eubacterium*, *Parabacteroides*, *Ruminococcus* occurred. For propionate, a strong positive correlation was observed with *Anaeroglobus*, *Bilophila*, *Eisenbergiella*, *Enterococcus*, *Holdemania*, *Hungatella*, *Lachnospiraceae* UCG-004, *Marvinbryantia*, *Megasphaera*, *Methanobrevibacter*, *Parasutterella*, *Phascolarctobacterium*, *Subdoligranulum*, UCG-005 and *Veillonella* occurence. The highest positive correlation for butyric acid was with *Alistipes*, *Butyricicoccus*, *Eubacterium*, *Lactobacillus*, *Parabacteroides*, *Pseudomonas*

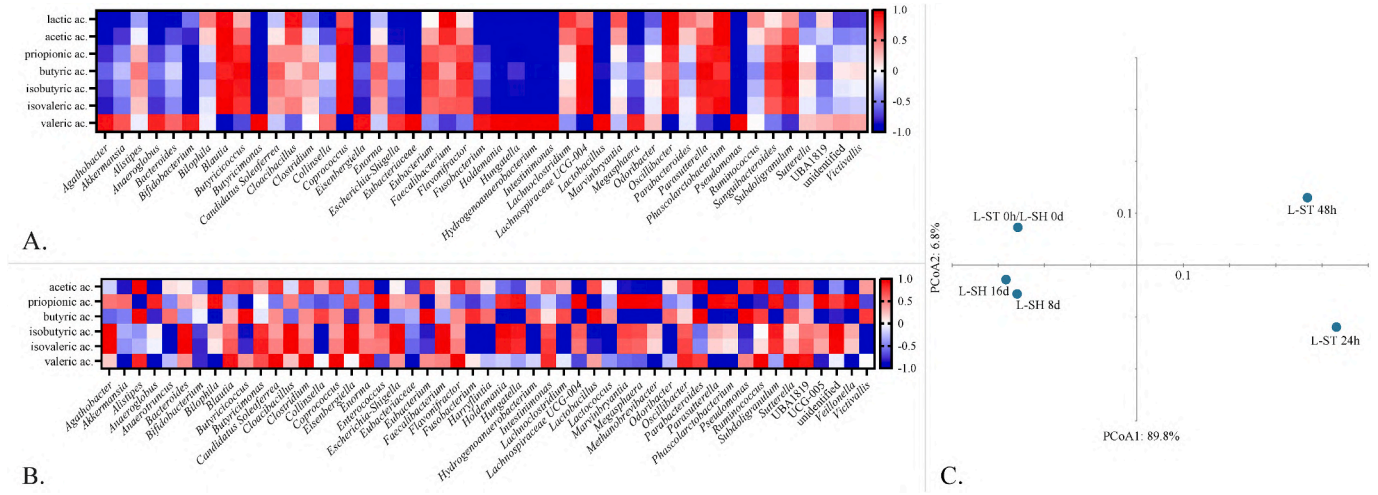

Fig. 4. Correlation between microbial taxa relative abundance and metabolites in the batch fermentation model (A) and dynamic fermentation model (B); Principal Coordinates Analysis (PCoA) of the tested colon fermentation models based on the Bray–Curtis distance (C); "L-ST" means batch fermentation model; "L-SH" means SHIME®; "h" mean hours, "d" mean days.

and *Victivallis*. Within isobutyric acid and isovaleric acid, correlation pairs for individual microorganisms were similar and these were *Agathobacter*, *Bacteroides*, *Cloacibacillus*, *Eisenbergiella*, *Escherichia-Shigella*, *Faecalibacterium*, *Holdemania* and *Hungatella*. In the case of valeric acid, the strongest positive correlations were shown by *Blautia*, *Candidatus Soleaferrea*, *Clostridium*, *Coprococcus*, *Enorma*, *Flavonifractor*, *Ruminococcus* and *Sutterella*. In the case of SHIME®, a greater variation in correlations was observed than in the batch fermentation system. This was related to the bacterial diversity, the relative abundance of taxa in the samples and the dynamics of changes in both *in vitro* systems.

The extensive distance between the batch fermentation (L-ST) and SHIME® (L-SH) indicates the low similarity of these *in vitro* models (Fig. 4C). These results show the overall variability under the influence of SBG supplementation in both models. In batch fermentation, differences were more significant. In SHIME® the changes between the start and other measurement points were smaller. The transformations in SHIME® reflected a greater influence of SBG on the gut microbiota and its metabolites.

4. Discussion

4.1. Chemical composition of spent brewery grains

SBG is a complex by-product that could be processed into a valuable food ingredient due to its high nutritional value, particularly the fibre, protein and phenolic compounds. The literature provides similar data on SBG protein, NSP, β-glucan, lignin and AX content (Ikram et al., 2017; Jackowski et al., 2020; Lynch et al., 2016). Relatively new data in this study pertain to maltotriose, maltose, and glucose. These sugars remain in the SBG after the lautering process. Only the study by Jin et al. (2022) has described the content of fermentable sugars so far (total content 8.2 ± 5.8 g/100 g), and the results are comparable across both studies. However, most of the studies have focused on the effects of acidic or enzymatic hydrolysis treatment of polysaccharides rather than sugars naturally present in SBG (Rojas-Pérez et al., 2022; Sganzerla et al., 2022). The greatest variation in published data relates to polyphenol content (Birsan et al., 2019; Macias-Garbett et al., 2021). This deviation is due to the differences in analytical methodologies and especially sample preparation. Like in the present study, SBG dried at high temperatures has a lower concentration of phenolic compounds than freeze-dried (Guido & Moreira, 2017). On the other hand, high-temperature treatment is less expensive, inactivates bacteria and moulds, and can be used without special equipment. In addition, a more complex polyphenol extraction method gives better quantification results (Guido & Moreira, 2017). The most commonly reported polyphenols in SBG are ferulic acid, p-coumaric acid, vanillic acid, caffeic acid, sinapic acid, salicylic acid, chlorogenic acid, quercetin, and kaempferol (Guido & Moreira, 2017; Macias-Garbett et al., 2021). In this study, high-temperature drying most probably caused the degradation of some phenolic compounds (Table 1) (Antony & Farid, 2022; Y. Zhang & Liu, 2022). Moreover, the level of ferulic acid, the most abundant polyphenol in SBG, was below the detection limit in our study. This was probably due to the temperature and time of dehydration. This phenolic acid could also not be extracted well due to the stable chemical bond with AX (Bento-Silva et al., 2018; Macias-Garbett et al., 2021). We did not use hydrolysis during extraction to release ferulic acid from chemical bonds with AX. Nonetheless, the content of the rest of the detected polyphenols aligns with the literature evidence (Birsan et al., 2019; Macias-Garbett et al., 2021). On the other hand, polyphenols exert their prebiotic effects through several molecular mechanisms involving interactions with gut microbes, mostly *Bifidobacterium,* LAB and the host (Alves-Santos et al., 2020). In summary, the content of the above ingredients in SBG, in terms of the definition of a prebiotic, indicates that it is a raw material rich in indigestible fractions of dietary fibre (AX and lignin) and polyphenolic compounds. However, SBG also contains digestible ingredients such as proteins and sugars.

4.2. Fermentation dynamic of SBG and prebiotic scores

Regarding the characteristics of prebiotic substances, the study was initially focused on the fermentation potential of SBG by defined probiotic bacteria. The observed bacterial growth (Fig. 2C) in SBG indicates its suitability for fermentation by *B. animalis* BB-12 and L. *rhamnosus* GG. The literature described SBG as a useful fermentation material with many potential applications. Many microorganisms, such as LAB, *Propionibacterium, Bacillus subtilis*, yeasts and moulds grew rapidly and produced typical metabolites in SBG (Neylon et al., 2021; Shetty et al., 2023). The effects of SBG fermentation include valorisation of this raw material into organic acids, alcohols, and bioactive peptides (*e.g.* bacteriocins), obtaining proteins with higher nutritional value, improving digestibility or increasing technological utility. The valorisation of SBG has been studied for various purposes, in the synthesis of active ingredients, in animal feed and in the production of supplements and food (Fernandes et al., 2021; Jackowski et al., 2020; Neylon et al., 2021; Shetty et al., 2023).

Regarding fermentation of SBG by L. *rhamnosus* GG and *B. animalis*

BB-12, typical changes in the SBG chemical composition included mainly a reduction in the levels of sugars (glucose, maltose, maltotriose). The catabolism of these sugars was associated with the presence of α-1,4-glycosidase and α-glucosidase bacterial enzymes, which can degrade disaccharides and maltotriose to simple sugars (Gänzle & Follador, 2012; Pokusaeva et al., 2011). The organic acid profile indicates the correct formation of typical metabolites such as lactic acid and acetic acid (Pokusaeva et al., 2011; Suissa et al., 2023). Malonic and succinic acids according to the metabolic pathways were not utilised by *B. animalis* BB-12. A different phenomenon was observed in L. *rhamnosus* GG, where succinic acid was metabolised by succinate dehydrogenase in the incomplete citric acid cycle (Suissa et al., 2023). Furthermore, the unchanged propionic acid content confirms the metabolic changes, as it is not a typical metabolite for L. *rhamnosus* GG and *B. animalis* BB-12 (Pokusaeva et al., 2011; Suissa et al., 2023).

Apreb and *Ipreb* are indicators of prebiotic activity typically used in *in vitro* studies of polysaccharides and oligosaccharides. The higher the values of these indicators, the greater the prebiotic properties of the tested substance. If the *Ipreb* value is greater than one, the substance stimulates the growth of microorganisms compared to the control carbohydrate, here glucose (Figueroa-González et al., 2019; Huebner et al., 2007). To our knowledge, no data have been published on these indicators in SBG. Nevertheless, Paesani et al. (2019) showed that the *Aperb* for wheat AXOS was 0.36 for *Lactobacillus* and *Bifidobacterium*, while the *Ipreb* was 4.09. On the other hand, the values obtained for inulin by Paesani et al. (2019) were also measurably lower than in our studies (Fig. 2E, F). In another study, unprocessed cereal drinks such as barnyard, foxtail and kodo millet were tested and the authors obtained an *Apreb* value of 0.45 (Arya & Shakya, 2021). In addition, the authors studying AX and AXOS found a strong correlation between the chemical structure of AX and the rate of fermentation by probiotic bacteria. *Ipreb* was observed to increase when AX was treated with the enzyme xylanase, resulting in the formation of more xylose compared to untreated AX. This reaction increased the number of saccharides available to the bacteria and stimulated their growth. It was also shown that a high degree of polymerisation of AX reduced the dynamics of fermentation compared to AX with a low degree of polymerisation (Pollet et al., 2012; Wang et al., 2020). On the other hand, when testing unprocessed food raw materials such as SBG in this method, the prebiotic activity of polysaccharides may be masked by nutrients such as sugars, proteins or lipids used in metabolic pathways by bacteria. Nevertheless, these methods provide a general comparison of the growth of *Enterobacteriaceae* and probiotics in the presence of a specific substance.

4.3. Gut microbiota, SCFA and BCFA modulation

The classification of prebiotic substances, as defined by ISAPP, includes non-digestible substrates utilised by host microorganisms and confer a health benefit (Gibson et al., 2017). The most important feature of prebiotic substances is their ability to be fermented by intestinal microbiota, expressed, among others, by desired changes in SCFA and BCFA content.

Currently, there is limited evidence regarding the response of gut microbiota, SCFA, and BCFA to SBG. Researchers so far have focused mainly on the prebiotic properties of isolated AX or AXOS fractions from SBG or other cereal materials. In a study by Lynch et al. (2021), SBG was tested as one of the samples in a batch fermentation system under controlled bioreactor conditions with continuous pH monitoring (pH 6.8). The composition of the fermented sample included 40 % rice along with barley malt. A significant decrease in the relative abundance of *Bacteroides*, *Blautia* and *Faecalibacterium* was observed, along with an increase in *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Agathobacter*, *Subdoligranulum*, *Phascolarctobacterium*, *Eubacterium* and *Escherichia-Shigella*. In SCFA synthesis a non-significant increase in the acetate, propionate, and butyrate content was observed. For BCFA, a significant decrease in isovaleric acid content and an increase in valeric acid content were noted. Calvete-Torre et al. (2023) examined four different SBGs with varying proportions of barley malt with other grains such as rice, unmalted barley, and rice flour. This experiment was conducted in batch fermentation without pH stabilisation. An increased number of readings for *Bilophila*, *Parabacteroides*, *Phascolarctobacterium*, *Senegalemassilia*, *Collinsella*, *Coprococcus*, *Lachnoclostridium*, *Clostridium* and *Escherichia-Shigella* were observed. The content of SCFA and BCFA significantly increased for all analysed acids. In the study by Bonifácio-Lopes et al. (2022), the impact of SBG flour on gut microbiota was investigated. The study was conducted under *in vitro* batch fermentation without acidity stabilisation. The authors observed increased copy numbers in RT-PCR assays for *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Escherichia-Shigella*. Significant increases were noted in the contents of succinate, lactate, acetate, propionate and butyrate. The authors did not analyse BCFA. No further studies testing SBG were found. Two other studies examined the modulation of gut microbiota by AX and AXOS fractions isolated from SBG (Gómez et al., 2015; Reis et al., 2014). Both research used batch fermentation without acidity control. The researchers observed a significant increase in *Bifidobacterium*, *Enterococcus*, *Bacteroides*, *Prevotella*, *Clostridium*, *Eubacterium* and an overall increase in the total bacteria copy number. Both studies observed significantly increased SCFA concentration, but BCFA was not analysed.

Comparing literature data on SBG, AX, and AXOS with our batch fermentation results reveals several similarities in microbiota changes and SCFA and BCFA concentrations. In the presented study, no stimulation of the development of *Bifidobacterium* or *Lactobacillus* genera was observed (Fig. 3C). However, the increase in the relative abundance of *Subdoligranulum, Faecalibacterium, Eubacterium*, family and *Phascolarctobacterium*, but the decrease of the *Bacteroides* and *Escherichia-Shigella* genera were observed, what remains consistent with the findings of Harris et al. (2019) and Lynch et al. (2021). Other research utilizing batch fermentation observed a similar trend to this study in changes in SCFA and BCFA when the fermentation acidity was not regulated. The lack of pH monitoring led to significant intense fatty acid synthesis. Such results were reported in all the mentioned research concerning SCFA and BCFA in batch fermentation (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023; Gómez et al., 2015; Reis et al., 2014).

There is a notable gap in the current knowledge on the prebiotic properties of SBG in advanced, dynamic *in vitro* systems such as SHIME®, animal models or human trials. The presented analysis on the SHIME® model is similar to the investigation by Lynch et al. (2021), which used controlled pH conditions in batch fermentation. When comparing SHIME® and Lynch et al. (2021) results, a similar increase in the relative abundance of *Bifidobacterium*, *Lactobacillus* and *Enterococcus* and a decrease in *Bacteroides* was observed (Fig. 3F). However, the similarity between batch fermentation and SHIME® remains low due to variations in experiment duration, system parameters and differences in nutrient and prebiotic feeding (Isenring et al., 2023; Roupar et al., 2021). On the other hand, extensive data has been documented on the prebiotic properties of AX and AXOS in dynamic systems, animal models or human studies. These studies highlight the ability of AX and AXOS to promote the increase in relative abundance of *Bacteroides*, *Bifidobacterium*, *Blautia*, *Dorea*, *Eubacterium*, *Lactobacillus*, *Faecalibacterium*, *Prevotella*, *Roseburia* and *Enterococcus* (François et al., 2012; Kjølbæk et al., 2020; Schupfer et al., 2021, 2023; Walton et al., 2012; Zambrana et al., 2019). Other research has reported significant reductions in *Campylobacter, Clostridium* and *Escherichia-Shigella* (Schupfer et al., 2021). In addition, an increase in butyrate-producing bacteria was observed, whose relative abundance also increased in our study (*Butyricicoccus*, *Bilophila*, *Marvinbryantia*, *Odoribacter*, *Lachnoclostridium*) (Damen et al., 2011; Schupfer et al., 2023; Van den Abbeele et al., 2011). Conversely, another research group observed a reduction in alpha biodiversity due to AX's promotion of the growth-selected species of bacteria like *Bifidobacterium* (Müller et al., 2020). In summary, AX and AXOS studies often observed a moderate bifidogenic effect, supported *Bacteroides* growth, and simultaneously retained *Firmicutes* and *Bacteroides* proper ratio.

However, a clear and specific influence on certain microbiota taxa cannot be consistently attributed to all AX and AXOS. This effect depends on the structure and degree of branching of AX and AXOS, which is determined by the origin of the raw material (Z. Chen et al., 2019; Neyrinck et al., 2018). The molecular weight of AX and the number of side chains, which vary between raw materials, further influence these properties. For example, AX from barley has a higher molecular weight than AX from rice, resulting in smaller and less branched molecules that are more easily fermented by the gut microbiota (Z. Chen et al., 2019; Wang et al., 2020). Notably, the hydrolysis products of AX, namely AXOS, showed more substantial bifidogenic effects than AX due to its less branched structure, shorter chemical forms and lower molecular weight (Broekaert et al., 2011).

The presented research indicates that SBG supports the growth of *Bifidobacterium*, LAB, *Akkermansia*, *Lachnospiraceae*, and cellulose-degrading bacteria in SHIME® (Fig. 3F). The increase in the relative abundance of cellulose-degrading bacteria is often observed when analysing the impact of minimally processed high-fibre raw materials with complex chemical characteristics, such as bran and whole grain foods (So et al., 2018). SBG also contains other dietary fibre fractions besides AX, primarily cellulose and lignin. Although these are not considered substances with direct prebiotic effects. Noteworthy, according to recent reports, cellulose stimulates the growth of bacteria from the *Ruminococcaceae* family (*Ruminococcus*, *Faecalibacterium*, *Subdoligranulum*, *Oscillibacter*, formerly *Blautia*), which have a positive impact on host health (Moraïs et al., 2024). Cellulose-degrading bacteria are also frequently associated with the mucosal layer of the intestine, contributing to its integrity (Di Vincenzo et al., 2024). In addition, a high-fibre diet increases the relative abundance of *Akkermansia* (Ramos Meyers et al., 2022; Vinelli et al., 2022; Zhang, Hu, et al., 2022). *A. muciniphila* degrades mucin and produces propionate and acetate. It has also been observed that although *A. muciniphila* degrades mucus, it increases the expression of the Muc2 gene. This enhances mucus production, potentially improving the mucin layer, its bacterial community, and the intestinal wall, thereby positively influencing gut-associated lymphoid tissue and supporting the maintenance of homeostasis (Ramos Meyers et al., 2022). Furthermore, in the absence of dietary fibre, *A. muciniphila* significantly reduces mucus levels in the gut, decreasing its relative abundance and increasing intestinal inflammation (Zhang, Hu, et al., 2022). Researchers focusing on the consumption of unprocessed fibre sources noted an increase in *Lachnospiraceae* count (Yao et al., 2022), which was documented in our study as well. Bacteria from this family are commensal microorganisms involved in the synthesis of SCFAs, mainly propionate and acetate. However, in pathological conditions they are associated with obesity, diabetes, inflammatory bowel disease and other disorders (Vacca et al., 2020). Various dietary fibre fractions such as AX, AXOS, β-glucans, xylooligosaccharides, cellulose, and lignin in unprocessed (high-fibre) foods have broad effects on the microorganisms that establish the gut microbiota (So et al., 2018; Vacca et al., 2020). However, the literature often highlights the deep incorporation of the prebiotic fraction of dietary fibre into chemical structures with other molecules that limits the bioavailability of these molecules for microbes (Makki et al., 2018). Consequently, fibre-rich foods stimulate a slower increase in *Bifidobacterium* and LAB than isolated prebiotic fractions. On the other hand, these foods stimulate cellulose-degrading bacteria such as, *Lachnospiraceae* and others that affect the mucosal layer, and produce metabolites that have beneficial effects on the organism (Makki et al., 2018). However, the time required to observe these effects is typically seen with long-term habitual consumption of fibre-rich foods (Makki et al., 2018; Zhang, Fan, et al., 2022).

Literature data on SCFA and BCFA concerning SBG use suggest an increasing content of both fatty acids groups. However, these results refer to batch fermentation systems without pH regulation (Bonifácio-Lopes et al., 2022; Calvete-Torre et al., 2023). In contrast, where pH regulation was applied to batch fermentation, the results were similar to those presented in our SHIME® experiment. They showed no effect on SCFA and reduction in isovalerate concentration (Lynch et al., 2021). On the other hand, studies investigating isolated AX or AXOS fractions observed an increase in SCFA concentration and a decrease in specific BCFA concentrations in dynamic models, animal studies or human studies (Nguyen et al., 2020; So et al., 2018). However, the differences in chemical composition between the pure AX and AXOS and the SBG are too extensive to extrapolate these results to the SBG. A systematic review by Vinelli et al. (2022) found no significant effect of dietary fibre from different unprocessed food sources on changes in SCFA *in vivo*. Numerous studies suggest that the gut microbiota community composition is modulated without affecting SCFA levels (O. Chen et al., 2021; Müller et al., 2020; Vinelli et al., 2022; Wilms et al., 2021). This phenomenon is primarily attributed to the interdependencies between gut microbiota microorganisms and their metabolites, including SCFA and BCFA. It means that healthy gut microbiota's metabolites are constantly used and produced by microbes in this community, ensuring homeostasis (Peterson et al., 2022; Vinelli et al., 2022). In contrast, a greater response in fatty acid synthesis was observed in studies using selective dietary fibre fractions that allowed high levels of SCFA production by targeted groups of microorganisms (Vinelli et al., 2022). The key factors that modulate the level of SCFA production seem to be fibre dose, type, degree of processing and chemical structure (Yao et al., 2022). The effect of dietary fibre on BCFA production varies, but most studies suggest that high-fibre diets reduce BCFA levels (Vinelli et al., 2022). Thus, our study results are consistent with the others, given the reduction in BCFA content during the SBG intervention period and the stable SCFA levels in SHIME®. An important finding in this study is the return of the BCFA content to the baseline level after the washout period in SHIME®. This suggests a direct modulatory effect of SBG. Only the synthesis of propionic acid increased after the washout period (L-SH 16d) probably due to the increase in abundance of the *Lachnospiraceae* family and synthesis through the acrylate pathway by *Akkermansia* (El Hage et al., 2019; Kirmiz et al., 2020; Zaplana et al., 2024). However, metabolic pathways for propionate production involve many taxa of microorganisms and pathways of this fatty acid need to be elucidated.

Regarding the correlation of microbial taxa and fatty acids (Fig. 4A, B). In most studies, *Akkermansia*, *Bacteroides*, *Bifidobacterium*, *Prevotella*, *Ruminococcus*, *Blautia*, *Clostridium*, *Streptococcus*, *Phascolarctobacterium*, *Dialister* and *Veillonella* are closely correlated with acetate synthesis. Bacteria associated with propionate synthesis include *Bacteroides*, *Coprococcus*, *Megasphaera*, *Roseburia*, *Ruminococcus*, *Akkermansia*, *Veillonella* and *Propionibacterium*. For butyrate, the key bacteria are *Bacteroides*, *Anaerostipes*, *Coprococcus*, *Eubacterium*, *Faecalibacterium*, *Roseburia*, *Ruminococcus* and *Lachnospiraceae*. The bacterial groups associated with BCFA synthesis mainly include *Bacteroides*, *Prevotella*, *Megasphaera*, *Escherichia-Shigella* and *Clostridium* (Lange et al., 2023; Morrison & Preston, 2016; Ramos Meyers et al., 2022; Rios-Covian et al., 2020; Salazar et al., 2022). In the case of batch fermentation, the correlations did not broadly align with the literature data. This was due to the high variability in the batch fermentation, which resulted in a loss of alpha-biodiversity in this model. However, the SHIME® results were more consistent with the *in vivo* studies. Essentially, data linked with SCFA and BCFA to specific taxonomic units in the human gut microbiota most often come from animals and human trials that are not free from variability and heterogeneity in microbiota due to differences among populations influenced by lifestyle, diet, diseases, geographic region, development level, and other factors (Hou et al., 2022).

4.4. Difference between in vitro systems

Many differences have been observed between used *in vitro* systems. Other authors have highlighted differences between batch fermentation and colon models such as SHIME®, TIM-2 or less commonly used models. The most common differences indicate lower stability, inability to stabilise microbiota, short-term responses, and the influence of environmental conditions that degrade microbiota biodiversity in batch

fermentation (Isenring et al., 2023; Roupar et al., 2021). In the research presented, a decrease in alpha biodiversity was also observed in batch fermentation. This was most likely due to changes resulting from the characteristics of batch fermentation and exposure of the gut microbiota to altered environmental conditions during the laboratory procedures. This occurred despite the highest possible timed and environmentally rigorous procedures used. These procedures included sampling, transfer to anaerobic conditions and temperature changes (Isenring et al., 2023). In addition, the movement and periodic refreshing of the bacterial feed media that occurs in SHMIE® is not practised in batch fermentation. This leads not only to pH changes in the latter but also to changes in substrate availability for fermentation and metabolic products of the microbiota between these two systems fermentation (Isenring et al., 2023; Roupar et al., 2021). Hence, the lack of similarity was shown in the PCoA analysis (Fig. 4C). The differences between the *in vitro* models have practical implications for the prebiotic properties analysis. Thus, a higher degree of stability was achieved in SHIME®, and these results can be to a higher degree discussed and extrapolated to animal and human studies.

5. Conclusion

The study demonstrated the prebiotic potential of SBG. Substances with prebiotic properties were identified in SBG, including dietary fibre fractions and polyphenols. SBG was also shown to contain digestible nutrients - sugars and protein. The results indicated that SBG is a suitable growth medium for probiotic bacteria. In addition, SBG has a high *Ipreb* and *Apreb* and modulated intestinal microbiota. However, changes in the microbiological community were dependent on the *in vitro* system used. In the SHIME®, SBG showed a moderate bifidogenic effect, stimulating the growth of *Akkermansia*, LAB and reducing the relative abundance of *Bacteroides*, *Clostridium* and *Escherichia-Shigella*. Stabilisation of the SCFA content and reducing the BCFA content were demonstrated as well in SHIME®. However, the effect of BCFA decrease was not maintained after the end of SBG supplementation. A different gut microbiota response was observed in batch fermentation with no *Bifidobacterium* or LAB stimulation effect. Batch fermentation resulted in increased SCFA and BCFA synthesis. The observed differences between the *in vitro* systems indicate the greater utility of SHIME® for research on the prebiotic properties of fibre-rich food ingredients due to similarities with fibre-microbiota interactions reported *in vivo*.

This study provided new insights into the properties of SBG and its effects on the gut microbiota, SCFA and BCFA. It is also the first study that comprehensively describes the aspects of the chemical composition, fermentation capabilities and gut microbiota response under the influence of SBG in different *in vitro* systems. In addition, the same initial composition of the gut microbiota in both *in vitro* models allowed a direct comparison of the systems used and the identification of specific differences between them. This study had several limitations that should be addressed in the future. Only one SBG from one brewery was tested. The short duration of SBG supplementation in the SHMIE can be extended to observe changes in microbiota, SCFA and BCFA over time. Nevertheless, the results indicate that SBG is a promising by-product that could be used to create valuable dietary supplements and functional foods with prebiotic properties. Future standardisation of SBG will be required to achieve greater analysis repeatability and a consistent chemical composition.

The research provides a substantial basis for further analysis into the prebiotic potential of SBG in more complex *in vitro* models, *e.g.* cell lines, and further *in vivo* in animals and humans. This study demonstrated moderate prebiotic effects of SBG in *in vitro* models. Moreover, future studies on the prebiotic activity of SBG should prioritize evaluating its impact on the microbiome through long-term supplementation and *in vivo* models. Additionally, conducting cohort population studies could provide insights into the microbiota response within intervention groups and reveal potential health benefits associated with SBG consumption.

Ethical statement

Before the study, consent was obtained from the Research Ethics Committee (decision KE-U/12/2022).

Declaration of generative AI in scientific writing

The authors declare no use of AI technology.

CRediT authorship contribution statement

Marcin Kruk: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Piotr Lalowski:** Writing – review & editing, Formal analysis. **Magdalena Płecha:** Writing – review & editing, Methodology, Data curation. **Alicja Ponder:** Writing – review & editing, Methodology. **Agnieszka Rudzka:** Writing – review & editing, Resources. **Dorota Zielińska:** Writing – review & editing, Supervision. **Monika Trząskowska:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

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

Data availability

The data are available in an open repository at the following link: https://doi.org/10.18150/EI2LQX

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141254.

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Food by-product valorisation in nutrients through spent brewer's yeast bioprocessing with *Propionibacterium freudenreichii*

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ABSTRACT

This study investigated the potential of *Propionibacterium freudenreichii* to enhance the nutritional quality of spent brewer's yeast (SBY) by producing short-chain fatty acids (SCFAs) and vitamin B_{12} (B_{12}). SBY was also evaluated as a growth medium for *P. freudenreichii*. Four SBY samples from different breweries were utilized and compared to microbiological yeast extract (YE). The tested SBY samples exhibited similar chemical compositions in terms of protein content, free amino acids (FAAs), SCFAs, carbohydrates, riboflavin, and cobalt, whereas YE was richer in most of these substances. However, *P. freudenreichii* growth was hindered by polyphenols in SBY, as evidenced by better growth in samples with lower polyphenol levels and the control sample (YE). SCFA synthesis was efficient in the SBY sample, resulting in 1.3 mg mL^{-1} of acetic acid and 1.70 mg mL^{-1} of propionic acid. Furthermore, *P. freudenreichii* fermentation increased essential free amino acids (FAAs) in all tested samples. SBY was found to be a suitable medium for supporting the production of the active form of B_{12}, with a concentration of 25.2 ng mL^{-1}. However, YE outperformed SBY as a medium for B_{12} synthesis, yielding a concentration of 214.3 ng mL^{-1} after fermentation. The results indicate that both SBY and YE can serve as one-component sustainable growth media, valorised with valuable compounds through *P. freudenreichii* fermentation. In conclusion, SBY offers a novel opportunity for the biotechnological synthesis of SCFAs, FAAs, and B_{12} through *P. freudenreichii* fermentation.

1. Introduction

Spent brewer's yeast (SBY) is a by-product of beer wort fermentation. In 2022, European Union (EU) countries produced nearly 34.3 billion litres of beer, with Germany, Spain and Poland contributing 44.7% (EUROSTAT, 2023). SBY production ranges from 2 to 4 kg per 100 L of beer, totalling approximately 0.7–1.3 million tons annually (Jaeger et al., 2020; Puligundla et al., 2020; San Martin et al., 2020). In brewing, only a fraction of the generated yeast is reused due to the accumulation of dead cells, impacting beer quality (Jaeger et al., 2020; Marson et al., 2020). SBY finds applications in animal feed, yeast extract, vitamins, and protein concentrate production, as well as in the baking industry (Jaeger et al., 2020; Schlabitz et al., 2022). The chemical composition of SBY includes proteins, B group vitamins, excluding B_{12}, β-D-glucan, minerals (including cobalt), and organic acids (Puligundla et al., 2020).

Propionibacterium freudenreichii is a well-known bacterium with GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety) status. In biotechnology, food biotechnology, and science, *P. freudenreichii* is widely used for synthesizing short-chain fatty acids (SCFAs) like propionic and acetic, B_{12} vitamin (B_{12}), Swiss-type cheese production, and certain strains exhibit probiotic activities (Piwowarek et al., 2018b; Thierry et al., 2011; Zahed et al., 2021). While *P. freudenreichii* is commonly employed in industrial propionic acid synthesis and cheese production (Ranaei et al., 2020), it can also synthesize active and pseudo forms of B_{12}. In the absence of appropriate factors, including 5,6-dimethylbenzimidazole (DMBI) and oxygen, pseudo forms of B_{12} are produced (Chamlagain et al., 2015; Deptula et al., 2017a). Chemical synthesis of B_{12} is time-consuming, and microbial fermentation by bacteria lacking GRAS status (*Pseudomonas denitrificans*, *Bacillus megaterium*, or *Salmonella typhimurium*) requires subsequent chemical purification that can be avoided by using *P. freudenreichii* (Mohammed et al., 2014; Piwowarek et al., 2018b).

Exploring the potential of utilizing SBY for *P. freudenreichii* growth and the production of nutritionally valuable metabolites, including B_{12}, presents an opportunity for direct application in food processes

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Abbreviations

SBY	spent brewer's yeast
SCFAs	short-chain fatty acids
YE	yeast extract
FAAs	free amino acids
P. f	Propionibacterium freudenreichii
GRAS	Generally Recognized as Safe
QPS	Qualified Presumption of Safety
DMBI	5,6-dimethylbenzimidazole
YEL	lactate agar and broth
RI	refractive index detector
PDA	photodiode array detector
ICP-MS	inductively coupled plasma mass spectrometry
CFU	colony-forming units
UHPLC	Ultra-High-Performance Liquid Chromatography
HPLC	High-Performance Liquid Chromatography
TPC	total polyphenol content
GABA	gamma-aminobutyric acid
His	Histidine
Ser	Serine
Arg	Arginine
Gly	Glycine
Asp acid	Aspartic acid
Glu acid	Glutamic acid
Thr	Threonine
Ala	Alanine
Pro	Proline
Cys	Cysteine
Lys	Lysine
Tyr	Tyrosine
Met	Methionine
Val	Valine
Ile	Isoleucine
Leu	Leucine
Phe	Phenylalanine
H-D Pro	D-proline
Asn	Asparagine
Gln	Glutamine
Orn	Ornithine
Trp	Tryptophan
EAA	essential amino acids
Disacch	disaccharides
Glu	glucose
Suc a	Succinic acid
Lac a	Lactic acid
Ace a	Acetic acid
Prop a	Propionic acid

(Gagnaire et al., 2015; Jaeger et al., 2020; Marson et al., 2020; Thierry et al., 2011). This approach aligns with the goals of sustainable development, addressing the needs of consumers with specific nutrient deficiencies, such as vegans and those facing B_{12} scarcity (Nawaz et al., 2020; Van Mierlo et al., 2017). Moreover, SBY utilization could serve as an animal-free growth medium for *P. freudenreichii*, contributing to a low-cost, sustainable source for the efficient synthesis of valuable metabolites. (Jaeger et al., 2020; Puligundla et al., 2020). This bioprocessing of SBY may lead to cleaner production of chemical compounds, reducing energy consumption and minimizing waste generation. It aligns with the sustainable development goals by addressing the management of post-production waste and promoting the recycling

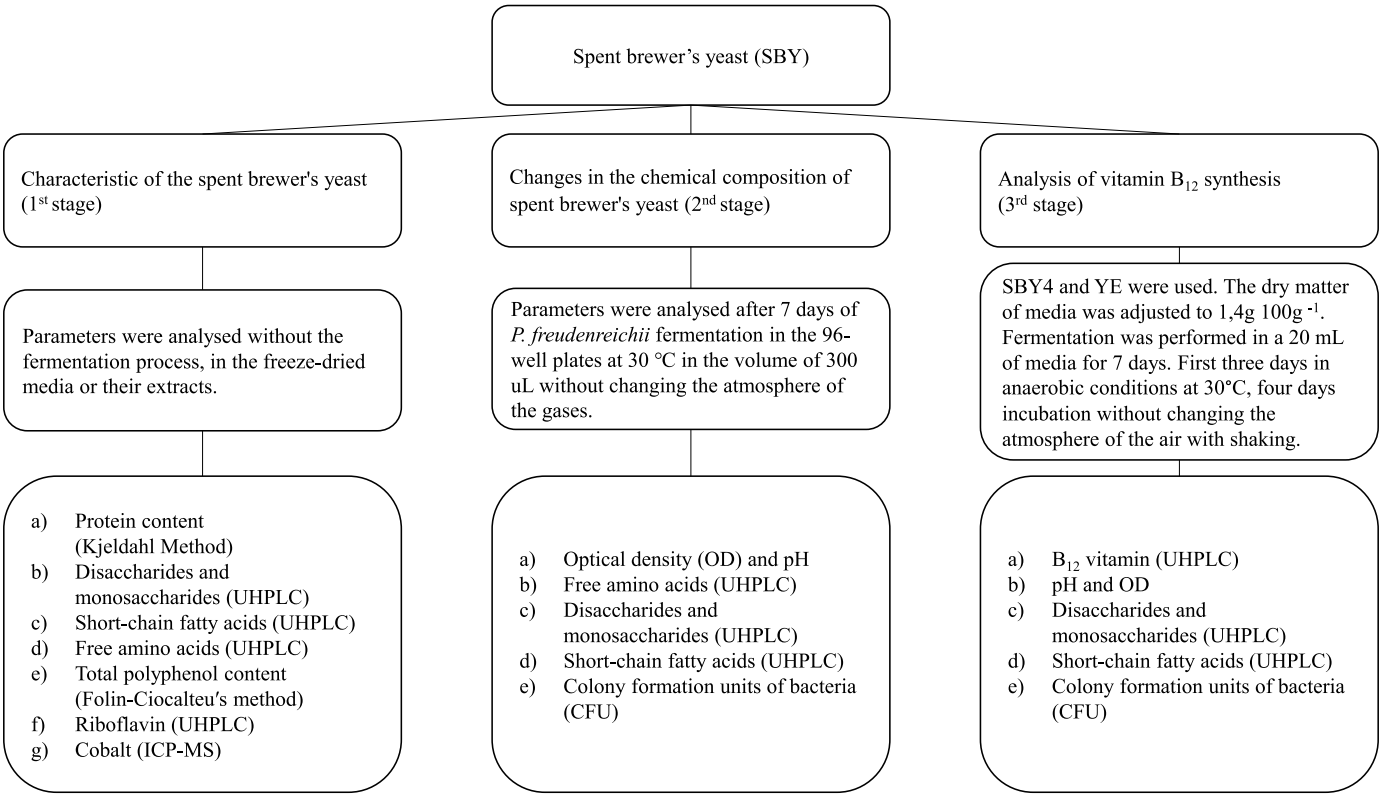

Fig. 1. Experiment scheme and stages of research.

of raw materials (Nassary and Nasolwa, 2019; Yin et al., 2023; Spinelli et al., 2019; Strøm-Andersen, 2020; Van Raamsdonk et al., 2023).

Innovatively, the study aims to investigate *P. freudenreichii*'s potential to valorize SBY into nutritionally valuable metabolites and explore the viability of using SBY as a growth medium. The study unfolds in three stages: characterizing SBY chemical composition, evaluating SBY changes under *P. freudenreichii* influence, and analyzing B_{12} synthesis potential in selected SBY along with identifying influencing factors.

2. Materials and methods

2.1. Plan of the experiments

The research plan comprised three stages, as depicted in Fig. 1 and detailed below.

In the 1st stage, we aimed to characterise the SBY by determining the content of specific chemical compounds. Unfermented material was used, with the primary goal of presenting essential features of the samples.

The 2nd stage involved assessing *P. freudenreichii* growth on SBY extracts and YE, along with changes in chemical composition due to the fermentation. The fermentation process in this research stage was optimized by preliminary experiments (data not published). Proper analyses included optical density of culture, colony formation units, pH value, and transformations in initial chemical compounds, assessing FAAs, SCFAs, disaccharides, and monosaccharides. This stage was performed on a microscale in 96-well microplates. This part of the study aimed to analyse the metabolic changes and identify the most suitable SBY for the 3rd stage.

The 3rd stage focused on assessing B_{12} synthesis on the selected media that performed best in supporting *P. freudenreichii* growth. Besides analyzing B_{12} content, parameter changes during the fermentation process were studied, including pH value, optical density, colony formation units, disaccharides, monosaccharides, and SCFAs. Optimized culture conditions from previous studies about B_{12} synthesis (Chamlagain et al., 2016; Deptula et al., 2017a; Piwowarek et al., 2018a) were used for more comparable results.

The experiment was performed in 3 independent biological replicates.

2.2. Propionibacterium freudenreichii strains and growth medium

P. freudenreichii strains DSM 4902 and DSM 20271 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH; Germany) representing subspecies *shermanii* and *freudenreichii*, respectively, were employed in this study as key type strains for *P. freudenreichii* research (Deptula et al., 2017b). These strains, known for their genetic and phenotypic distinctions, serve to exemplify the general features of the *P. freudenreichii* species (Deptula et al., 2017b). Cultivation involved routine propagation in yeast extract sodium lactate agar and broth (YEL). The YEL medium composition comprised microbiological tryptone 10 g L^{-1} (Millipore, Merck, Germany); yeast extract 10 g L^{-1} (Gibco; Thermofisher; Waltham; MA; USA), 20% sodium lactate 16,7 g L^{-1} (Thermofisher; Waltham; MA; USA); K_2HPO_4 3275 g L^{-1} (SAFC, Merck, Germany); $MNSO_4$ 56 mg L^{-1} (Supelco; Merck; Germany), and optionally bacteriological agar 10 g L^{-1} (Millipore, Merck, Germany) for solidification. The medium was sterilized (121 °C for 15 min). Bacteria activation from −80 °C involved a loop culture on YEL agar plates, followed by anaerobic incubation at 30 °C for 4 days. A single colony was selected, transferred to YEL broth, and cultured for an additional 4 days at 30 °C. These prepared *P. freudenreichii* cultures were then utilized for the research.

2.3. Spent brewer's yeast

The study utilized four distinct spent brewer's yeast (SBY) from various Polish breweries, collected in 2021: SBY1 (*Saccharomyces pastorianus* W34/70, Ciechan brewery, Ciechanów, Poland), SBY2 (*Saccharomyces cerevisiae* 3878, Jabłonowo brewery, Jabłonowo, Poland), SBY3 (*S. cerevisiae* US-05 and *Saccharomyces cerevisiae* WLP066, Palatum brewery, Warsaw, Poland) and SBY4 (*S. pastorianus* W34/70, Perła brewery, Lublin, Poland). Samples were directly obtained from the fermentation tank, deep-frozen at −80 °C, and subsequently freeze-dried using Labconco (MO, USA) freeze-drier (−45 °C, 0.11 mBar) for 6 days, without light exposure. Freeze-dried samples were stored tightly closed at −80 °C. The control sample was a microbial yeast extract from Gibco Thermo Fisher (MA, USA) obtained from autolyzed *S. cerevisiae* cells.

2.4. Spent brewer's yeast extraction and yeast extract sample preparation

To obtain transparent and user-friendly mediums, SBYs were extracted by Bzducha-Wróbel et al. (2014) method with adaptation. Briefly, 3 g of SBY and 90 mL of MilliQ-water (Merck; Germany) were sonicated in 4 cycles of 5 min using the horn sonicator with power 300 W and 20 kHz (Branson Digital Sonifier 450; Thermofisher; Waltham; MA; USA) under continuous ice cooling. The samples were cooled to 6 °C before each cycle, and the maximal reached temperature was 25 °C at the end cycle. After sonication, the pH of the extracts was adjusted to 7.0 with hydrochloric acid (Sigma-Aldrich; Merck, Germany) and sodium hydroxide (Sigma-Aldrich; Merck, Germany). The extracts were filled to 100 mL and centrifuged (12000 rpm, 25 min, 5 °C) using Eppendorf Centrifuge 5804 R (Eppendorf, Germany), microfiltered (0.22 μm, Nalgene Rapid-Flow; Thermofisher; Waltham; MA; USA), and stored at 4 °C in the dark. To prepare the control medium, 3 g of the yeast extract was dissolved in MilliQ-water, adjusted to the pH 7.0, filled to 100 mL and filtered similarly.

2.5. Fermentation conditions in the 2nd and 3rd stages of the research

In the 2nd stage, fermentation was conducted for seven days in 96-well plates (Thermofisher; Waltham; MA; USA) at 30 °C in 300 μL volume, and without changing the atmosphere of the air. Media were inoculated with 3 μL (1%, approximately 6 log CFU mL^{-1} in the initial culture) from a fresh *P. freudenreichii* culture. The plates were lidded by the producer lid and covered by a Parafilm® M to protect them against liquid evaporation. After assessment of the growth of *P. freudenreichii* in the different types of SBY extracts, SBY4 and YE as control sample were used to perform 3rd stage of the experiment. Media dry matter was adjusted to 1.4g 100 g^{-1} by adding sterile MilliQ water. Dry matter was measured using the weight method before standardizing the substance for the 3rd stage experiment. SBY extracts and YE water solution were weighted on an analytical scale. Empty glass weighing dishes were filled with 5 g of extracts, dried at 100 °C for 12 h in a laboratory dryer and weighed again after cooling in an exicator for 12 h. The obtained values were converted to dry matter content, and the results are in the supplementary materials.

In the 3rd stage, 200 μL (1%, approximately 6 log CFU mL^{-1} in the initial culture) from a 4-day-old *P. freudenreichii* culture in YEL broth at 30 °C was added to inoculate all media. The fermentation occurred in falcon tubes (50 mL) with 20 mL of media for 7 day at 30 °C. The initial three days were under anaerobic conditions without shaking, followed by four days with constant shaking at 100 rpm under a normal atmosphere at 30 °C.

2.6. Protein content

The protein content in freeze-dried SBY samples and YE was evaluated using the Kjeldahl method according to ISO 1871:2009 with a Kjeltec digestor and analyzer units (FOSS analytical A7S Hilleröd, Denmark). 100 mg of each sample was weighed, and one Kjeldahl tablet (FOSS, Denmark) was added and digested in 18 mL of concentrated (95–97%) sulfuric acid (Sigma-Aldrich; Merck, Germany) at 400 °C for

1 h. After cooling and the addition of 75 mL MilliQ the hydrolyzed samples were analysed for nitrogen content, and the nitrogen-to-protein conversion factor 6.25 was used for calculation of protein content. The analysis was performed in triplicate.

2.7. Short-chain fatty acids (SCFAs) and sugars

The SBY extracts and YE water solution, prepared following the procedures outlined in section 2.4 "*SBY extraction and YE sample preparation*" were used for analysis, along with the supernatant obtained after the fermentation during the 2nd and 3rd stages of the experiment. All analysed samples were centrifuged for 10 min at 5 °C and 10000 rpm using Eppendorf Centrifuge 5804 R (Eppendorf; Germany). Subsequently, 1 mL of the media was filtered by a 0.45 μm syringe filter into vials. The analysis conditions were according to Xie et al. (2011) with modifications. Succinic, lactic, acetic, and propionic acid, as well as sugars (disaccharides and glucose), were analysed using an HPLC system (Waters Alliance separation module e2695, Milford, MA, USA) connected to a refractive index detector (RI; Waters 2414) and a photodiode array detector (PDA; Waters 2996. An Aminex HPX-87H column 300 × 7.8 mm; Bio-Rad, Hercules; CA; USA) maintained at 40 °C with a flow rate of 0.5 mL min^{-1}, and a mobile phase of 10 mM H_2SO_4, was employed for compound separation. Quantification relied on RI detection and external standard curves ranging from 0.12 to 40 μg per injection for each analyte.

2.8. Free amino acids (FAAs)

Free amino acids were analysed in the SBY extracts and YE water solution, prepared as outlined in the chapter "SBY extraction and YE sample preparation". Additionally, analysis was performed on the supernatant after fermentation by *P. freudenreichii* DSM 4902 from the 2nd stage of the experiment. All analysed samples were centrifuged for 15 min at 5 °C and 10000 rpm using the Eppendorf Centrifuge 5804 R (Eppendorf; Germany). Subsequently, the supernatants were diluted in MilliQ water (SBY 1:1 v/v; YE 1:5 v/v) ultra-filtered in a 10-kDa ultrafiltration centrifuge tube (Amicon, Merck Millipore, Germany). FAAs were analysed in the filtered extract after pre-derivatisation using an Acquity UPLC system (Waters AccQ-Tag TM Ultra, Waters, Milford, MA, USA) described by Tuccillo et al. (2022). Amino acids were identified by their retention times, and quantifications were based on the internal standard method, utilizing L-norvaline (Waters, Milford, MA, USA) as the internal standard. Amino acid standard hydrolysate mixture (Waters, Milford, MA, USA) containing 2.5 mM of each amino acid (except cysteine 1.25 mM) was diluted to appropriate concentrations. Additionally, a 2.5 mM stock solution from asparagine, glutamine, ornithine, γ-aminobutyric acid, and tryptophan (UPS; Merck, Germany) was prepared in water and diluted. A six-point linear calibration curve (from 0.75 to 12.5 pmol $μl^{-1}$) for each amino acid was established by calculating the ratio of peak to the area of the internal standard norvaline.

2.9. Total polyphenol content

The method described by Attard (2013) was employed for the determination of the total polyphenol content. Briefly, 100 mg of SBY and YE were combined with 5 mL of 80% methanol (Supelco; Merck; Germany). Subsequently, the samples underwent extraction in an ultrasonic bath (Branson Ultrasonics™; Thermofisher; Waltham; MA; USA) at 30 °C for 10 min. Post-extraction, the samples were centrifuged using the Eppendorf Centrifuge 5804 R (Eppendorf; Germany) for 10 min at 10000 rpm and 0 °C. The supernatant was utilized for analysis, with sample extracts being appropriately diluted in Milli-Q water. For the analysis, 20 μL of the prepared sample dilution was dispensed into a 96-well plate (Thermofisher; Waltham; MA; USA), followed by the addition of 100 μL of Folin-Ciocalteu's reagent (Supleco; Merck; Germany). The plate was then incubated for 5 min at room temperature in the absence of light exposure. Subsequently, 80 μL of the 7.5% sodium carbonate (Supelco; Merck; Germany) solution in MilliQ water was added to the wells. The plate was mixed at 150 rpm for 5 min and incubated for 2 h in the dark. Measurements were taken at a wavelength (λ) of 750 nm using the Multiskan EX reader (Thermofisher; Waltham; MA; USA). Before measurement, the plate underwent a 1-min shaking by the auto-shaker of the Multiskan EX reader. The obtained values were calculated using a standard curve constructed with gallic acid (Sigma-Aldrich; Merck, Germany). The results are expressed as the equivalent of gallic acid (GAE).

2.10. Riboflavin (B_2 vitamin) content

Riboflavin was extracted and analysed with a UHPLC method described by Edelmann et al. (2019). In brief, 0.5 g of the freeze-dried SBY and YE were extracted with 15 mL of 0.1 M hydrochloric acid (Supelco; Merck; Germany) in a boiling water bath for 60 min, and the pH was adjusted to 4.5 with 2.5 M sodium acetate (Supelco; Merck; Germany) after cooling the samples on ice. The samples were then incubated with the enzymes Taka-Diastase (Sigma-Aldrich; Merck, Germany) (50 mg; 100 U mg^{-1}) and β-amylase (Sigma-Aldrich; Merck, Germany) (5 mg; 750 U mg^{-1}) in a water bath for 24 h at 37 °C with constant shaking. After centrifugation (10 min; 8000 rpm Eppendorf Centrifuge 5804 R; Eppendorf; Germany), the supernatant was collected and the final volume was adjusted to 25 mL with 0.02 M sodium acetate (Sigma-Aldrich; Merck, Germany). The samples were filtered (0.22 μm) into vials and analysed with a UPLC system (Waters, Milford, MA, USA) connected to a FLR detector set at a wavelength of 444 nm (excitation) and 520 nm (emission). Riboflavin was separated on a BEH C18 column (2.1 mm x 100 mm; 1.7 μm; Waters, Bedford, MA, USA) with an isocratic flow (0.2 mL min^{-1}) of 20 mM ammonium acetate (Supelco; Merck; Geramany) in 30% aqueous methanol (Supelco; Merck; Geramany) at a temperature of 30 °C. The injected volume was 10 μL. Quantification was based on an external standard method using a multilevel (n = 6) standard curve of riboflavin (0.01–1.0 ng/μL).

2.11. Cobalt content

The cobalt contents of SBYs and YE were determined by the Finnish Environmental Institute using a method based on microwave-assisted digestion and inductively coupled plasma mass spectrometry (ICP-MS) quantification (Nóbrega et al., 2012).

2.12. Optical density

The optical density (OD) of the bacterial cultures was measured by the Multiskan EX reader (Thermofisher; Waltham; MA; USA) in the 96-well plates (Thermofisher; Waltham; MA; USA) at wavelength λ595 nm. The volume always used for measurement was 300 μL. An unfermented sample of the respective SBY or YE was used as a blank sample. If the OD of the culture was higher than 0.6, the sample was diluted appropriately to get the absorbance from 0.0 to 0.6, and the outcome was multiplied by the dilution rate to obtain the authentic OD of the analysed culture.

2.13. Bacteria enumeration

The number of bacteria was analysed according to Naghili et al. (2013). The culture of the bacteria was serially diluted in the sterile phosphate buffer (PBS) (Sigma-Aldrich; Merck, Germany). All dilutions were cultured on the surface YEL agar media on the Petri dishes using the micro drop method (5 μL). The samples were incubated anaerobically at 30 °C for 4 days. After that, the obtained colonies were counted. The results are presented as logarithm colony-forming units per millilitre (log CFU mL^{-1}).

2.14. Vitamin B_{12} content

Vitamin B_{12} was determined as cyanocobalamin using an ultra-high performance liquid chromatography (UHPLC) method according to Chamlagain et al. (2015). The 20 mL cultures of the *P. freudenreichii* strains in the SBY4 and YE were centrifuged using an Eppendorf Centrifuge 5804 R (Eppendorf; Germany) for 15 min at 10000 rpm and 5 °C. Obtained bacterial pellets were placed in a boiling water bath for 30 min after mixing with 15 mL of extraction buffer (20.7 mmol/L acetic acid and 8.3 mmol/L sodium hydroxide, pH 4.5; Sigma-Aldrich; Merck, Germany) and 100 μl of sodium cyanide (Sigma-Aldrich; Merck, Germany) in the 1% concentration (1g 100 mL^{-1}). The solutions were cooled on ice. Samples were centrifuged for 10 min at 10000 rpm, and the supernatants were collected in the 20 mL volumetric flask. The extracts were filtered by 0.22 μm into UHPLC vials. A Waters UHPLC system (Milford, MA, USA) equipped with a PDA (361 nm) and an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 μm) was used for analysis. The mobile phase was a gradient flow of acetonitrile (Sigma-Aldrich; Merck, Germany) and Milli-Q water with 0.025% trifluoroacetic acid (0.32 ml min^{-1}) (Sigma-Aldrich; Merck, Germany). An external calibration curve for quantitation was prepared by injecting six cyanocobalamin standards (0.015–0.75 ng/μL) in duplicate.

2.15. pH value

All performed measurements of the pH values were obtained by a Mettler Toledo MP225 pH meter (Mettler-Toledo, Switzerland).

2.16. Statistical analysis

The results were checked for the homogeneity of variance by the Brown–Forsythe test, and the normality test Shapiro–Wilk was performed. One-way ANOVA or Student's t-test was performed. Significant differences between the samples were determined by the Tukey HSD post-hoc test. The data before creating the variability graph (Fig. 5) of the studied parameters were standardised. Principal component analysis (PCA) was performed based on the correlation matrix. All statistical analyses and graph preparation were performed using Statistica 13 (StatSoft, Poland). The difference was considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Characteristic of the chemical composition of the spent brewer's yeast (1st stage of study)

The chemical composition of the tested SBY and control sample (YE, microbiological yeast extract) is presented in Table 1. YE contained the highest protein (64.55 g 100 g^{-1}) among the tested samples (31.3–40.3 g 100 g^{-1}). Literature suggests that SBY typically have total protein in the range of 40–49 g 100 g^{-1} (Marson et al., 2020). However, the autolysis of yeast cells can influence the content of various compounds in the SBY, including protein levels at 18–45 g 100 g^{-1} (Alves et al., 2021). Spent brewer's yeast is usually affected by the autolysis process (Jaeger et al., 2020; Marson et al., 2020); therefore, the protein content of the samples was generally lower.

In the samples, different levels of the disaccharides were observed. Maltose is presumed to be the main disaccharide in SBY, and sucrose in YE due to the beer and yeast extract production process (Li et al., 2020; Tomé, 2021). These disaccharides presumably occurred with trehalose, found in yeast cells (François and Parrou, 2001). However, a comprehensive separation of the disaccharides was not feasible under the employed analysis conditions.

In the case of glucose, its highest content was observed in the SBY4 sample. In other samples, the content of this compound was significantly lower ($p < 0.05$) (Table 1). No prior studies were identified on the content of glucose in SBY. The elevated glucose content in the SBY4 sample may be attributed to the enzymatic lysis of trehalose and glycogen, which serve as reserve carbohydrates for yeast (François and Parrou, 2001). Other possible explanations include the low metabolic state of the yeast post-beer wort fermentation and serial repitching, ultimately slowing down glucose catabolism and leading to its accumulation in the SBY (Wang et al., 2019; Xia et al., 2022).

The YE sample was the richest in succinic and acetic acid, whereas lactic acid was the most abundant in the SBY3 and SBY4 samples. These detected organic acids are characteristic compounds for brewer's yeast metabolism (Li and Liu, 2015). Propionic acid was not detected in any of the samples, and it is not a specific metabolite for yeast (Ding et al., 2022; Gonzalez-Garcia et al., 2017).

For total polyphenol content, the highest was in the SBY3 sample (12.7 mg GAE 100 g^{-1}) and the lowest in the SBY4 (0.7 mg GAE 100 g^{-1}). The concentration of polyphenols in SBY depends on the content of hops added to the wort and, consequently, the migration of phenolic compounds (Bryant and Cohen, 2015).

YE had the highest concentration of vitamin B_2 (7.9 mg 100 g^{-1}), while the SBY samples ranged from 0.5 to 0.7 mg 100 g^{-1}, consistent with the literature (Marson et al., 2020). Notably, vitamin B_2 serves as the precursor for DMBI, the lower ligand of biologically active B_{12} (Deptula et al., 2017a).

Cobalt levels were highest in YE (127.8 μg 100 g^{-1}), with SBY samples ranging from 8.8 (SBY1) to 14.2 μg 100 g^{-1} (SBY3), aligning with published data (Marson et al., 2020). Cobalt is essential for B_{12} synthesis by *P. freudenreichii* (Piwowarek et al., 2018b).

The free proteogenic and non-proteogenic amino acids (FAAs) profile (including GABA, hydroxyproline, and ornithine) is shown in Fig. 2 and Supplementary Table 1. The individual FAA concentrations varied among the samples ($p < 0.05$). Tryptophan, D-proline (absent in YE), and cysteine had the lowest concentrations. Arginine, alanine, proline, and glutamic acid were generally abundant, except for glutamic acid in SBY3, which was low (191.6 mg 100 g^{-1}). The reduced glutamic acid

Table 1
Content of selected chemical compounds in the tested SBY and YE; n = 3; Co, n = 1.

		SBY1	SBY2	SBY3	SBY4	YE
Protein	g $100g^{-1}$	40.3^{a}±0.3	38.9^{ab} ± 1.2	38.6^{b} ± 0.9	31.3^{c}±0.1	64.6^{d} ± 0.8
Disaccharides		1.7^{a}±0.1	0.9^{b} ± 0.1	0.7^{b} ± 0.1	5.0^{c}±0.3	8.5^{d} ± 0.5
Glucose		1.4^{a}±0.1	1.2^{a}±0.1	1.1^{a}±0.1	2.8^{b} ± 0.4	0.9^{a}±0.2
Succinic ac.		0.2^{a}±0.0	0.2^{a}±0.0	0.3^{a}±0.0	0.1^{a}±0.0	1.6^{b} ± 0.3
Lactic ac.		1.5^{a}±0.0	1.9^{b} ± 0.0	2.7^{c}±0.0	2.4^{c}±0.1	2.1^{b} ± 0.2
Acetic ac.		nd	nd	0.3^{a}±0.0	0.3^{bc}±0.0	0.3^{c}±0.0
Propionic ac.		nd	nd	nd	nd	nd
TPC	mg GAE $100g^{-1}$	7.6^{a}±0.1	3.3^{b} ± 0.1	12.7^{c}±0.1	0.7^{d} ± 0.1	1.8^{e}±0.5
Riboflavin	mg $100g^{-1}$	0.6^{a}±0.0	0.7^{a}±0.0	0.7^{a}±0.0	0.5^{a}±0.0	7.9^{b} ± 0.2
Co	μg $100g^{-1}$	8.8	8.3	14.2	9.2	127.8

Explanatory notes: SBY1, SBY2, SBY3, and SBY4 mean the samples of the spent brewer's yeast and YE means microbiological yeast extract; letters a, b, c, d, and e mean the statistical difference between the samples in the posthoc Tukey's test ($p < 0,05$); the value behind ± indicates the standard deviation; nd – not detected.

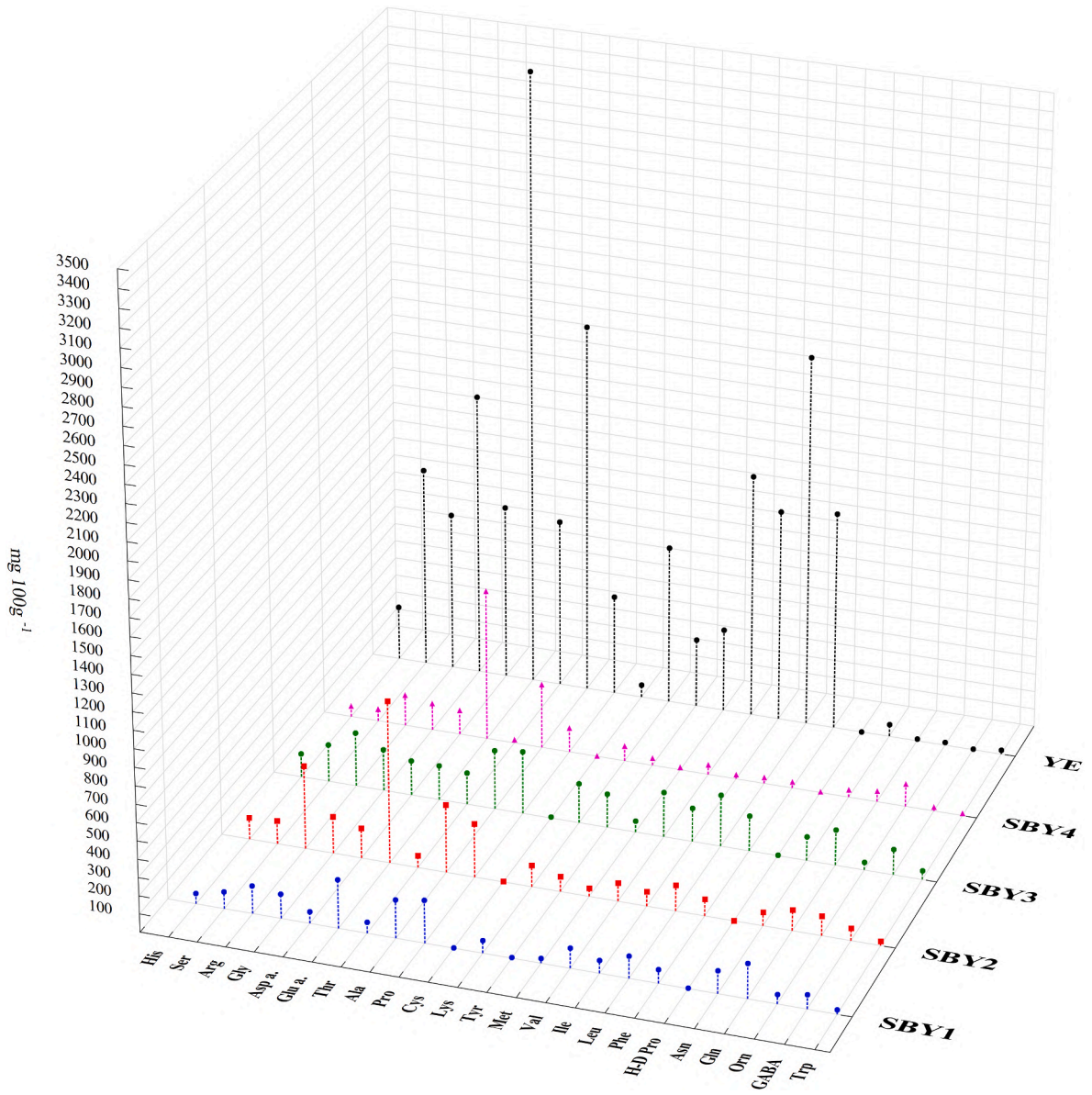

Fig. 2. Free amino acids (FAAs) profile of the tested SBY and control sample (yeast extract; YE); n = 3 Explanatory notes: SBY1, SBY2, SBY3, and SBY4 - the samples of the spent brewer's yeast; YE - yeast extract.

content in SBY3 may result from its conversion to GABA. SBY3 contained the highest GABA concentration, suggesting the possible transformation of glutamic acid through autolysis or lactic acid bacteria activity. Both reactions involve l-glutamate-1-carboxylase, catalysing the decarboxylation of glutamic acid to GABA (Jacob et al., 2019a, 2019b; Yogeswara et al., 2020). It is essential to note that SBY3, from a small craft brewery with less stringent hygienic monitoring (Rodríguez-Saavedra et al., 2020), may be prone to contamination by lactic acid bacteria.

Despite significant differences ($p < 0.05$) between the content of individual FAAs in the samples, a consistent profile of these compounds is observed for all analysed samples. There is substantial evidence in the literature regarding the FAA composition of brewer's yeast. Several papers have tested different models for extracting FAAs from SBY (Jacob et al., 2019a, 2019b; Stănilă et al., 2018). In the study conducted by Jacob et al. (2019a), a comparable method was used to isolate FAAs, resulting in a slightly higher concentration of FAAs compared to the levels reported here. However, the extraction takes 10 min longer, and the power of the sonication was 100 W higher in the Jacob et al. (2019a) study. In the same study, various extraction techniques of the FAAs from SBY were checked, such as cell mill or autolysis. Statistical differences were obtained between these extraction methods. In other research, the extraction was performed by sonication with solvent change (Stănilă et al., 2018). It was found that the type of solvent affects the final recovery of amino acids from SBY. In conclusion, the FAA content in the different studies depends on the production method. On the other hand, variations between SBY samples occurred. These differences can be due to yeast species and strain (Berlowska et al., 2017; Vieira et al., 2016), yeast physiological conditions dependent on growth medium, nutrients and access to oxygen (Granata et al., 2021; Jacob et al., 2019b), age of the culture (Aris et al., 2012), environmental stress (Kieliszek et al., 2019) and level of autolysis (Bianchi et al., 2019; Jacob et al., 2019a). In the presented results, the main differences focused on the origin of SBY from different breweries, differences in species, age of cultures, and types of beers for which they were used. All these factors influenced the content of FAAs in the analysed samples.

3.2. - Changes in the nutrient composition of spent brewer's yeast (2nd stage of study)

The changes in FAA contents before and after fermentation are depicted in Fig. 3. The FAAs profile changed after one week of fermentation in all tested samples. In SBY1, the content decreased for D-proline, asparagine, glutamine, GABA, and ornithine. In SBY2, the content of aspartic acid, asparagine, glutamine, ornithine, and GABA decreased. In SBY3, D-proline, asparagine, and GABA were reduced. In SBY4, the amount of serine, glycine, aspartic acid, glutamic acid, alanine, D-proline, asparagine, glutamine, ornithine, and GABA decreased. In the YE sample, the following FAAs were reduced: serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, cysteine, asparagine, glutamine, and GABA. Other unlisted FAAs increased compared to the unfermented samples. An interesting observation is a variation in levels of different FAAs between the SBY and YE

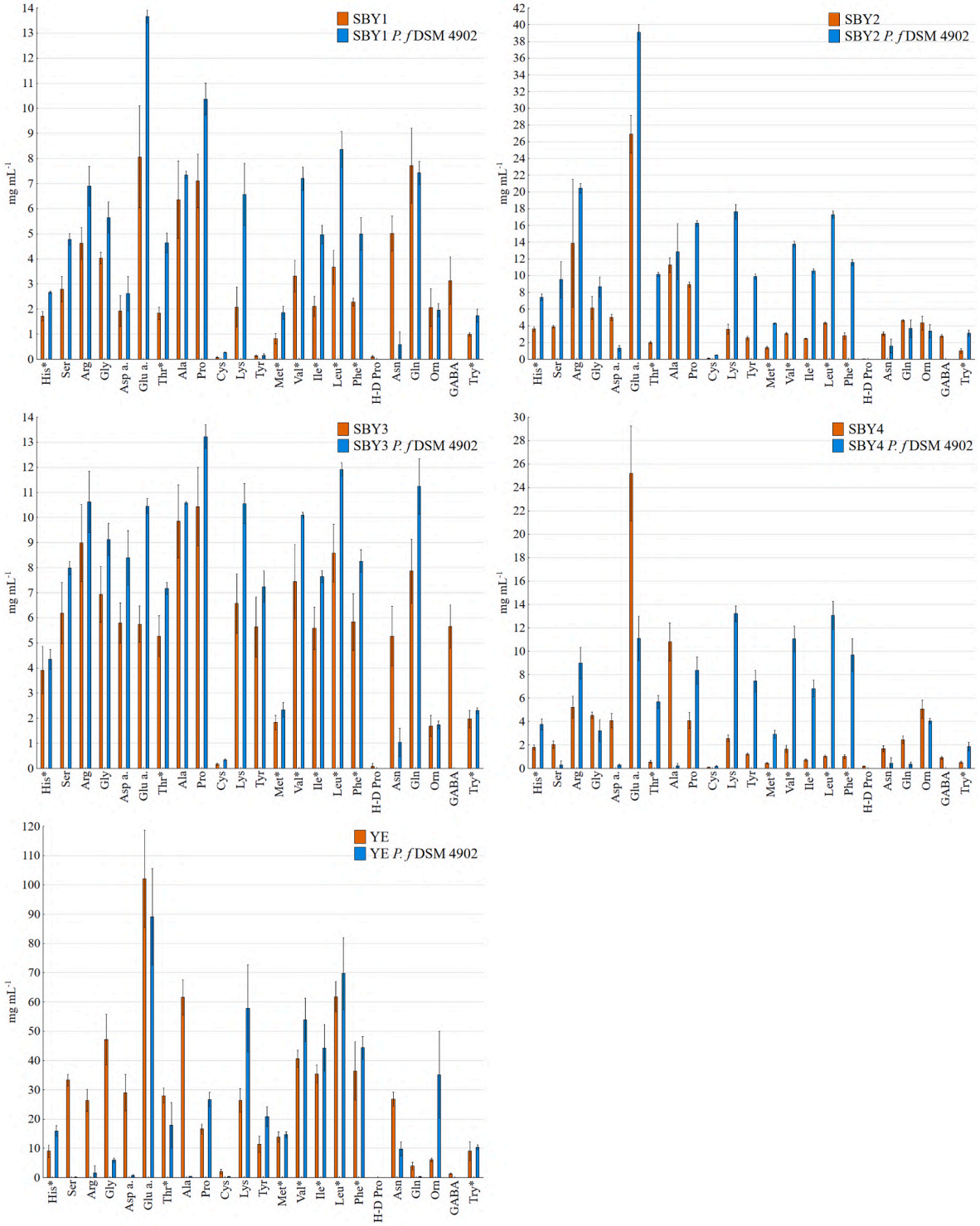

Fig. 3. Free amino acids (FAAs) profile changes before and after *P. freudenreichii* DSM 4902 fermentation of the tested SBY sample and the YE; n = 3 Explanatory notes: SBY1, SBY2, SBY3, and SBY4 - the samples of the spent brewer's yeast; YE - yeast extract; * - essential amino acids; error bars represent the confidence interval ($p < 0.05$). This result was confirmed by a *t*-test.

samples. Specifically, in the SBY samples, the content of a few tested FAAs increased after the fermentation period. These differences were probably due to the activity of proteolytic enzymes released from yeast cells as a result of autolysis and mechanical disintegration (Jacob et al., 2019a; Marson et al., 2020). *S. pastorianus* and *S. cerevisiae* produce several proteolytic enzymes like carboxypeptidase Y and S, aminopeptidase I and Y, proteinases A and B, and dipeptidyl aminopeptidase. Due to the lack of thermal inactivation of SBY, the proteins contained in the SBY culture media were degraded into smaller peptides and ultimately into amino acids (Hecht et al., 2014).

P. freudenreichii possesses several types of peptidases, including aminopeptidase, iminopeptidase (PepI), X-prolyl dipeptidyl aminopeptidase (PepX), and endopeptidase. However, *P. freudenreichii's* contribution to proteolysis can be limited because its proteolytic activity is lower than that of yeast's autolytic enzymes (Thierry et al., 2011). The catabolism of amino acids may differ, as *P. freudenreichii* has notable abilities in metabolizing amino acids, which ultimately break down into flavour compounds, as demonstrated in the process of making Swiss-type cheeses (Thierry et al., 2004). In the study by Aburjaile et al. (2016), the FAA profile was analysed after 3, 9, and 11 days of *P. freudenreichii* fermentation in YEL media. The changes observed in the FAA profile were similar to those obtained in this study. The content of the following amino acids: aspartic acid, threonine, serine, glutamic acid, glutamine, glycine, alanine, cysteine, and arginine varied differently compared to the study of Aburjaile et al. (2016). These variations occurred especially in SBY3, but also to a lesser extent in SBY1, and SBY2. Nonetheless, the variabilities in SBY4 and YE were similar to those observed in the previous study of Aburjaile et al. (2016).

Based on the conducted analysis, alterations appear to be attributed to the polyphenol content in the SBY medium. Notably, SBY3 exhibited the highest total polyphenol content (Table 1). Polyphenols are recognized for their antimicrobial effects, potentially affecting bacterial cell metabolism or causing damage (Daglia, 2012). The impact of polyphenols on the final cell densities of *P. freudenreichii* in the SBY medium is reflected in the results (Table 2). Samples with a higher content of polyphenols displayed lower *P. freudenreichii* colony-forming units, correlating with reduced metabolic activity.

The FAAs that are commonly reported in literature to be consumed from the environment by *P. freudenreichii*, are aspartic acid, asparagine, glycine, alanine, and serine. Asparagine metabolizes to aspartic acid, further converted to fumarate or succinate, generating energy-an assumed intermediate energy substrate for *P. freudenreichii* (Piveteau, 1999; Thierry et al., 2011).

Glycine reduction SBY4 and YE, along with increased proline, is likely attributed to yeast proteolytic enzymes from SBY and *P. freudenreichii* synthesized proline iminopeptidase (Pip) (Neviani et al., 2002). Glycine and proline play a protective role against osmotic stress in the metabolism of *P. freudenreichii* (Gagnaire et al., 2015).

Alanine and serine notably decreased in SBY4 and YE, possibly converted into pyruvate, an energy source for *P. freudenreichii* (Dalmasso et al., 2012). Remarkably, GABA reduction in all fermented samples challenges the common belief of its extracellular secretion, hinting at a possible transformation into succinic acid via the Wood-Werkman cycle (Guan et al., 2013; Liu et al., 2021). Noteworthy is the similarity in the FAA transformations between YE and SBY4 samples.

In SBY4, the lower final pH (4.6) compared to other SBY samples (not less than 5.2) likely explains the difference in FAA profiles. The proteolytic activity originating from brewer's yeast autolysis probably accounts for the variations in FAA contents in the *P. freudenreichii* fermented SBY samples. These proteolytic enzymes have reported optimal activities within a pH range of 5.0–7.0 (Vieira and Delerue-Matos, 2020). The highly acidic conditions in SBY4 likely inhibited the activity of proteolytic enzymes, resulting in a reduced rate of protein degradation. Consequently, the conversion of FAAs in SBY4 was comparable to that in the YE sample, where enzyme activity was absent.

Crucially, no studies have explored the use of SBY or YE medium alone as a nutrient source for *P. freudenreichii*. Adapting biochemical metabolic pathways to generate essential metabolites reflects the observed differences in FAA metabolism (Aburjaile et al., 2016; Dalmasso et al., 2012; Gagnaire et al., 2015).

An intriguing observation is the increase in free essential amino acids (EAA) in SBY, attributed to *P. freudenreichii* and yeast autolytic enzymes. This aligns with YE results, except for threonine, suggesting a direct impact of *P. freudenreichii* on EAA formation. Literature points to the effects of EAA on human regulatory functions, including muscle protein synthesis, muscle loss prevention, and control of glucose metabolism (Rondanelli et al., 2011; Xiao and Guo, 2022).

Sugars and SCFAs fermentation in the tested samples (Fig. 4) reveals comparable metabolism by *P. freudenreichii* strains, except for increased propionic acid production in SBY4 ($p < 0.05$). Disaccharides increase in SBY1 ($p < 0.05$) and SBY3, possibly originating from maltose-based polysaccharides. Chromatograms of non-fermented SBY samples showed peaks at the retention times characteristic of oligosaccharides (data not shown), reduced in fermented samples (unpublished results). Precise identification was impossible under applied analytical conditions. The second factor for increasing disaccharides in these samples might be trehalose synthase in environmentally stressing conditions by *P. freudenreichii*, later converted to maltose (Cardoso et al., 2007; Ruhal et al., 2013). This phenomenon is perhaps associated with a high content of polyphenols, which were most abundant in these samples. In SBY2 and SBY4, disaccharide content decreased after fermentation ($p < 0.05$). Glucose content in all SBY samples increased during fermentation compared to non-fermented samples ($p < 0.05$). Disaccharide content in YE did not change during fermentation ($p > 0.05$), while glucose was metabolized totally by *P. freudenreichii*. It is essential to note that *P. freudenreichii* lacks enzymes for the degradation of carbohydrates presumably present in examined samples (e.g. trehalose and maltose), and sucrose metabolism is limited (Piveteau, 1999; Piwowarek et al., 2018a). Nevertheless, *P. freudenreichii* catabolizes oligosaccharides characteristic of malt, such as maltotriose (Cardoso et al., 2007), leading to an increase in disaccharide and glucose content. The second aspect affecting the disaccharides and glucose quantity was the presence of autolytic amylolytic enzymes in SBY, possibly causing the decomposition of maltose and oligosaccharides characteristic of beer (Londesborough, 2001; Piddocke et al., 2009; Rautio and Londesborough, 2003).

The lactic acid content was significantly reduced in all samples post-fermentation. Lactic acid serves as a primary energy source for *P. freudenreichii*, converting to pyruvate in later metabolism, entering the Wood-Werkman cycle (Piveteau, 1999; Thierry et al., 2011). Succinic acid levels in all SBY samples notably decreased due to fermentation ($p < 0.05$). Succinic acid, a crucial substrate in the Wood-Werkman

Table 2
OD λ 595, colony formation units (CFU) and pH value after fermentation of the tested SBY sample and the control sample (YE); n = 3.

Sample	OD λ 595	CFU log mL^{-1}	pH
SBY1 *P. f* DSM 4902	$0.2^{a}\pm0.0$	7.7^{a}	$5.6^{a}\pm0.4$
SBY1 *P. f* DSM 20271	$0.1^{a}\pm0.0$	7.7^{a}	$5.3^{a}\pm0.1$
SBY2 *P. f* DSM 4902	$0.2^{a}\pm0.0$	8.4^{b}	$5.2^{ab}\pm0.0$
SBY2 *P. f* DSM 20271	$0.2^{a}\pm0.0$	8.3^{b}	$5.0^{abc}\pm0.1$
SBY3 *P. f* DSM 4902	$0.1^{a}\pm0.0$	6.7^{c}	$5.8^{ad}\pm0.3$
SBY3 *P. f* DSM 20271	$0.02^{a}\pm0.0$	6.7^{c}	$5.9^{ade}\pm0.1$
SBY4 *P. f* DSM 4902	$3.6^{b}*\pm0.5$	9.2^{d}	$4.7^{c}\pm0.0$
SBY4 *P. f* DSM 20271	$1.7^{c}*\pm0.2$	8.7^{e}	$4.7^{c}\pm0.0$
YE *P. f* DSM 4902	$1.5^{c}\pm0.2$	9.5^{f}	$6.3^{de}\pm0.0$
YE *P. f* DSM 20271	$1.3^{c}\pm0.3$	9.6^{f}	$6.4^{e}\pm0.2$

Explanatory notes: SBY1, SBY2, SBY3, and SBY4 - the samples of the spent brewer's yeast; YE - yeast extract; letters a, b, c, d, e and f mean the statistical difference between the samples in the post-hoc Tukey's test ($p < 0,05$); the value behind ± indicates the standard deviation; * - precipitation of sediment from the medium after fermentation.

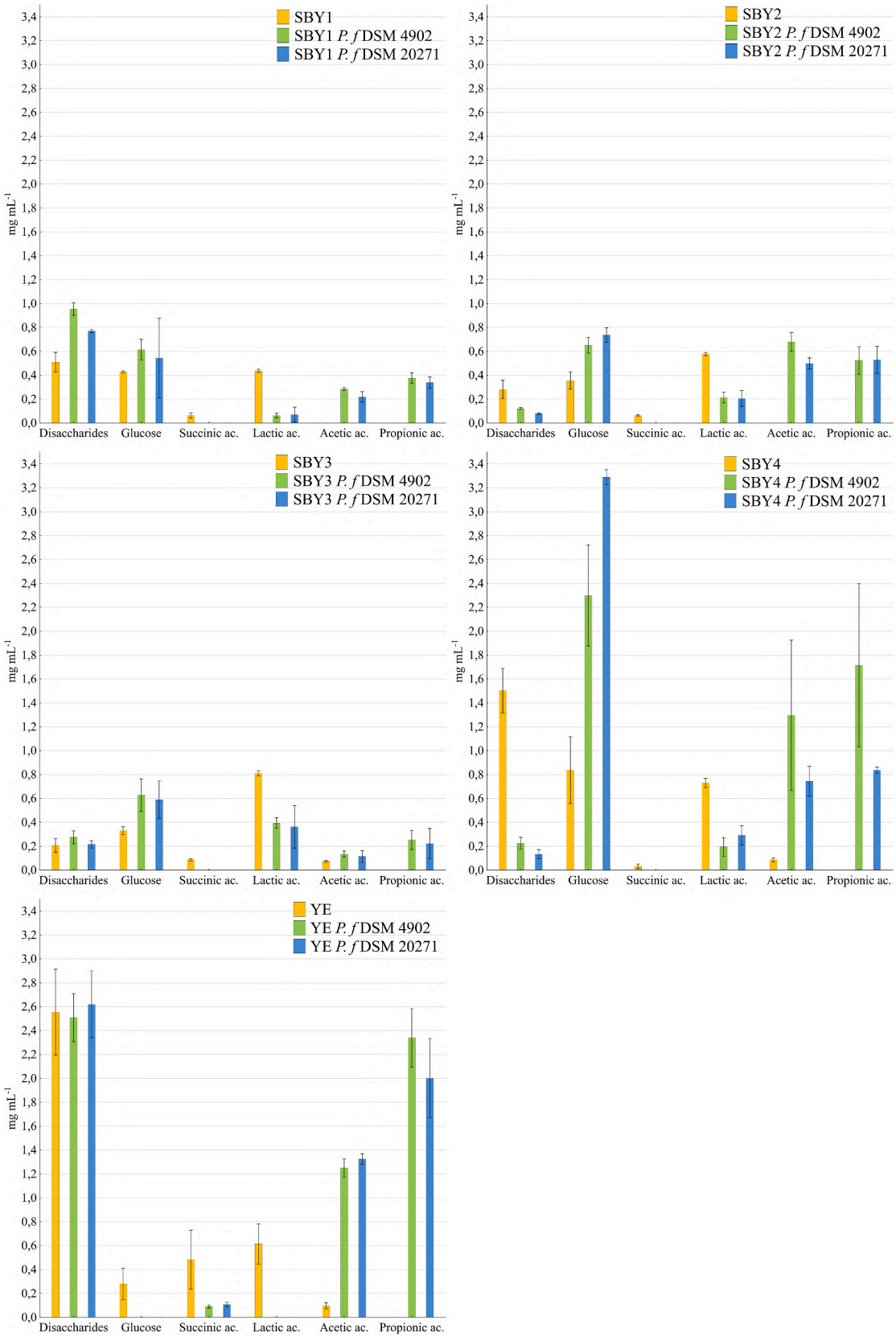

Fig. 4. Sugars and short-chain fatty acids (SCFAs) changes before and after *P. freudenreichii* fermentation of the tested SBY sample and YE; n = 3 Explanatory notes: SBY1, SBY2, SBY3, and SBY4 - the samples of the spent brewer's yeast; YE - yeast extract; error bars represent the confidence interval ($p < 0.05$). This result was confirmed by Tukey's HSD post hoc test after ANOVA analysis.

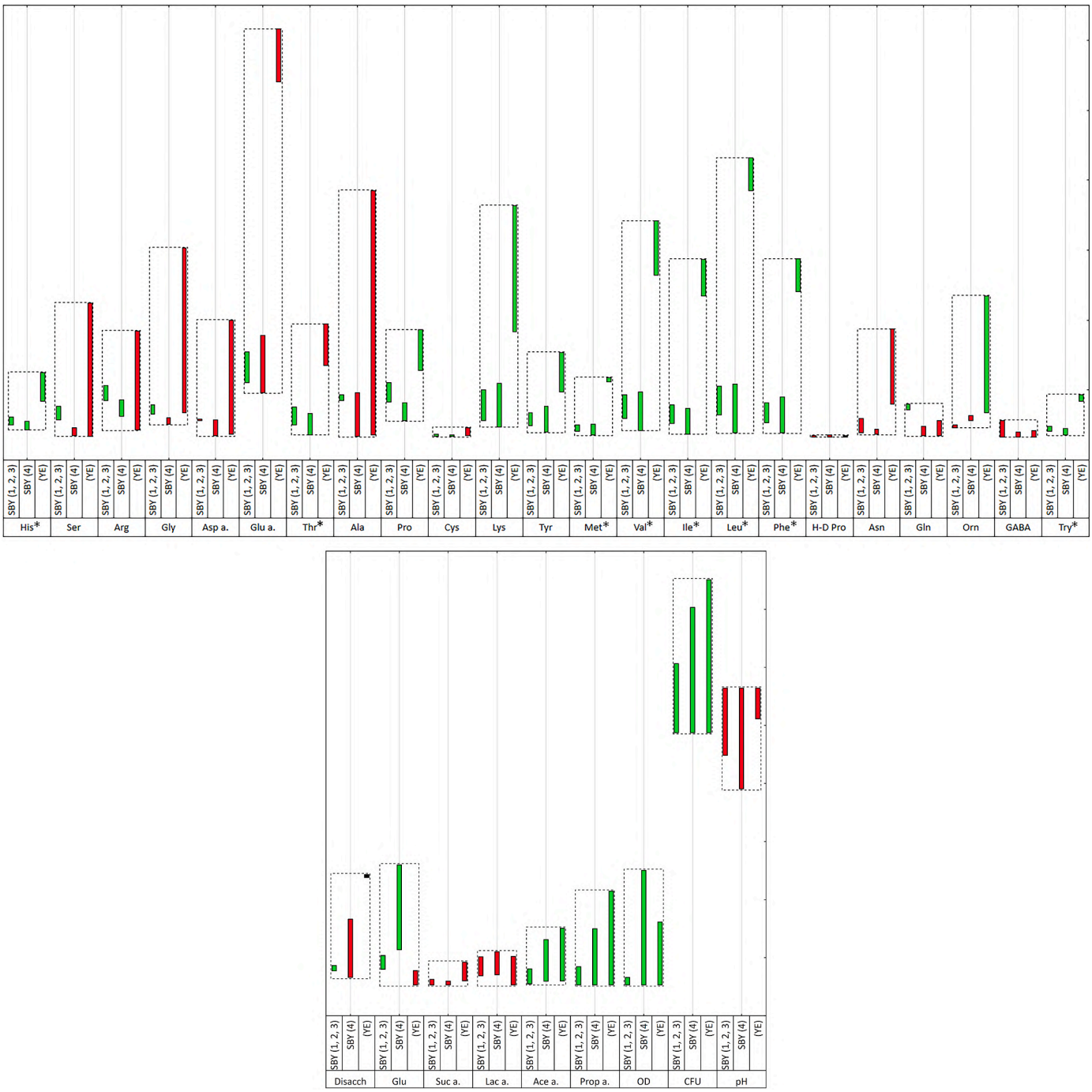

Fig. 5. Graphs of the variability of the tested chemical compounds and parameters by *P. freudenreichii* fermentation separately for SBY4, YE, and SBY (1, 2, 3) samples

Explanatory notes: SBY1, SBY2, SBY3, and SBY4 - the samples of the spent brewer's yeast; YE - yeast extract. Each bar in the chart indicates the variability of an analysed parameter or chemical compound. The longer the bar, the greater the volatility. The location of the bar on the graph indicates the level of the given parameter in the tested materials. If the bar is green, it means that the value of the tested substance or parameter increased after the fermentation period. A black bar means that there was no change, and a red bar means that the value decreased. The data used to prepare the graph has been normalized; * - essential amino acids; disacch - disaccharides. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cycle, decreased in all samples, converting to propionic acid (Feng et al., 2010; Thierry et al., 2011). Acetic and propionic acid concentrations significantly increased in all analysed samples post-fermentation ($p < 0.05$), with the highest increase observed in SBY4 and YE samples (Fig. 4). Propionic and acetic acid, as short-chain fatty acids (SCFAs), are potential dietary postbiotics, offering health benefits (Salminen et al., 2021; Shimizu et al., 2019). They play roles in energy regulation, immunostimulation, and cardiovascular health by modulating triglyceride concentrations (Xiong et al., 2022). Acetic acid, produced by reducing pyruvate for bacterial redox balance (Dalmasso et al., 2012; Thierry et al., 2011) varies based on substrate, environmental conditions, and strain properties (Thierry et al., 2011). Differences in propionic acid synthesis, particularly in the SBY4 sample, likely stem from strain-specific adaptations to the media and variations in resistance to environmental stress (Gaucher et al., 2019; Tarnaud et al., 2020). *P. freudenreichii* fermentation capacity and acid production depend on

environmental factors and strain-specific characteristics. Despite strain-specific variations, the metabolic changes in sugars and acids in SBY and YE align with *P. freudenreichii* characteristics (Thierry et al., 2011). All essential metabolic features for *P. freudenreichii*, including lactic acid catabolism, succinic acid, and propionic and acetic acid anabolism, were evident (Aburjaile et al., 2016; Dank et al., 2022; Farhadi et al., 2013). The results demonstrate SBY's potential for propionic and acetic acid synthesis. Previous studies indicated propionic and acetic acid production from different by-products, like sweet sorghum bagasse hydrolysate and apple pomace, with higher fermentable sugar levels (Ammar et al., 2020; Piwowarek et al., 2022). However, these findings provide a basis for further research in this area.

Table 2 presents the optical density, colony formation units, and pH values after fermentation of the tested SBY sample and the YE. The highest bacterial cell densities were observed in the YE media, and SBY4, and the lowest in SBY3 and SBY1. After fermentation, the lowest pH values were detected in the SBY4 samples and the highest in the YE sample ($p < 0.05$). In the SBY4 samples, precipitation of sediment from the medium occurred during fermentation, impacting the high value of OD measurement. This sediment was likely due to protein denaturation in the acidic environment.

A direct correlation between the growth of *P. freudenreichii* and the polyphenol content in the SBY samples was observed. Higher levels of phenolic compounds (likely from hops) in the SBY media inhibited the growth of *P. freudenreichii* significantly. As previously mentioned, polyphenolic compounds have an extremely broad antimicrobial activity based on numerous mechanisms (Daglia, 2012). Among the tested media, YE provided the best conditions for *P. freudenreichii* growth.

Despite intensive growth in the YE samples, the pH values decreased only slightly (from pH 7.0 to 6.3–6.4). Proteins and amino acids, with buffering properties, act as weak acids or bases to control pH (Karow et al., 2013). Phosphate salts also play a significant role in pH stabilization (Poznanski et al., 2013). YE had the highest protein and FAAs content (Table 1, Fig. 2), with evidence of a high phosphorus salt content (Christ and Blank, 2019). Since yeast extract is produced as a concentrate, the concentration of these substances is increased (Tao et al., 2023). The lower fermentable sugar content (glucose) in the YE sample compared to the SBY samples could influence pH change during fermentation.

Results in Fig. 5 summarize changes in chemical compounds and parameters in the second stage of experiments. The variability of the tested compounds and parameters is separately presented for YE and SBY4 samples, and collectively for SBY1, SBY2, and SBY3 samples due to their similar changes. This presentation method facilitates the observation of general shifts in the composition of the growth media throughout the fermentation period. The variation in the analysed compounds was more consistent in the SBY1, SBY2, and SBY3 samples. Notably, sample SBY4 exhibited a distinct profile of the tested parameters compared to all other SBY samples, resembling the profile changes seen in the YE sample. It is crucial to highlight the novel alterations in metabolites, observed for the first time in SBY and YE during the growth of *P. freudenreichii*.

3.3. Analysis of vitamin B_{12} synthesis (3rd stage of study)

Table 3 presents B_{12} analysis in SBY4 and YE fermented by *P. freudenreichii* and the following parameters: optical density, colony formation units, and pH. SBY4 was chosen for further study due to *P. freudenreichii*'s highest final cell density among the tested SBY media. B_{12} synthesis in YE samples exceeded that in SBY4 by nearly eight times. The low pH (around 4.5) in SBY4 imposed a significant constraint on B_{12} synthesis, attributed to reduced enzyme activities and slowed metabolism of *P. freudenreichii* under acidic conditions (Ahmadi et al., 2017). Optimal B_{12} production necessitates maintaining a neutral pH environment. Importantly, the produced B_{12} was in its active form, not a pseudo-vitamin, a distinction extensively elucidated by Chamlagain et al. (2015). Additionally, it's noteworthy that the tested bacterial strains exhibited exceptional growth under the specified conditions and media.

Table 3
Vitamin B_{12} content, OD λ 595, colony formation units (CFU) and pH values after fermentation of the SBY4 and the YE samples in the condition applied to the B_{12} synthesis; n = 3.

Sample	B_{12} vitamin (ng mL^{-1})	OD λ 595	CFU log mL^{-1}	pH
YE *P. f* DSM 4902	214.3^{a}±25.2	1.5^{ab} ± 0.2	9.4^{a}	8.1^{a}±0.1
SBY4 *P. f* DSM 4902	23.5^{b} ± 4.3	2.3^{c*}±0.1	9.0^{ab}	4.5^{b} ± 0.0
YE *P. f* DSM 20271	188.5^{a}±13.8	1.2^{a}±0.2	9.4^{ab}	7.9^{a}±0.4
SBY4 *P. f* DSM 20271	25.2^{b} ± 2.4	1.6^{b*}±0.1	9.4^{b}	4.6^{b} ± 0.0

Explanatory notes: SBY4 - the sample of the spent brewer's yeast; YE - yeast extract; letters a and b mean the statistical difference between the samples in the posthoc Tukey's test ($p < 0,05$); the value behind ± indicates the standard deviation; * - precipitation of sediment from the medium after fermentation.

This study presents the first application of SBY and YE for B_{12} synthesis. However, other researchers have used other by-products. Assis et al. (2020) investigated the impact of liquid protein residue from soybean as a substrate addition, enhancing B_{12} synthesis efficiency (0.6 mg g^{-1} of B_{12} in dw of biomass). This approach involved cobalt and DMBI supplementation, yielding notable B_{12} synthesis (Chamlagain et al., 2017). Wang et al. (2020) similarly utilized corn stalk in conjunction with cobalt (0.05 mg mL^{-1} B_{12} of culture), achieving substantial synthesis. Piwowarek et al. (2022) compared apple pomace and potato wastewater, achieving optimal B_{12} content in a 1:1 ratio (289.8 μg 100 g-1 wet biomass). Single by-product media resulted in half the synthesis (126.6 and 159.9 μg 100 g^{-1} wet biomass). Chamlagain et al. (2017) reported values comparable to SBY in cereal products, varying with the strain used (9–37 ng g^{-1} of food matrix), with no by-product additive. YE samples surpassed literature yields, excluding the cobalt and DMBI supplementation studies. Additionally, two-step fermentation conditions (anaerobic followed by aerobic) are most often used in B_{12} synthesis studies (Chamlagain et al., 2015, 2016; Deptula et al., 2017a; Piwowarek et al., 2018a). Microaerophilic fermentation optimisation warrants future investigation.

Fig. 6 illustrates the variation in sugars and SCFAs in SBY4 and YE samples before and after fermentation under conditions employed for B_{12} synthesis. In SBY4 samples, disaccharides, glucose, lactic acid, and succinic acid levels decreased, while acetic and propionic acids increased after fermentation. In the YE sample, disaccharide content was slightly reduced, glucose was metabolized, and lactic and succinic acid content decreased. Acetic acid content remained relatively stable ($p > 0.05$). *P. freudenreichii* metabolism produced propionic acid. These changes differed from those in the 2nd stage of the experiment with different fermentation conditions. Attention should be given to glucose metabolism and its precursors, like disaccharides (maltose, trehalose), released into SBY4 samples. The decrease in disaccharide content in SBY4 samples may be due to yeast-derived glucosidases, described in the 2nd stage of the study. High glucose increased SCFA production, impacting the acidification of the growth environment (Piwowarek et al., 2018a). Anaerobic growth led to rapid acidification, hindering *P. freudenreichii* metabolism and impeding B_{12} synthesis in SBY4, resulting in a lower yield (Thierry et al., 2011). This phenomenon did not occur in YE samples due to the low glucose content and buffering properties of yeast extract.

Switching from anaerobic to aerobic fermentation conditions with oxygen access led to the oxidation of propionic and acetic acids, in line with the reversal of the Wood-Werkman cycle (Dank et al., 2021; Thierry et al., 2011). *P. freudenreichii* was previously shown to use acetic and

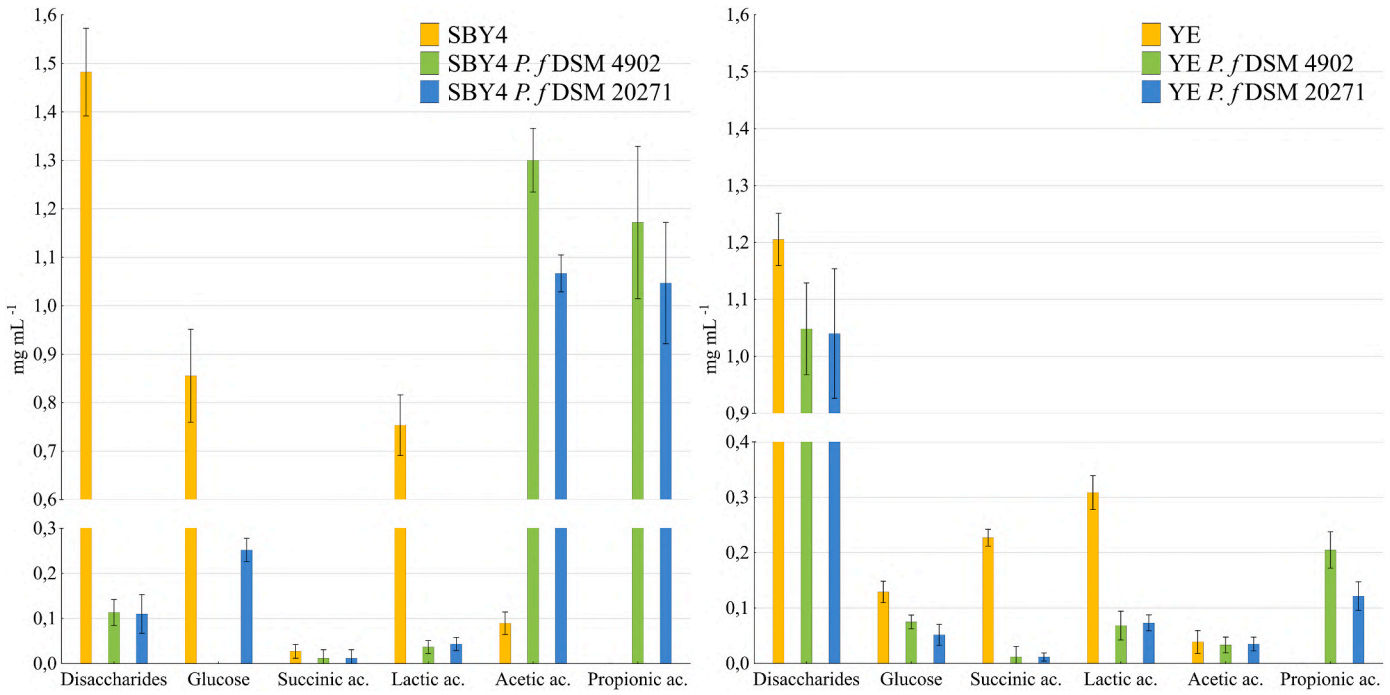

Fig. 6. Sugars and short-chain fatty acids (SCFAs) change before and after fermentation of the SBY4 sample and the yeast extract in the condition applied to the vitamin B_{12} synthesis; n = 3 Explanatory notes: SBY4 - the sample of the spent brewer's yeast; YE - yeast extract; error bars represent the confidence interval ($p < 0.05$). This result was confirmed by Tukey's HSD post hoc test after ANOVA analysis.

propionic acid as a carbon source under conditions of limited access to simple sugars in the nutrient-poor growth media (Dank et al., 2022). Here, metabolic conversions increased pH, promoting *P. freudenreichii* growth and abundant B_{12} synthesis in the YE medium, aligning with observations in the literature (Dank et al., 2022). Another study suggested that sucrose content might have a positive impact on B_{12} synthesis, in contrast to glucose which serves as a precursor for SCFAs synthesis (Piwowarek et al., 2018a).

The PCA analysis (Fig. 7) depicted factors influencing B_{12} synthesis and sample similarity. SBY4 and YE differed in SCFAs, sugars, riboflavin, cobalt, colony formation units, pH, and optical density. Significant factors for increased B_{12} synthesis were cobalt, riboflavin, pH, disaccharides, lactic and succinic acid. Acetic and propionic acids directly affected B_{12} synthesis. Glucose, colony formation units, and OD had no direct effect on PCA results, but glucose was linked to environment acidification by *P. freudenreichii*.

Results align with the literature—cobalt, vitamin B_2, lactic and succinic acids are essential for B_{12} synthesis (Chamlagain et al., 2017; Dank et al., 2021, 2022; Deptula et al., 2017a; Thierry et al., 2011). Disaccharides like sucrose may enhance B_{12} synthesis, and pH levels significantly affect *P. freudenreichii* growth and B_{12} synthesis (Ahmadi et al., 2017; Piwowarek et al., 2018a).

4. Conclusion

The study revealed novel insights in reutilizing SBY and innovative applications of YE. *P. freudenreichii* valorised SBY into free EAAs, SCFAs, and active B_{12}. No prior scientific evidence existed for using SBY and YE in *P. freudenreichii* culturing and metabolite synthesis, addressing an information gap. The findings propose SBY as a sustainable one-component growth medium for *P. freudenreichii*, highlighting its potential in by-product utilization. Moreover, the research identified diverse biotechnological and microbiological applications of SBY for managing post-production raw materials.

The study yielded significant outcomes, such as enhanced growth of *P. freudenreichii* and B_{12} synthesis in microbiological YE. SBY was successfully valorised as a source of essential nutrients for humans. However, limitations were observed, including methodological constraints in profiling transformations of chemical compounds in SBY and YE due to a single growth atmosphere in different experiment stages. Yeast autolytic enzymes may have affected *P. freudenreichii* metabolism,

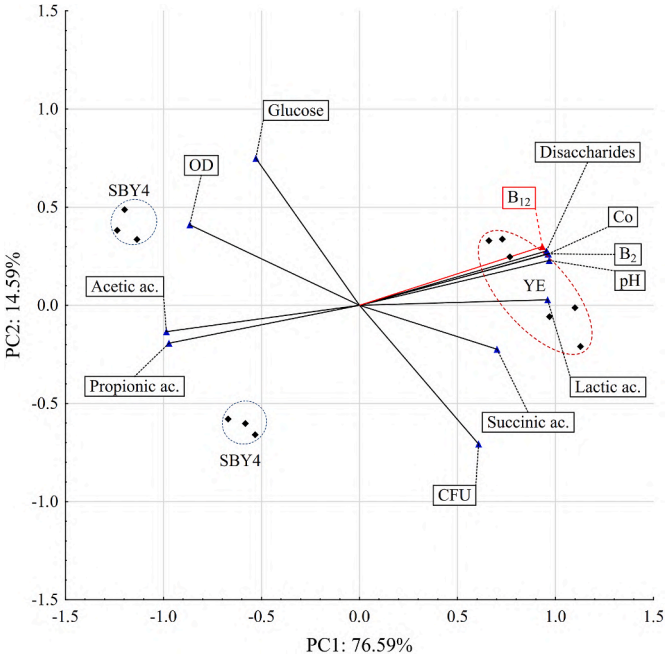

Fig. 7. Principal component analysis (PCA) of the selected chemical compounds and tested parameters influencing the synthesis of vitamin B_{12} in samples SBY4 and YE Explanatory notes: SBY4 - the sample of the spent brewer's yeast; YE - yeast extract; projection of variables (analysed chemical compounds and the) and cases (tested samples presented as circles) onto the plane of the principal components (PC1 and PC2).

and using only one SBY type limited a comprehensive understanding of its role in B_{12} synthesis.

Despite these limitations, the study offers promising avenues. Analysing various SBY types and extraction methods is crucial for maximizing precursor components for *P. freudenreichii* growth and valuable metabolite synthesis. Exploring factors influencing metabolism, such as microaerophilic conditions, polyphenols, and precursor metabolism, is essential. Proper SBY processing and controlled fermentation may elevate metabolite grade, particularly B_{12}, comparable to microbiological YE levels.

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Ethical statement

For this study ethical statement is not necessary.

Declaration of generative AI in scientific writing

The authors declare no use of AI technology.

CRediT authorship contribution statement

Marcin Kruk: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Resources. **Pekka Varmanen:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing, Resources. **Minnamari Edelmann:** Formal analysis, Funding acquisition, Investigation, Methodology, Writing – review & editing, Resources, Validation. **Bhawani Chamlagain:** Funding acquisition, Methodology, Validation, Writing – review & editing. **Monika Trząskowska:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2023.140102.

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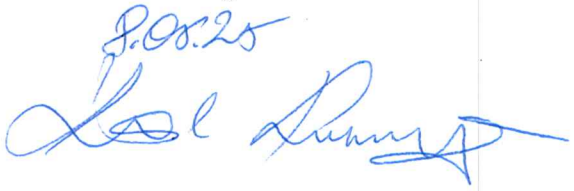

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Niniejszym oświadczam, że w pracy „Kruk, M., Lalowski, P., Płecha, M., Ponder, A., Rudzka, A., Zielińska, D., & Trząskowska, M. (2025). Prebiotic potential of spent brewery grain – In vitro study. Food Chemistry, 463, 141254." mój indywidualny udział w jej powstaniu polegał na przygotowywaniu koncepcji pracy, nadzorze badań laboratoryjnych, korekcie wersji roboczej artykułu naukowego oraz odpowiedzi na uwagi recenzentów.

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My individual contribution in the development thereof involved, conceptualization, funding acquisition, investigation, methodology, project administration, supervision, review & editing and resources.

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My individual contribution in the development thereof involved formal analysis, funding acquisition, investigation, methodology, review & editing, resources, validation.

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